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(57) Abstract

The invention provides methods for screening potential therapeutic compounds useful in the treatment of metabolic diseases, e.g., diabetes. In particular, the invention provides methods of identifying compounds which modulate the activity of a mitochondrial anion carrier (MAC) which include contacting assay vesicles of the present invention with test compounds, determining the ability of the test compounds to modulate the transport of a MAC substrate across the lipid bilayer of the assay vesicle, and identifying the test compound as a modulator of MAC activity. The assay vesicles of the present invention include, for example, one two or three MACs, wherein the activity of at least one MAC is coupled to a detectable readout (e.g., a change in membrane potential and/or pH). The invention also provides novel assay vesicles as well as novel methods of preparing assay vesicles.

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NOVEL METHODS FOR IDENTIFYING MODULATORS OF MITOCHONDRIAL ANION CARRIER ACTIVITY

Government Rights

This invention was made at least in part with government support under grant: DK46200 awarded by the National Institutes of Health. The government has certain rights in this invention.

Background of the Invention

Approximately 17 million persons in the United States are affected by type II diabetes, also known as adult-onset or non-insulin dependent diabetes mellitus (NIDDM). Type II diabetes is a chronic and incurable disease that accounts for 90% to 95% of all diagnosed cases of diabetes. It is a leading cause of death in the United States. This condition is a complex disorder involving a combination of factors, including the inability of certain tissues to respond to insulin, referred to as "insulin resistance" and an inability of the pancreas to produce appropriate levels of insulin. Insulin resistance occurs when insulin-stimulated uptake of glucose into peripheral cells is impaired and the inhibitory effect of insulin on glucose production in the liver is reduced. This condition can lead to excessive blood levels of glucose (hyperglycemia). Hyperglycemia in most diabetics is accompanied by, and may even be preceded by, increases in the blood levels of triglycerides, free fatty acids, and other lipids. Hyperglycemia over an extended period of time is believed to damage the walls of blood vessels, causing complications characteristic of diabetes, including blindness from microvascular deterioration in the retina, loss of circulation in the extremities leading to amputation, and kidney failure. In addition, the high blood levels of triglycerides, free fatty acids, and total cholesterol pose serious health risks to the diabetic and are believed to lead to cardiovascular disease including coronary heart disease.

Obesity is also a significant health problem in the United States. About 60 million Americans, or 45% of the adult population, are overweight and about 35 million, or 26% of the adult population, are obese. Approximately 30% of obese Americans are Type II diabetics, and many of the others are considered to be in pre-diabetic states with

varying levels of insulin resistance or impaired glucose tolerance. Obesity is also considered a risk factor for cardiovascular disease, gall bladder disease and osteoarthritis.

There is no cure for obesity or diabetes. After diagnosis of Type II diabetes, the first course of therapy is to try to control hyperglycemia through diet and exercise. If this dietary and exercise approach fails, patients typically begin medication to achieve glycemic control. Most patients initially take sulfonylureas, which work by stimulating the production of insulin in the pancreas. However, over time, many Type II diabetics taking sulfonylureas lose their ability to control glucose levels, and others lose their ability to secrete insulin. This occurs mainly in patients who are obese and do not lose weight following diagnosis of the disease. When sulfonylureas become ineffective, the Type II diabetic must then begin daily insulin injections.

Reduction of obesity provides the most effective treatment of non-insulin dependent diabetes. Control of body weight is a complex interaction involving, at a minimum, signals from peripheral fat stores to the central nervous system (CNS) and messages from the CNS to the periphery. Previous product development in the area of obesity has targeted neurotransmitter handling by the brain. Active agents have had only small effects on body composition, and their mechanisms are not yet fully understood. Some of these agents are reported to have potentially serious side effects.

CNS regulating signals from adipose tissue to the CNS, such as leptin, have been recently identified and may serve as potential targets for regulating body mass. By contrast, energy-regulating signals from the CNS to the periphery are not yet known but must ultimately regulate the efficiency of energy metabolism at the level of the cell. Thus, effective treatment of obesity may be achieved by modulating the efficiency of energy production such that more fuel is burned to make a set amount of energy.

In human physiology, the mitochondria of a cell play a key role in energy metabolism. Mitochondrial energy metabolism is dependent on and controlled by various transport proteins, including a family of mitochondrial transport proteins known as the mitochondrial anion carriers (MACs) that span the inner mitochondrial membrane. There are at least nine anion carrier proteins located in the mitochondrial inner membrane. In intact mitochondria the ADP/ATP carrier (AAC), also known as the

adenine nucleotide translocator (ANT), and the phosphate carrier (PiC), are absolutely essential in all tissues for the process of oxidative phosphorylation that generates most of the cellular ATP. The remaining anion carriers frequently play a dual role participating in two or more metabolic processes, often in a tissue-specific manner. Thus, the pyruvate carrier (PYC) supplies pyruvate to the mitochondrial matrix during the catabolic phase of cell metabolism to fuel the citric acid cycle, and during the anabolic phase to initiate glucose synthesis in the liver and kidney. The aspartate/glutamate carrier (AGC) and the α-ketoglutarate carrier (KGC), also known as the oxoglutarate carrier (OGC), play a vital role in glucose catabolism in cytosolic-mitochondrial shuttle systems responsible in some systems for regenerating NAD^+ for glycolysis. They also participate in glucose synthesis and in nitrogen metabolism. The dicarboxylate carrier (DIC) is best known for its role in glucose synthesis but also participates in urea synthesis, whereas the citrate carrier (CIC) plays an essential role in fatty acid and lipid biosynthesis. The glutamate carrier (GC) plays a role in urea synthesis and in some other processes related to nitrogen metabolism, mainly in liver. Finally, the ATPMg/P_i carrier (APC) is evidently responsible for "charging" the mitochondrial matrix with adenine nucleotides during early stages of development, thus inducing a variety of processes including ATP and glucose synthesis.

Of particular interest are MACs which have a secondary role as proton transporters. For example, the phosphate carrier (PiC) serves a dual role as a carrier of inorganic phosphate as well as catalyzing P_i/H^+ symport (or P_i/OH^- antiport). (See Wohlrab, H. (1986) *Biochim. Biophys. Acta* 853:8170-8173 and Wehrle, J. P. and Pedersen, P. L. (1989) *J. Membr. Biol.* 111:199-213). By contrast to the pH neutral transport processes of other MACs (e.g., the ATPMg/P_i carrier (APC), the oxoglutarate carrier (OGC)), transport by the PiC results in a pH change in the intramitochondrial milieu.

In addition to the nine MACs described above, it is now known that a family of proteins called the uncoupling proteins (UCPs) function to transport free fatty acid anions (FAA^-s) across the inner mitochondrial membrane. Three UCP family members have been identified to date. UCP-1 is a mitochondrial uncoupling protein which plays an important role in generating heat and burning calories in brown adipose tissue. In the

mitochondria of most cells, energy generated from the oxidation of NADH and $FADH_2$ is harnessed by an electron transport chain embedded in the mitochondrial inner membrane, which pumps protons out of the matrix to create a transmembrane electrochemical gradient. This gradient, which includes contributions from both a membrane potential and a pH difference is in turn used by a protein complex in the inner membrane to synthesize ATP (respiration, or oxidation, is said to be coupled to the synthesis of ATP.) By contrast, in the mitochondria of brown adipose cells, mitochondrial uncoupling proteins create a pathway that allows dissipation of the proton electrochemical gradient across the inner mitochondrial membrane without coupling to any other energy-consuming process. As a result of this uncoupling, respiratory energy is dissipated as heat rather than being stored as high energy ATP.

UCP-2 is a close homologue of UCP-1 which is widely expressed in adult human tissues, including tissues rich in macrophages, and is upregulated in white fat in response to fat feeding. As there is little brown adipose tissue present in adult large-size animals and humans, it is believed that UCP-2 (having 59% amino acid identity to UCP-1) functions in human adult tissues in heat generation, thermoregulation, and body weight regulation. (Fleury et al. (1997) *Nature Genetics*, 15:269-272.) This belief is strengthened by the knowledge that the UCP-2 gene maps to regions of chromosome 11 and mouse chromosome 7 that have been linked to hyperinsulinemia and obesity. Furthermore, UCP-2 mRNA is upregulated in the white adipose tissue of *ob/ob* and *db/db* mice, two genetic mouse models of obesity. (Gimeno et al. (1997) *Diabetes*, 46:900-906.) This increased expression is believed to be a compensatory change in which UCP-2 induction is attempting to act against powerful genetically-induced obesity.

UCP-3 has also been identified as a new member of the mitochondrial transport protein family having 73% amino acid identity with UCP-2 and 57% identity with UCP-1 (Boss et al. (1997) *FEBS Letters*, 408(1):39-42 and Vidal-Puig et al. (1997) *Biochem. Biophys. Res. Commun.*, 235(1):79-82.) UCP-3 is highly expressed in skeletal muscle in humans as well as in brown adipose tissue in rodents. (Id.) Furthermore, UCP-3 mRNA levels are regulated by both hormonal (e.g., dexamethasone, leptin, or

hypo/hyperthyroidism) and dietary manipulations (e.g., starvation). (Gong at al. (1997) *J. Biol. Chem.*, 272(29):24129-24132.)

With the identification of these additional classes of mitochondrial anion carriers, obesity therapies have been proposed that focus on the regulation of expression of the genes encoding these carriers, such as the uncoupling protein genes, UCP-1, UCP-2, and UCP-3. However, the prevailing strategy of manipulating gene expression without an understanding of their basic regulatory mechanisms will be inadequate. Therefore, there exists a need to develop obesity therapeutics which target mechanisms other than the regulation of expression of obesity-related enzymes and transporters.

Summary of the Invention

The present invention is based on the understanding that regulation of the activity of mitochondrial anion carriers (MACs) may be more influential than regulation of their abundance. As such, the present invention provides methods for identifying pharmaceutically effective compounds that modulate the activity of mitochondrial anion carriers (MACs). The invention is based on the knowledge that compounds that modulate such mitochondrial anion carriers (MACs) may also modulate energy efficiency and provide a means for controlling body mass.

Accordingly, in one embodiment, a method of the present invention involves contacting a MAC with a test compound and a MAC substrate which is capable of being transported by the MAC; determining the ability of the test compound to modulate transport of the MAC substrate; and identifying the test compound as a modulator of MAC activity. In a preferred embodiment, the MAC is formulated within a barrier separating a first and a second compartment (e.g., a biological or structural barrier) under conditions such that transport of the MAC substrate across the barrier occurs. In another embodiment, the barrier includes more than one MAC. In another embodiment, the first or second compartment further comprises an indicator of MAC activity. In yet another embodiment, the first and the second compartment include an indicator of MAC activity and/or an indicator of MAC-independent activity (e.g., barrier "leakiness").

In a preferred embodiment, a method of the present invention involves contacting an assay vesicle, preferably an alkaline assay vesicle, which includes at least one MAC and an indicator of MAC activity (e.g., a pH indicator or a potentiometric probe) with a test compound and a MAC substrate which is capable of being transported across the lipid bilayer of the assay vesicle; determining the ability of the test compound to modulate transport of the MAC substrate across the lipid bilayer of the assay vesicle; and identifying the test compound as a modulator of MAC activity. The MAC can be selected, for example, from the group consisting of an ADP/ATP carrier (AAC), a phosphate carrier (PiC), a pyruvate carrier (PYC), an aspartate/glutamate carrier (AGC), an α-ketoglutarate carrier or oxoglutarate carrier (KGC/OGC), a dicarboxylate carrier (DIC), a citrate carrier (CIC), a glutamate carrier (GC), an ATPMg/P_i carrier (APC), and an uncoupling protein (UCP). In one embodiment, the MAC is a pyruvate carrier (PYC) and the MAC substrate is pyruvate. In another embodiment, the MAC is a phosphate carrier (PiC) and the MAC substrate is inorganic phosphate (Pi). In yet another embodiment, the MAC is a glutamate carrier (GC) and the MAC substrate is glutamate. In yet another embodiment, the MAC is a mitochondrial uncoupling protein (UCP) and the MAC substrate is a free fatty acid anion (FAA^-). For example, the mitochondrial UCP can be selected from the group consisting of uncoupling protein 1 (UCP1), uncoupling protein 2 (UCP2) and uncoupling protein 3 (UCP3).

The present invention also involves a method for identifying a compound which modulates the activity of a mitochondrial anion carrier (MAC) comprising contacting an assay vesicle which includes at least one MAC, a first MAC substrate, and an indicator of MAC activity (e.g., a pH indicator or a potentiometric probe) with a test compound and a second MAC substrate, wherein the first and second MAC substrates are capable of being transported across the lipid bilayer of the assay vesicle; determining the ability of the test compound to modulate transport of the MAC substrates across the lipid bilayer of the assay vesicle, wherein determining the ability of the test compound to modulate transport of the MAC substrates across the lipid bilayer of the assay vesicle comprises determining a change in the indicator of MAC activity; and identifying the test compound as a modulator of MAC activity. In one embodiment, the MAC is an ADP/ATP carrier (AAC), the MAC substrates are ADP and ATP. In another

embodiment, the MAC is an aspartate/glutamate carrier (AGC), and the MAC substrates are aspartate and glutamate. In another embodiment, the MAC is an α-ketoglutarate carrier or oxoglutarate carrier (KGC/OGC) and the MAC substrates are α-ketoglutarate and malate. In another embodiment, the MAC is a dicarboxylate carrier (DIC) and the MAC substrates are inorganic phosphate (Pi) and a dicarboxylate (e.g. malate or succinate). In another embodiment, the MAC is a citrate carrier (CIC) and the MAC substrates are malate and citrate. In another embodiment, the MAC is an ATPMg/P_i carrier (APC) and the MAC substrates are ATP-Mg^{++} and inorganic phosphate (Pi). In yet another embodiment, the MAC is an uncoupling protein (UCP) and the MAC substrates are a free fatty acid anion (FAA^-) and a free fatty acid.

The present invention also involves a method for identifying a compound which modulates the activity of a mitichondrial anion carrier (MAC) comprising contacting an assay vesicle which comprises a first MAC, a second MAC, a second MAC substrate, and an indicator of activity of the second MAC (e.g., a pH indicator or a potentiometric probe) with a test compound and a first MAC substrate, wherein the MAC substrates are capable of being transported across the lipid bilayer of the assay vesicle; determining the ability of the test compound to modulate transport of the first MAC substrate across the lipid bilayer of the assay vesicle, wherein determining the ability of the test compound to modulate transport of the first MAC substrate across the lipid bilayer of the assay vesicle comprises determining a change in the indicator of activity of the second MAC; and identifying the test compound as a modulator of MAC activity. Accordingly, in one embodiment, the first MAC is an aspartate/glutamate carrier, the second MAC is a glutamate carrier (GC), the first MAC substrate is aspartate and the second MAC substrate is glutamate. In another embodiment, the first MAC is an ATPMg/Pi carrier (APC), the second MAC is a phosphate carrier (PiC), the first MAC substrate is $ATPMg^{++}$, and the second MAC substrate is inorganic phosphate (Pi). In yet another embodiment, the first MAC is a dicarboxylate carrier, the second MAC is a phosphate carrier, the first MAC substrate is a dicarboxylate (e.g., malate or succinate), and the second MAC substrate is inorganic phosphate (Pi).

The present invention also involves a method for identifying a compound which modulates the activity of a mitichondrial anion carrier (MAC) comprising contacting an assay vesicle which comprises a first MAC, a second MAC, a third MAC, a second MAC substrate, a third MAC substrate, and an indicator of activity of the third MAC (e.g., a pH indicator or a potentiometric probe) with a test compound and a first MAC substrate, wherein the MAC substrates are capable of being transported across the lipid bilayer of the assay vesicle; determining the ability of the test compound to modulate transport of the first MAC substrate across the lipid bilayer of the assay vesicle, wherein determining the ability of the test compound to modulate transport of the first MAC substrate across the lipid bilayer of the assay vesicle comprises determining a change in the indicator of activity of the third MAC; and identifying the test compound as a modulator of MAC activity. Accordingly, in one embodiment, the first MAC is an α-ketoglutarate carrier or oxoglutarate carrier (KGC/OGC), the second MAC is a dicarboxylate carrier, the third MAC is a phosphate carrier (PiC), the first MAC substrate is α-ketoglutarate, the second MAC substrate is malate, and the third MAC substrate is inorganic phosphate (Pi). In another embodiment, the first MAC is a citrate transporter (CIC), the second MAC is a dicarboxylate transporter (DIC), the third MAC is a phosphate transporter, the first MAC substrate is citrate, the second MAC substrate is malate, and the third MAC substrate is inorganic phosphate (Pi).

Assay vesicles are also intended to be within the scope of the present invention. In one embodiment, an assay vesicle of the present invention contain a phospholipid, at least one MAC, and an indicator of MAC activity (e.g., a pH indicator or a potentiometric probe). For example, the phospholipid can be selected from the group consisting phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylserine. Furthermore, the MAC can be selected from the group consisting an ADP/ATP carrier (AAC), a phosphate carrier (PiC), a pyruvate carrier (PYC), an aspartate/glutamate carrier (AGC), an oxoglutarate carrier (KGC/OGC), a dicarboxylate carrier (DIC), a citrate carrier (CIC), a glutamate carrier (GC), an ATPMg/P_i carrier (APC), and an uncoupling protein (UCP). In one embodiment, the assay vesicle further comprises a lysophospholipid. For example, the lysophospholipid can be selected from the group consisting of lysophosphotidate, lysophosphatidylcholine (e.g., lyso 1-acyl

phosphatidylcholine), lysophosphatidylethanolamine, lysophosphatidylinositol, and lysophosphatidylserine. In another embodiment, the assay vesicle further includes a fatty acid. For example, the fatty acid can be selected from the group consisting of oleic acid, myristic acid, palmitic acid, lauric acid, stearic acid, and arachidonic acid.

Furthermore, assay vesicles having an indicator of MAC-independent activity (e.g., vesicle "leakiness" or movement of MAC substrates across the lipid bilayer which occurs in a manner independent of the MAC) are intended to be within the scope of the invention.

Brief Description of the Drawings

Figure 1 is a schematic representation of exemplary embodiments of the present invention. The diagram depicts assay vesicles comprising one, two and three mitochondrial anion carriers (MACs) as well as the MAC substrates transported by the enumerated carriers.

Figure 2 is an alignment of the coding sequences for the human MACs, UCP-1, UCP-2, and UCP-3.

Detailed Description of the Invention

The present invention provides, *inter alia*, rapid, effective assays for screening and identifying pharmaceutically effective compounds that specifically act as modulators of mitochondrial anion carriers (MACs). The present invention is based, at least in part, on the fact that mitochondrial metabolism is dependent on MACs including, for example, the ADP/ATP carrier (AAC), also known as the adenine nucleotide translocator (ANT), the phosphate carrier (PiC), the pyruvate carrier (PYC), the aspartate/glutamate carrier (AGC), the oxoglutarate carrier (KGC/OGC), the dicarboxylate carrier (DIC), the citrate carrier (CIC), the glutamate carrier (GC), and the ATPMg/P_i carrier (APC). The invention is also based on the discovery that a key role of at least one class of MACs, the mitochondrial uncoupling proteins (UCPs), appears to be transport of fatty acid anions to induce cyclical proton movement across the inner mitochondrial membrane. This transport is tightly related to oxidation (i.e. burning) of

fatty acids in the mitochondrion, thereby converting fatty acids into energy rather than storing fatty acids. As such, the instant assays provide a convenient format for discovering compounds which have therapeutic potential in the treatment of obesity and diabetes, as well as other diseases and anomalies which result, at least in part, from aberrant cellular metabolism. In one example, the assays of the present invention can be utilized to identify compounds which enhance transport activity of the MACs. Compounds which enhance or promote transport (e.g., MAC agonists or activators) are beneficial to increase the energy expenditure of a cell, particularly a cell of an individual that would benefit from enhanced expenditure of energy (e.g., an obese subject). Alternatively, compounds which reduce or inhibit transport (e.g., MAC antagonists or inhibitors) are beneficial to decrease the energy expenditure of a cell, particularly a cell of an individual that would benefit from reduced energy expenditure (e.g., an aging subject or subject afflicted with cancer).

Accordingly, in one embodiment, the present invention involves a method for identifying a compound which modulates the activity of a mitochondrial anion carrier (MAC) which involves contacting a MAC with a test compound and a MAC substrate which is capable of being transported by the MAC; determining the ability of the test compound to modulate transport of the MAC substrate; and identifying the test compound as a modulator of MAC activity. In a preferred embodiment, the MAC is formulated within a barrier separating a first and a second compartment (e.g., a biological or structural barrier) under conditions such that transport of the MAC substrate across the barrier occurs. In an exemplary embodiment, the MAC substrate is transported from the first compartment to the second compartment. In another exemplary embodiment, the MAC substrate is transported from the second compartment to the first compartment. In another embodiment, the first or second compartment further comprises an indicator of MAC activity.

Also according to the present invention, the activity of a MAC can be evaluated in an assay system in which a MAC is reconstituted in an assay vesicle and an indicator of MAC activity (e.g., a pH indicator or a membrane potential indicator) is included within the vesicle. The activity of a MAC in a reconstituted assay vesicle of the present invention, can be measured by measuring the transport of a MAC substrate across the

lipid bilayer of the assay vesicle. For example, the transport of a MAC substrate from the inside of an assay vesicle to the outside of an assay vesicle can be measured. Alternatively, the transport of a MAC substrate from the outside of an assay vesicle to the inside of an assay vesicle can be measured.

Also, according to the present invention, a barrier or assay vesicle can include more than one MAC. For example, a barrier or assay vesicle of the present invention can contain a first MAC and a second MAC which operatively cotransport at least one MAC substrate. Furthermore, a barrier or assay vesicle of the present invention can contain more than two MACs (e.g., a first, second, and third MAC, wherein the first and second MAC operatively co-transport at least one MAC substrate and wherein the second and third MACs operatively cotransport at least one MAC substrate.

MACs formulated within barriers as well as assay vesicles reconstituted with at least one MAC and containing an indicator of MAC activity can be used to test the effects of compounds on the transport activity of MAC. The measurements of MAC activity are straightforward and quick, thus allowing the rapid testing of many compounds. Compounds can be tested in the assays of the present invention singly or, more preferably, as members of libraries of compounds. Thus, the subject assays enable rapid screening of large numbers of compounds or a library of compounds to identify those compounds which modulate the activity of a MAC.

Before further description of the invention, certain terms employed in the specification, examples and appended claims are, for convenience, collected here.

As used herein, the term "compounds" (e.g., as in "test compound") is intended to include test agents, usually in the form of a library of agents, which in certain embodiments, will be contacted with the MACs or "assay vesicles" of the invention to determine the ability of such compounds to modulate the activity of a "mitochondrial anion carrier (MAC)" which exists in a barrier or lipid bilayer of an assay vesicle. The compounds can be peptidic or non-peptidic in nature. Compounds of the non-peptidic nature often include small organic and/or non-organic molecules. Furthermore, it is contemplated that compounds of the present invention can be naturally-occurring compounds such as nucleic acid molecules, proteins, sugars, lipids, or derivatives thereof. Test compounds include compounds for which the structure and/or physical

characteristics are known but for which a MAC modulatory function has not been demonstrated, as well as compounds for having a yet undetermined structure and/or physical characteristics. Furthermore, compounds for which an activity other than a MAC modulatory activity is known, can be identified as modulators of MAC activity using the methods of the present invention.

As used herein, the term "contacting" (*i.e.*, contacting an assay vesicle with a compound) is intended to include incubating the compound or agent and the assay vesicle together *in vitro* (*e.g.*, adding the agent to assay vesicles in a suitable vessel) such that the agent has the potential, for example, to interact with and/or bind to a mitochondrial anion carrier (MAC) which exists in the lipid bilayer of the assay vesicle such that the activity of the MAC is modulated.

As used herein, the term "mitochondrial anion carrier (MAC)" is intended to include a class of proteins that exist, in their natural state (e.g., in the intact mitochondria of a cell), in the inner mitochondrial membrane of the mitochondria of a cell. The MACs commonly contain a characteristic 3-fold repeated sequence approximately 100 amino acids in length, each repeated 100 amino acid element being composed of two membrane-spanning alpha-helices linked by an extensive hydrophilic domain. For example, MACs of the present invention include the ADP/ATP carrier (AAC), also known as the adenine nucleotide translocator (ANT) (e.g., isoforms ANT1, ANT2, and ANT3, also known as T1, T3, and T2), the phosphate carrier (PiC), (e.g., phosphate carrier isoform A (PiC A) and phosphate carrier isoform **B** (PiC B)), the pyruvate carrier (PYC), the aspartate/glutamate carrier (AGC), the oxoglutarate carrier (KGC/OGC), the dicarboxylate carrier (DIC), the citrate carrier (CIC), the glutamate carrier (GC), the ATPMg/P_i carrier (APC), and the mitochondrial uncoupling proteins (UCPs), (e.g., uncoupling protein 1 (UCP-1), uncoupling protein 2 (UCP-2), and uncoupling protein 3 (UCP-3)).

In general, the "activity of a MAC" is to shuttle or transport "MAC substrates" from one side of an inner mitochondrial membrane or lipid bilayer of an assay vesicle (e.g., the outside of the membrane or lipid bilayer) to the other side of an inner mitochondrial membrane or lipid bilayer of an assay vesicle (e.g., the inside of the membrane or lipid bilayer). For a detailed review of the activities of the various MACs

of the present invention, see, for example, Wehrle and Pedersen (1989) *J. Membrane Biol.* 111:199-213; Azzi et al. (1993) *J. Bioenerg. Biomembr.* 25:515-524; Walker and Runswick (1993) *J. Bioenerg. Biomembr.* 25:435-446; Ferreira and Pedersen (1993) *J. Bioenerg. Biomembr.* 25:483-492; Kaplan and Mayor (1993) *J. Bioenerg. Biomembr.* 25:503-514; Palmieri et al. (1993) *J. Bioenerg. Biomembr.* 25:493-501; Klingenberg (1993) *J. Bioenerg. Biomembr.* 25:447-457; Aprille (1993) *J. Bioenerg. Biomembr.* 25:473-481; Wohlrab (1986) *Biochem. Biophys. Acta* 853:115-134; Gimeno et al. (1997) *Diabetes* 46:900-906; Fleury et al. (1997) *Nature Genet.* 15:269-272; Vidal-Puig et al. (1997) *Biochem. Biophys. Res. Commun.* 235:79-82; Boss et al. (1997) *FEBS Lett.* 408:39-42; and Gong et al. (1997) *J. Biol. Chem.* 272:24129-132. Thus, according to the present invention, a MAC substrate is "capable of being transported across the lipid bilayer of a vesicle".

In their natural state, the activity of a MAC may be coupled to a mitochondrial process such as oxidative phosphorylation that generates cellular energy in the form of ATP (e.g., ACC/ANT and PiC). Alternatively, the activity of a MAC may play a dual role in two or more metabolic processes (e.g. PYC, AGC, KGC/OGC, DIC, CIC, and GC.) Alternatively, the activity of the MAC may be uncoupled (e.g., the UCPs), in which case oxidation results in the generation of heat rather than cellular energy.

The phrase "modulates" or "modulating the activity of a mitochondrial anion carrier (MAC)" is intended to include enhancing, promoting, or inducing the activity of a MAC as well as or reducing or inhibiting the activity of a MAC, such that the activity of the MAC is detectably enhanced, promoted, induced, reduced, or inhibited after contacting the reconstituted lipid bilayer vesicle containing the MAC with the test compound or agent as compared to the activity of the MAC detected prior to contacting the reconstituted lipid bilayer vesicle with the test compound or agent.

Also as used herein, the term "MAC substrate" refers to the molecule or molecules which are transported from one side of the inner mitochondrial membrane or "reconstituted lipid bilayer" to the other side of the inner mitochondrial membrane or "reconstituted lipid bilayer". For example, a MAC substrate can be transported from the outside of the inner mitochondrial membrane or vesicle bilayer to the inside of the inner mitochondrial membrane or vesicle bilayer. Alternatively, a MAC substrate can be

transported from the inside of the inner mitochondrial membrane or vesicle bilayer to the outside of the inner mitochondrial membrane or vesicle bilayer.

A particular MAC is associated with specific MAC substrate, such specificity most likely being determined by unique amino acid sequences attributed to a particular MAC (e.g., the sequences that are not commonly shared among the class of MAC proteins). For example, the PiC specifically transports the MAC substrate, inorganic phosphate (P_i or HPO_4^{2-}) across the mitochondrial membrane (e.g., from the outside of the inner mitochondrial membrane to the inside of the inner mitochondrial membrane). The PiC is also referred to herein and in the art as both a Pi/H^+ symporter (e.g., cotransports Pi and protons (H^+s) from the outside of the inner mitochondrial membrane to the inside of the inner mitochondrial membrane) or as a Pi/OH^- antiporter (e.g., exchanges Pi for hydroxyl ions (OH^-) across the inner mitochondrial membrane). The ACC or ANT specifically transports the MAC substrate, ADP^{3-}, across the inner mitochondrial membrane (e.g., from the outside of the inner mitochondrial membrane to the inside of the inner mitochondrial membrane), and transports the MAC substrate, ATP^{4-}, across the inner mitochondrial membrane (e.g., from the inside of the inner mitochondrial membrane to the outside of the inner mitochondrial membrane). The PYC specifically transports the MAC substrate, pyruvate, across the inner mitochondrial membrane (e.g., from the outside of the inner mitochondrial membrane to the inside of the inner mitochondrial membrane). Like PiC, the PYC is also referred to herein and in the art as both pyruvate/H^+ symporter (e.g., cotransports pyruvate and protons (H^+s) from the outside of the inner mitochondrial membrane to the inside of the inner mitochondrial membrane) or as a pyruvate/OH^- antiporter (e.g., exchanges pyruvate for hydroxyl ions (OH^-) across the inner mitochondrial membrane). The glutamate carrier specifically transports the MAC substrate, glutamate, across the inner mitochondrial membrane (e.g., from the outside of the inner mitochondrial membrane to the inside of the inner mitochondrial membrane). Like PiC and PYC, the GC is also referred to herein and in the art as both glutamate/H^+ symporter (e.g., cotransports glutamate and protons (H^+s) from the outside of the inner mitochondrial membrane to the inside of the inner mitochondrial membrane) or as a glutamate/OH^- antiporter (e.g., exchanges glutamate for hydroxyl ions (OH^-) across the inner mitochondrial membrane).

The AGC specifically exchanges the MAC substrates, aspartate and glutamate, from across the inner mitochondrial membrane. The KGC/OGC specifically transports the MAC substrate, α-ketoglutarate (also referred to herein as 2-oxoglutarate), across the inner mitochondrial membrane (e.g., from the outside of the inner mitochondrial membrane to the inside of the inner mitochondrial membrane), and transports the MAC substrate, malate (or other dicarboxylic acids), across the inner mitochondrial membrane (e.g., from the inside of the inner mitochondrial membrane to the outside of the inner mitochondrial membrane). The DIC specifically transports the MAC substrates, dicarboxylate (e.g., malate and succinate) and inorganic phosphate (Pi), across the inner mitochondrial membrane (e.g., the DIC catalyzes an electroneutral Pi:dicarboxylate or Pi:Pi exchange across the inner mitochondrial membrane). In particular, the dicarboxylate exchanger catalyzes the transport of respiratory substrates (e.g., malate and succinate) into the mitochondria and may facilitate translocation of dicarboxylates into the cytoplasm for gluconeogenesis. The CIC specifically transports the MAC substrate, citrate (e.g., tricarboxylate), from one side of the inner mitochondrial membrane (e.g., from the inside of the inner mitochondrial membrane to the outside of the inner mitochondrial membrane. In particular, the CIC catalyzes a 1:1 electroneutral exchange across the inner mitochondrial membrane of a tricarboxylate (e.g., citrate, threo-D_s-isocitrate, *cis*-aconitate) plus a proton for either another tricarboxylate/H^+ , a dicarboxylate (e.g., malate, succinate), D- and L- tartarate, or phosphoenolpyruvate.

The APC specifically transports the MAC substrate, adenine nucleotides (e.g., $ATPMg^{2-}$), across the inner mitochondrial membrane (e.g., from the outside of the inner mitochondrial membrane to the inside of the inner mitochondrial membrane), and transports the MAC substrate, inorganic phosphate (P_i or HPO_4^{2-}), across the inner mitochondrial membrane (e.g., from the inside of the inner mitochondrial membrane to the outside of the inner mitochondrial membrane). Transport via the APC is completely reversible and nonproductive exchanges also occur (e.g., $ATPMg^{2-}$:$ATPMg^{2-}$ and P_i:P_i). Finally, the UCPs specifically transport the MAC substrate, fatty acid anions, across the inner mitochondrial membrane (e.g., from the inside of the inner mitochondrial membrane to the outside of the inner mitochondrial membrane). In particular, long chain (C>10), unesterfied fatty acid anions with an optimum response at

14 carbons (myristic acid anion) and increasing activity with degree of unsaturation. Transport of palmitate anion and laurate anion has also been demonstrated. (Jezek *et al.* (1996) *J. Biol. Chem.* 271:6199-6205 and references contained therein). Transport of fatty acid anions is also reffered to as fatty acid activation of proton (H^+) transport or proton (H^+) conductance.

Transfer of the above-described MAC substrates can be recapitulated in artificial or reconstituted systems. For example, in an assay system involving a MAC as a component of a barrier separating a first and second compartment, the MAC activity involves transport of the MAC substrate from, for example, the first compartment to the second compartment. Also for example, activity of the MACs in the inner mitochondrial matrix can be readily reconstituted in lipid bilayer vesicles utilizing methods known in the art as well as those described in detail in Examples 2-3. Although the naturally occurring MACs have a particular sidedness or orientation as they exist in the mitochondria, MACs in a reconstituted lipid bilayer can have either a "right-side-in" orientation (e.g., existing in the reconstituted vesicle in the same orientation as they exist in the intact mitochondria) or, alternatively, can have an "in-side-out" orientation (e.g., existing in a reverse or the opposite orientation as they exist in the intact mitochondria.) Accordingly, a MAC substrate that is transported from the inside of the inner mitochondrial membrane to the outside of the inner mitochondrial membrane in the intact membrane can be transported form the outside of a reconstituted vesicle to the inside when the MAC is inserted in the "in-side-out" orientation.

Furthermore, as used herein, the phrase "modulate transport of the MAC substrate" is intended to include enhancing, promoting, or inducing the transport of a MAC as well as or reducing or inhibiting the transport of the MAC substrate such that the transport of the MAC substrate is detectably enhanced, promoted, induced, reduced, or inhibited after contacting the assay vesicle containing the MAC with a test compound or agent as compared to the transport of the MAC substrate prior to contacting the assay vesicle with a test compound or agent. "Determining the ability of a test compound to modulate transport of a MAC substrate" includes determining a change in the level of a MAC substrate on one side of the mitochondrial membrane or assay vesicle bilayer which occurs as a result of contacting the MAC-containing mitochondrial membrane or

assay vesicle bilayer with a test compound. "Determining the ability of a test compound to modulate transport of a MAC substrate" can involve either a direct determination or an indirect determination of MAC activity. In one embodiment, transport of the MAC substrate results in a change in the internal pH of the lipid vesicle (e.g., an acidification or net increase in proton (H^+) concentration, or an alkalinization or net decrease in proton (H^+) concentration). Accordingly, compounds which modulate transport of a MAC substrate can be identified as compounds which effect the change in intravesicular pH caused by transport of the MAC substrate. In one embodiment, transport of the MAC substrate results in a change in the internal pH of the lipid vesicle (e.g., an acidification or net increase in proton (H^+) concentration, or an alkalinization or net decrease in proton (H^+) concentration). Accordingly, compounds which modulate transport of a MAC substrate can be identified as compounds which effect the change in intravesicular pH caused by transport of the MAC substrate. For example, transport of the MAC substrate, inorganic phosphate (P_i), from the outside of an assay vesicle to the inside of a PiC-containing assay vesicle results in a concomitant decrease in intravesicular pH. A test compound which modulates transport of P_i by the PiC can be identified as a compound which effects this pH change (e.g., a compound which effects (increases or decreases) either the magnitude or rate of the pH change). Likewise, transport of the MAC substrate, pyruvate, from the outside of an assay vesicle to the inside of a PYC-containing assay vesicle results in a concomitant decrease in intravesicular pH. A test compound which modulates transport of pyruvate by the PYC can be identified as a compound which effects this pH change (e.g., a compound which effects (increases or decreases) either the magnitude or rate of the pH change). Likewise, transport of the MAC substrate, glutamate, from the outside of an assay vesicle to the inside of a GC-containing assay vesicle results in a concomitant decrease in intravesicular pH. A test compound which modulates transport of glutamate by the GC can be identified as a compound which effects this pH change (e.g., a compound which effects (increases or decreases) either the magnitude or rate of the pH change). Furthermore, transport of the MAC substrate, free fatty acid anion (FAA^-), from the outside of an assay vesicle to the inside of a UCP-containing assay vesicle results in a concomitant decrease in intravesicular pH. A test compound which modulates transport

of FAA^- by the UCP can be identified as a compound which effects this pH change (e.g., a compound which effects (increases or decreases) either the magnitude or rate of the pH change).

In another embodiment, transport of the MAC substrate results in a change in the membrane potential of the assay vesicle (e.g., an increase or decrease in membrane potential). Accordingly, compounds which modulate transport of a MAC substrate can be identified as compounds which effect the change in membrane potential caused by transport of the MAC substrate. For example, transport of the MAC substrates, ADP^{3-} and ATP^{4-}, across the lipid bilayer of an AAC-containing assay vesicle results in a concomitant decrease in membrane potential. A test compound which modulates transport of ADP^{3-} and ATP^{4-} by the AAC can be identified as a compound which effects this change in membrane potential (e.g., a compound which effects (increases or decreases) either the magnitude or rate of change in membrane potential). Likewise, transport of the MAC substrates, asparate and glutamate, across the lipid bilayer of an AGC-containing assay vesicle results in a concomitant decrease in membrane potential. A test compound which modulates transport of aspartate and glutamate by the AGC can be identified as a compound which effects this change in membrane potential (e.g., a compound which effects (increases or decreases) either the magnitude or rate of change in membrane potential). Likewise, transport of the MAC substrate, FAA^- from the inside of a UCP-containing assay vesicle to the outside of the assay vesicle results in a concomitant change in membrane potential. A test compound which modulates transport of FAA^- by the UCP can be identified as a compound which effects this change in membrane potential (e.g., a compound which effects (increases or decreases) either the magnitude or rate of change in membrane potential).

In another embodiment, transport of the MAC substrate results in a change in the net charge inside the lipid vesicle (e.g., an increase or decrease in the concentration of a specific ion, for example FAA^-). Accordingly, compounds which modulate transport of a MAC substrate can be identified as compounds which effect the change in charge distribution (e.g., FAA^- concentration) resulting from transport of the MAC substrate. For example, transport of the MAC substrate, FAA^-, from the inside of an assay vesicle to the outside of a UCP-containing assay vesicle results in a concomitant decrease in

intravesicular FAA^- concentration and a decrease in membrane potential (ψ_m). A test compound which modulates transport of FAA^- by the UCP can be identified as a compound which affects this change in membrane potential (e.g., a compound which affects (increases or decreases) either the magnitude or rate of the membrane potential). The change in membrane potential is due to a change in charge distribution caused by uncompensated ion movement in one direction (e.g., FAA^- is transported out without a compensatory H^+ or exchange for OH^-).

In yet another embodiment, transport of the MAC substrate by a particular MAC substrate results in neither a change in intravesicular pH or in membrane potential. In such a situation it may be desirable to couple transport of that MAC substrate to a second MAC, wherein transport of the MAC substrate by the second MAC results in a detectable change in intravesicular pH or membrane potential. For example, transport of the MAC substrates, inorganic phosphate (P_i) and dicarboxylate (e.g., malate) by a DIC in an assay vesicle can be coupled to transfer of P_i by a PiC in the same assay vesicle, which results in a change in intravesicular pH. Likewise, transport of the MAC substrates, $ATPMg^{2-}$ and inorganic phosphate (P_i) by an APC in an assay vesicle can be coupled to transfer of P_i by a PiC in the same assay vesicle, which results in a change in intravesicular pH.

In yet another embodiment, it may be desirable to couple transport of the MAC substrate by a particular MAC substrate which results in a change in membrane potential to transport by a MAC which results in a change in pH. For example, transport of the MAC substrates, aspartate and glutamate by an AGC in an assay vesicle can be coupled to transfer of glutamate by a GC in the same assay vesicle, which results in a change in intravesicular pH. Such an embodiment may be preferred when a rapid detection (e.g., change in pH) is desired as compared to the relatively slower change in membrane potential.

In yet another embodiment, an assay vesicle comprises three MACs and transport of the respective MAC substrates is effectively coupled resulting in a detectable readout (e.g., a change in pH or membrane potential). For example, an assay vesicle containing KGC, DIC, and PiC can be utilized to assay for modulators of KGC activity in the following manner. Transport of the MAC substrates α-ketoglutarate and malate by the

KGC is coupled to transport of the MAC substrates malate and inorganic phosphate (P_i) by the DIC which is, in turn, coupled to transport of the substrate P_i by the PiC which results in a change in pH. In another embodiment, an assay vesicle containing CIC, DIC, and PiC can be used to assay for modulators of CIC activity in the following manner. Transport of the MAC substrates, citrate and malate, by the CIC is coupled to transport of the MAC substrates, malate and inorganic phosphate (P_i), by the DIC which is, in turn, coupled to transport of the MAC substrate, P_i, by the PiC which results in a change in pH.

The phrase "barrier" is intended to include any biological and/or artificial separator of two compartments which provides a favorable environment for the existence of a MAC. For example, barriers can comprise phospholipids, glycolipids, other lipidic molecules, non-lipid molecules, synthetic molecules, any or all of which may support the function of a transporter which exists, in its natural environment, as a component of an organelle membrane. Structural support such as liquid crystals, films, biological chips, emulsions, for example, are intended to form barriers according to the invention.

The phrase "assay vesicles" is intended to encompass unilamellar or multilamellar structures having both an outer lipid monolayer and an inner lipid monolayer. The "assay vesicles" of the present invention can be synthesized by a variety of means as described in Example 1. In general the assay vesicles of the present invention are reconstituted from purified or, at least, partially-purified components (e.g., phospholipid molecules, protein molecules) and can accordingly be termed "reconstituted lipid bilayer vesicles". The "assay vesicles" of the present invention can include only phospholipid molecules, or can further comprise, for example, one or more of the following: protein molecules (e.g., the MACs of the present invention or other non-MAC proteins, for example); cholesterol; fatty acids (e.g., oleic acid, myristic acid, palmitic acid, lauric acid, stearic acid, or arachidonic acid); phospholipids (e.g., phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, and/or phosphatidylserine; glycolipids (e.g., cerebroside or gangliosides); and/or lysophospholipids (e.g., lysophosphidate, lyso 1-acyl phosphatidylcholine (lyso-PC), lysophosphatidylethanolamine, lysophosphatidylinositol, or lysophosphatidylserine). In one embodiment, the assay vesicles of the present invention are "alkaline assay

vesicles". As used herein, an "alkaline assay vesicles" is an assay vesicle in which has an alkaline, intravesicular pH (e.g., a pH of greater than 7.4). "Alkaline assay vesicles" are particularly useful, for example, when utilizing pH change as a determinant of MAC activity. The benefit of utilizing alkaline assay vesicles as opposed, for example, to assay vesicles having a neutral pH, arises from the fact that the change in pH as a determinant of MAC activity is generally a decrease in pH (e.g., an acidification of the interior milieu of the vesicle. Hence, utilizing an assay vesicle with an alkaline intravesicular environment can result in a pH change of greater magnitude. Accordingly, in one embodiment, an alkaline assay vesicle has a pH of about 7.4 to 8.0. In another embodiment, an alkaline assay vesicle has a pH of about 8.0 to 9.0. In another embodiment, an alkaline assay vesicle has a pH of about 9.0 to 10.0. In yet another embodiment, an alkaline assay vesicle has a pH of about 10.0 to 11.0, 11.0 to 12.0, or greater.

Also, as used herein, the term "pH indicator" is intended to include a molecule or composition of molecules which produce a quantitative or qualitative change in response to a change in pH. A "pH indicator" of the present invention can be placed on the inside of a reconstituted lipid bilayer vesicle (an "internal pH indicator") and/or can be placed on the outside of a reconstituted lipid bilayer vesicle (an "external pH indicator"). A pH indicator dye can be, for example, a fluorescent dye (e.g., pyranine, SNARF, SNAFL, HPTS, BCECF, and/or fluoroscein and its derivatives) or a colorometric inhibitor. Examples of such pH indicators are described in detail in Table 2.

Also, as used herein, the term "membrane potential indicator" is intended to include a molecule or composition of molecules which produce a quantitative or qualitative change in response to a change in membrane potential. A "membrane potential indicator" of the present invention can be placed, for example, on the inside of a reconstituted lipid bilayer vesicle (an "internal membrane potential indicator") . Examples of membrane potential indicators are described in detail in Table 3.

Furthermore, assay vesicles having an indicator of MAC-independent activity (e.g., vesicle "leakiness" or movement of MAC substrates across the lipid bilayer which occurs in a manner independent of the MAC) are intended to be within the scope of the invention. For example, in one embodiment, an assay vesicle includes a pH indicator as

a determinant of MAC activity (e.g., an assay vesicle including PiC, PYC, GC, or UCP) can have a second pH indicator on the outside of the vesicle to determine movement or "leak" of protons from the inside of the vesicle to the outside of the vesicle.

Further according to the present invention, the methods for identifying compounds which modulate the activity of a mitichondrial anion carrier (MAC) may involve contacting an assay vesicle with an inhibitor of MAC activity. For example, in one embodiment, an assay of the present invention may involve contacting an assay vesicle which comprises at least one MAC and an indicator of MAC activity with an inhibitor of MAC activity, a test compound and an MAC substrate, wherein the MAC substrate is capable of being transported across the lipid bilayer of the assay vesicle; determining the ability of the test compound to modulate transport of the MAC substrate across the lipid bilayer of the assay vesicle, wherein determining the ability of the test compound to modulate transport of the MAC substrate across the lipid bilayer of the assay vesicle comprises determining a change in the indicator of MAC activity; and identifying the test compound as a modulator of MAC activity. As used herein, an "inhibitor of MAC activity" is a compound which, when added to a MAC-containing vesicle, reduces or inhibits the activity of the MAC such that the transport of a MAC substrate from one side of the MAC-containing vesicle to another side of the MAC-containing vesicle is measurably reduced or inhibited. For example, the rate of transport of a MAC substrate from one side of the MAC-containing vesicle to another side of the MAC-containing vesicle may be reduced by an inhibitor of MAC activity. In an alternative example, a higher concentration of MAC substrate may be necessary to effect the transport of a MAC substrate from one side of the MAC-containing vesicle to another side of the MAC-containing vesicle. An inhibitor of MAC activity is not restricted by a particular mechanism of inhibition. For example, the inhibitor may function as an allosteric inhibitor of MAC activity, as a competitive inhibitor of MAC activity (e.g., competing with a MAC substrate for binding to a MAC), or may function by inducing a conformational change on a MAC.

Accordingly, the inhibitor of MAC activity may be contacted with an assay vesicle of the present invention prior to contacting the assay vesicle with the MAC substrate, concurrent with contacting the assay vesicle with the MAC substrate, and/or

subsequent to contacting the assay vesicle with the MAC substrate. In another embodiment, the inhibitor of MAC activity can be trapped in an assay vesicle upon formation of the assay vesicle. Inhibitors of MAC activity are intended to include inhibitors that are known in the art as well as compounds whose inhibitory activity remains to be demonstrated. For example, the following inhibitors of MAC activity are known in the art: APC activity is inhibited by sulfhydryl reagents (e.g., mersalyl, *n*-ethylmaleimide (NEM)). AAC activity is inhibited by carboxyatractyloside, atractyloside, and bongkrekate. CIC activity is inhibited by 1,2,3-Benzenetricarboxylic acid (BTA) and is dicarboxylate transport by the CIC is weakly inhibited by *n*-butylmalonate and/or *n*-ethyl maleimide (NEM). DIC activity is inhibited by *n*-butylmalonate or phenylsuccinate (which have no effect on the H+/Pi cotransporter). DIC activity is completely inhibited by mercurial reagents, but is not inhibited by the sulfhydral alkylator NEM. Furthermore, long-chain acyl-CoA acts as an inhibitor of, for example, citrate transport, UCP-1, and ANT. Nucleotides also are known in the art to have inhibitory activity on MACs (e.g., purine nucleotides inhibit the activity of UCP).

I. General Overview of Assay

The methods of the invention generally involve contacting an assay vesicle (e.g., a reconstituted lipid bilayer vesicle having a mitochondrial anion carrier (MAC) as a component of the vesicle bilayer and containing an indicator of MAC activity) with a test compound, determining the ability of the test compound to modulate transport of a MAC substrate, and identifying the test compound as a modulator of MAC activity based on its ability to modulate the transport activity of the MAC.

Accordingly, in one embodiment, the invention provides a method for identifying a compound which modulates the activity of a mitochondrial anion carrier (MAC) comprising:

(a) contacting an assay vesicle which comprises at least one MAC and an indicator of MAC activity with a test compound and an MAC substrate, wherein the MAC substrate is capable of being transported across the lipid bilayer of the assay vesicle;

(b) determining the ability of the test compound to modulate transport of the MAC substrate across the lipid bilayer of the assay vesicle, wherein determining the ability of the test compound to modulate transport of the MAC substrate across the lipid bilayer of the assay vesicle comprises determining a change in the indicator of MAC activity; and

(c) identifying the test compound as a modulator of MAC activity.

In such an embodiment, the assay is based, at least in part, on the fact that a change in intravesicular pH or membrane potential is indicative of MAC activity and a modulation of MAC activity can effect, for example, the pH change (e.g., the magnitude of the pH change or the kinetics of the pH change) or change in membrane potential which results from contacting an assay vesicle with a MAC substrate.

The MAC can be selected, for example, from the group consisting of an ADP/ATP carrier (AAC), a phosphate carrier (PiC), a pyruvate carrier (PYC), an aspartate/glutamate carrier (AGC), an α-ketoglutarate carrier or oxoglutarate carrier (KGC/OGC), a dicarboxylate carrier (DIC), a citrate carrier (CIC), a glutamate carrier (GC), an ATPMg/P_i carrier (APC), and an uncoupling protein (UCP). In one embodiment, the MAC is a pyruvate carrier (PYC) and the MAC substrate is pyruvate. In another embodiment, the MAC is a phosphate carrier (PiC) and the MAC substrate is inorganic phosphate (Pi). In yet another embodiment, the MAC is a glutamate carrier (GC) and the MAC substrate is glutamate. In yet another embodiment, the MAC is a mitochondrial uncoupling protein (UCP) and the MAC substrate is a fatty acid anion (FAA^-). For example, the mitochondrial UCP can be selected from the group consisting of uncoupling protein 1 (UCP1), uncoupling protein 2 (UCP2) and uncoupling protein 3 (UCP3).

In another embodiment, the invention provides a method for identifying a compound which modulates the activity of a mitochondrial anion carrier (MAC) comprising:

(a) contacting an assay vesicle which comprises at least one MAC, a first MAC substrate, and an indicator of MAC activity with a test compound and a second MAC substrate, wherein the first and second MAC substrates are capable of being transported across the lipid bilayer of the assay vesicle;

(b) determining the ability of the test compound to modulate transport of the MAC substrates across the lipid bilayer of the assay vesicle, wherein determining the ability of the test compound to modulate transport of the MAC substrates across the lipid bilayer of the assay vesicle comprises determining a change in the indicator of MAC activity; and

(c) identifying the test compound as a modulator of MAC activity.

In one embodiment, the MAC is an ADP/ATP carrier (AAC), the MAC substrates are ADP and ATP. In another embodiment, the MAC is an aspartate/glutamate carrier (AGC), and the MAC substrates are aspartate and glutamate. In another embodiment, the MAC is an α-ketoglutarate carrier or oxoglutarate carrier (KGC/OGC) and the MAC substrates are α-ketoglutarate and malate. In another embodiment, the MAC is a dicarboxylate carrier (DIC) and the MAC substrates are inorganic phosphate (Pi) and a dicarboxylate (e.g. malate or succinate). In another embodiment, the MAC is a citrate carrier (CIC) and the MAC substrates are Malate and citrate. In another embodiment, the MAC is an ATPMg/P_i carrier (APC) and the MAC substrates are ATP-Mg^{++} and inorganic phosphate (Pi). In yet another embodiment, the MAC is an uncoupling protein (UCP) and the MAC substrate is a fatty acid anion (FAA^-).

In another embodiment, the invention provides a method for identifying a compound which modulates the activity of a mitichondrial anion carrier (MAC) comprising:

(a) contacting an assay vesicle which comprises a first MAC, a second MAC, a second MAC substrate, and an indicator of activity of the second MAC with a test compound and a first MAC substrate, wherein the MAC substrates are capable of being transported across the lipid bilayer of the assay vesicle;

(b) determining the ability of the test compound to modulate transport of the first MAC substrate across the lipid bilayer of the assay vesicle, wherein determining the ability of the test compound to modulate transport of the first MAC substrate across the lipid bilayer of the assay vesicle comprises determining a change in the indicator of activity of the second MAC; and

(c) identifying the test compound as a modulator of MAC activity.

Accordingly, in one embodiment, the first MAC is an aspartate/glutamate carrier, the second MAC is a glutamate carrier (GC), the first MAC substrate is aspartate and the second MAC substrate is glutamate. In another embodiment, the first MAC is an ATPMg/Pi carrier (APC), the second MAC is a phosphate carrier (PiC), the first MAC substrate is $ATPMg^{++}$, and the second MAC substrate is inorganic phosphate (Pi). In yet another embodiment, the first MAC is a dicarboxylate (e.g., malate or succinate), and the second MAC substrate is inorganic phosphate (Pi).

In another embodiment, the invention provides a method for identifying a compound which modulates the activity of a mitichondrial anion carrier (MAC) comprising:

(a) contacting an assay vesicle which comprises a first MAC, a second MAC, a third MAC, a second MAC substrate, a third MAC substrate, and an indicator of activity of the third MAC with a test compound and a first MAC substrate, wherein the MAC substrates are capable of being transported across the lipid bilayer of the assay vesicle;

(b) determining the ability of the test compound to modulate transport of the first MAC substrate across the lipid bilayer of the assay vesicle, wherein determining the ability of the test compound to modulate transport of the first MAC substrate across the lipid bilayer of the assay vesicle comprises determining a change in the indicator of activity of the third MAC; and

(c) identifying the test compound as a modulator of MAC activity.

Accordingly, in one embodiment, the first MAC is an α-ketoglutarate carrier or oxoglutarate carrier (KGC/OGC), the second MAC is a dicarboxylate carrier, the third MAC is a phosphate carrier (PiC), the first MAC substrate is α-ketoglutarate, the second MAC substrate is malate, and the third MAC substrate is inorganic phosphate (Pi). In another embodiment, the first MAC is a citrate transporter (CIC), the second MAC is a dicarboxylate transporter (DIC), the third MAC is a phosphate transporter, the first MAC substrate is citrate, the second MAC substrate is malate, and the third MAC substrate is inorganic phosphate (Pi).

In yet another embodiment, the present invention involves a method for identifying a compound which modulates the activity of a mitichondrial anion carrier (MAC) comprising:

(a) contacting an assay vesicle which comprises a first MAC, a second MAC, and an indicator of activity of the first and second MAC (e.g., a pH indicator or a potentiometric probe) with a test compound and a MAC substrate, wherein the MAC substrate is capable of being transported across the lipid bilayer of the assay vesicle by the first and the second MAC;

(b) determining the ability of the test compound to modulate transport of the MAC substrate across the lipid bilayer of the assay vesicle, wherein determining the ability of the test compound to modulate transport of the MAC substrate across the lipid bilayer of the assay vesicle comprises determining a change in the indicator of MAC activity; and

(c) identifying the test compound as a modulator of MAC activity.

For example, an assay vesicle including UCP-1 and UCP-2, UCP-2 and UCP-3, or UCP-1 and UCP-3 can be utilized according to the present embodiment. Furthermore, an assay vesicle can comprise more than two MACs (e.g., UCP-1, UCP-2, and UCP-3).

According to the methods of the present invention described herein, identifying the test compound as a modulator of MAC activity can further include the step of comparing the effect of the test compound as a modulator of the activity of a first MAC in a first assay vesicle to the effect of the test compound as a modulator of at least the activity of a second MAC in a second assay vesicle. For example, identifying the test compound as a modulator of UCP-1 activity can include comparing the effect of the compound as a modulator of UCP-1 activity in a first assay vesicle to the activity of the compound as a modulator of, for example, UCP-2 or UCP-3 activity, in a second assay vesicle. A compound which has activity in the first UCP-1 assay vesicle but shows no activity in the second UCP-2 or UCP-3 containing assay vesicle can be said to act "specifically" on UCP-1. Such a comparison facilitates the identification of specific MAC modulators from among, for example, tens of thousands of compounds being assayed for modulatory activity.

II. Reconstituted Lipid Vesicles

The methods of the invention are carried out using a lipid vesicle having as a component of its lipid bilayer at least one MAC. The vesicles of the present invention can be generated utilizing a variety of methods known in the art. For example, the assay vesicles can be prepared by sonication (see, for example, Hamilton and Small (1981) *Proc. Nat'l Acad. Sci. USA* 78:878-6882), by extrusion through membranes (see, for example, Kamp *et al.* (1995) *Biocehmistry* 34:11928-11957), by detergent dialysis (see, for example, Garlid *et al.* (1995) *Methods Enzymol.* 260:331-348) as well as according the methods described herein (Example 1). According to the present invention, the reconstituted lipid vesicles can have a weight ratio of protein/phospholipid of, for example, 0.01-0.40, preferably 0.02-0.30, more preferably 0.05-0.25, and more preferably 0.10-0.20; a molar ratio of MAC to phospholipid of, for example, 0.01-1.00, preferably 0.02-0.50, more preferably 0.05-0.25, more preferably 0.10-0.15, and more preferably 0.12 μmol MAC/mol phospholipid; an internal volume of, for example, 0.4-10, preferably 0.8-8, more preferably 1.0-5.0, and more preferably 1.5-2 μl/mg phospholipid; a vesicle diameter of, for example, 250-2000Å, preferably 300-1750Å, more preferably 350-1500Å, more preferably 400-1250Å, more preferably 500-1000Å and even more preferably 600-850Å; 3,700-6.6x10^5, preferably 4,000-6.0x10^5, 6000-1x10^5, 8000-0.5x10^5, 1x10^4-0.25x10^5, 2x10^4-5x10^4, 3x10^4-4x10^4 phospholipid molecules per vesicles; and 1-10, preferably 2-8, and more preferably 3-6 MAC molecules per vesicle. In another embodiment, a reconstituted lipid vesicle can have 0-2000 fatty acid molecules per vesicle (e.g., when the assay vesicle comprises UCP as at least one MAC of the vesicle).

The methods of the invention can further be carried out using essentially any MAC as a component of the reconstituted lipid vesicle bilayer. Preferred MACs are MACs which produce a change in intravesicular pH as a "read-out" of activity (e.g., PiC, GC, PYC, and/or UCP) due to the fact that change in pH as an indicator of MAC activity is a more rapid response when compared to change in membrane potential as an indicator of MAC activity.

Other examples of suitable MACs include, but are not limited to, AAC, AGC, OCG, DIC, CIC, and APC. In one embodiment, modulation of the activity of a MAC can be determined directly using a membrane potential indicator as an indicator of MAC activity. In an alternative embodiment, the activity of a MAC can be indirectly determined in an assay vesicle comprising a second MAC which produces a change in intravesicular pH as an indicator of MAC activity, wherein the first and second MAC operatively cotransport at least one MAC substrate. For example, in one embodiment, the first MAC is an aspartate/glutamate carrier, the second MAC is a glutamate carrier (GC), the first MAC substrate is aspartate and the second MAC substrate is glutamate. In another embodiment, the first MAC is an ATPMg/Pi carrier (APC), the second MAC is a phosphate carrier (PiC), the first MAC substrate is $ATPMg^{++}$, and the second MAC substrate is inorganic phosphate (Pi). In yet another embodiment, the first MAC is a dicarboxylate (e.g., malate or succinate), and the second MAC substrate is inorganic phosphate (Pi).

In yet another embodiment, the activity of such a MAC can be indirectly determined in an assay vesicle comprising a second MAC which produces a change in intravesicular pH as an indicator of MAC activity, wherein the first and second MAC operatively cotransport at least one MAC substrate. Table 1 details the activities of the various MAC proteins of the present invention, as the proteins are understood to function in the intact mitochondria, as well as indicating pairs of MACs which potentially cotransport at least one MAC substrate. For example, in one embodiment, the first MAC is an α-ketoglutarate carrier or oxoglutarate carrier (KGC/OGC), the second MAC is a dicarboxylate carrier, the third MAC is a phosphate carrier (PiC), the first MAC substrate is α-ketoglutarate, the second MAC substrate is malate, and the third MAC substrate is inorganic phosphate (Pi). In another embodiment, the first MAC is a citrate transporter (CIC), the second MAC is a dicarboxylate transporter (DIC), the third MAC is a phosphate transporter, the first MAC substrate is citrate, the second MAC substrate is malate, and the third MAC substrate is inorganic phosphate (Pi).

TABLE 1

MAC	transports in / out		Δ pH	Δ Ψ_m	cotrans-port with 2nd MAC	Unidirectional transport or Exchange	Specific Inhibitor
ADP/ATP carrier (AAC) or adenine nucleotide translocator (ANT)	ADP^{3-}	ATP^{4-}	no	yes ↓		Exchange	carboxy-attractyl-oside LC-CoA
phosphate carrier (PiC)	Pi^-	OH^-	yes	no		either Pi and H^+ cotransport or Pi/OH^- exchange	n-ethyl-maleimide (NEM)
pyruvate carrier (PYC)	$pyruvate^-$	OH^-	yes	no		either $pyruvate^-$ and H^+ cotransport or $pyruvate^-/OH^-$ exchange	α-cyano-4-hydroxy-cinnamate
aspartate/gluta-mate carrier (AGC)	$aspartate^-$	gluta-mate	no	yes ↓	GC		
oxoglutarate carrier (OGC)*	α-keto-$glutarate^-$	$malate^-$	no	no	DIC, PiC		
dicarboxylate carrier (DIC)	$malate^-$ or succinate	Pi^-	no	no	PiC		n-butyl-malonate phenyl-succinate
citrate carrier (CIC)	citrate	$malate^-$	no	no	Pic, DIC		1, 2, 3-benzene-tricarboxylate LC-CoA
glutamate carrier (GC)	glutamate	OH^-	yes	no		same as PiC, PyC	
ATPMg/P_i carrier (APC)	ATP Mg	Pi	no	no	PiC		sulfhydral reagents (e.g. NEM, mersalyl)
uncoupling proteins (UCPs)	unknown, if any	fatty acid anions	yes			coupled to simple diffusion of FFA and ionization	nucleotides LC-CoA

*α-keto-glutarate and 2-oxoglutarate are used interchangeably, herein

MACs of the present invention can be obtained, at least for example, as purified protein preparations from appropriate biological sources or as products of recombinant protein expression technologies. A "purified" or "isolated" protein is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the MAC is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of MAC protein in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations of MAC protein having less than about 30% (by dry weight) of non-MAC protein (also referred to herein as a "contaminating protein"), more preferably less than about 20% of non-MAC protein, still more preferably less than about 10% of non-MAC protein, and most preferably less than about 5% non-MAC protein. When the MAC protein is recombinantly produced, it is also preferably substantially free of culture medium, i.e., culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the protein preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations of MAC protein in which the protein is separated from chemical precursors or other chemicals which are involved in the synthesis of the protein. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations of MAC protein having less than about 30% (by dry weight) of chemical precursors or non-MAC chemicals, more preferably less than about 20% chemical precursors or non-MAC chemicals, still more preferably less than about 10% chemical precursors or non-MAC chemicals, and most preferably less than about 5% chemical precursors or non-MAC chemicals.

A general MAC purification scheme involves solubilization of MACs with a non-ionic detergent, chromatography on hydroxylapatite, followed in some cases, by SH-affinity chromatography, See, for example, Wohlrab, H. (1986) *Biochim. Biophys. Acta* 853:8170-8173; Wehrle, J. P. and Pedersen, P. L. (1989) *J. Membr. Biol.* 111:199-213; Kramer, R. and Palmeri, F. (1989) *Biochim. Biophys. Acta* 974:1-23; Kolbe, H. V.

J. *et al.* (1984) *J. Biol. Chem.* 259:9115-9120; Kaplan, R. S. *et al.* (1986) *J. Biol. Chem.* 261:12767-12773; and Guerin, B. *et al.* (1990) *J. Biol. Chem.* 265: 19736-19741. An alternative MAC purification scheme involves solubilization of MACs with a non-ionic detergent, chromatography on hydroxylapatite, followed by sequential chromatography on Matrex Gel Orange A™, Matrix Gel Blue B™, and Affi-Gel 501™. See Kaplan, R. S. *et al.* (1990) *J. Biol. Chem.* 265:13379-13385.

Examples of such non-ionic detergent solubilizing agents include n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether)$_n$, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

As mentioned previously, MAC proteins of the present invention can also be produced using recombinant protein expression technologies. For example, the MAC proteins of the present invention can be produce in organisms including bacteria (e.g., *Escherichia coli*) and yeast (e.g., *Saccharomyces cerevisiae* or *Candida parapsilosis*). Production of MAC proteins in *E. coli* can been accomplished with slight modification of standard recombinant expression procedures e.g., those used to recombinantly produce globular proteins) taking into account the fact that MACs are integral membrane proteins. For instance, it has been demonstrated that abundant expression of the MACs, KGC/OGC and PiC, can be obtained in *E. coli*. The anionic detergent, sarkosyl, can be further utilized to enable the extraction of the expressed transporter from inclusion bodies in a form that can be functionally reconstituted into lipid vesicles. See, for example, Fiermonte, G. *et al.* (1993) *Biochem. J.* 294:293-299 and Wohlrab, H. and Briggs, C. (1994) *Biochemistry* 33:9371-9375. Furthermore, one of skill in the art can apply the procedures of Kaplan, R. S. (1996) *J. Bioenerg. Biomembr.* 28:41-47, to express other members of the MAC protein family. For example, the gene encoding DIC from the yeast Saccharomyces cerevisiae was recently cloned and the protein recombinantly expressed in *E. coli*. Kakhniashvili, *et al.* (1997) J. Biol. Chem. 272(7):4516-4521.

In a preferred embodiment, the MAC of the present invention is selected from the group consisting of UCP-1, UCP-2, and UCP-3. Preferably, the MAC of the present invention is selected from the group consisting of human UCP-1 (SEQ ID NO:2), UCP-2 (SEQ ID NO:4), and UCP-3 (SEQ ID NO:6). In general, recombinant production of MACs can be accomplished by inserting the coding sequence of the gene encoding a MAC into a recombinant expression vector in the context of the appropriate regulatory elements, such that expression of the recombinant MAC protein is achieved. Such a procedure is described in detail, for example, in Example 2.

Alternatively, the MAC proteins of the present invention can be recombinantly expressed at high levels and purified from mammalian cells. Preferred mammalian cells for recombinant expression are those that can be efficiently transfected and express high levels of recombinant protein. Such cells include, but are not limited to, baby hamster kidney (BHK) cells (ATCC No. CCL 10); Ltk$^-$ cells (ATCC No. CCL 1.3); DG44 cells (see e.g., Chasin (1986) *Cell. Molec. Genet.* 12:555); human embryonic kidney (HEK) cells (ATCC No. CRL 1573); Chinese hamster ovary (CHO) cells (ATCC Nos. CRL 9618, CCL 61, CRL 9096); PC12 cells (ATCC No. CRL 1721); CV-1 cells (ATCC No. CCL 70); and the COS-7 monkey kidney cell line (see e.g., Gluzman (1981) *Cell* 23:175) (ATCC No. CRL 1651). Purification of MAC proteins from mammalian cells can be accomplished according to the procedures described herein for the isolation of MACs from appropriate biological sources.

As exemplary embodiments, the MACs of the present invention can have the amino acid sequences of any of the human UCPs, (e.g., human UCP-1, human UCP-2, or human UCP-3); the human PiCs, (e.g., PiC A, PiC B, or PiC C); the human glutamate carrier; or the human pyruvate carrier. The nucleic acid sequence that encodes human UCP-1 can be found, at least for example, as GenBank Accession Number U28480. The nucleic acid sequence that encodes human UCP-2 can be found, at least for example, as GenBank Accession Number U82819 or as GenBank Accession Number U76367. The nucleic acid sequence that encodes human UCP-3 can be found, at least for example, as GenBank Accession Number AF001787 or as GenBank Accession Number U84763. The nucleic acid sequence that encodes the human PiCs, (e.g., PiC A, PiC B, and PiC C;

three alternatively spliced forms of PiC) can be found, at least for example, as GenBank Accession Number X77337.

In an alternative exemplary embodiment, the MACs of the present invention can have the amino acid sequences of any of AAC, AGC, OCG, DIC, CIC, and APC. The nucleic acid sequences that encode the electroneutral carrier proteins can be found, at least for example, as GenBank Accession Numbers gi: 339919 (ANT1), gi: 178660 (ANT2), gi: 339722 (ANT3), gi: 23843 (OGC), gi: 950003 (CIC), for example.

Alternatively, it is within the scope of the invention that the exemplary MACs of described herein can be encoded by nucleic acid sequences other than those specified herein, due to the degeneracy of the genetic code. Furthermore, it is within the scope of the present invention that the MACs of the present invention can have an amino acid sequence having sufficient homology to one of the amino acid sequences described herein. As used herein, the term "sufficiently homologous" refers to a first amino acid sequence which contains a sufficient or minimum number of identical or equivalent (e.g., an amino acid residue which has a similar side chain) amino acid residues to a second amino acid sequence such that the first and second amino acid sequences share a common functional activity. For example, amino acid sequences that share at least 40%, preferably 50%, more preferably 60, 70, or 80% homology and share a common functional activity are defined herein as sufficiently homologous.

To determine the percent homology of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes (e.g., gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence and non-homologous sequences can be disregarded for comparison purposes). In a preferred embodiment, the length of a reference sequence aligned for comparison purposes is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence (e.g., when aligning a second sequence to the UCP-1 amino acid sequence of SEQ ID NO:2 having 307 amino acid residues, at least 92, preferably at least 123, more preferably at least 154, even more preferably at least 176, and even more preferably at least 215, 246 or 276 amino acid residues are aligned). The amino acid residues or nucleotides at

corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are homologous at that position (i.e., as used herein amino acid or nucleic acid "homology" is equivalent to amino acid or nucleic acid "identity"). The percent homology between the two sequences is a function of the number of identical positions shared by the sequences (i.e., % homology = # of identical positions/total # of positions x 100).

The comparison of sequences and determination of percent homology between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA 87:2264-68, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-77. Such an algorithm is incorporated into the NBLAST and XBLAST programs (version 2.0) of Altschul, et al. (1990) J. Mol. Biol. 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to STMST nucleic acid molecules of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to STMST protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Research 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS (1989). Such an algorithm is incorporated into the ALIGN program (version 2.0) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

For example, MACs having amino acid substitutions at "non-essential" amino acid residues can be used according to the present invention. A "non-essential" amino acid residue is a residue that can be altered from the wild-type sequence of the exemplary MACs described herein without altering the functional activity, whereas an "essential" amino acid residue is required for biological activity. For example, amino acid residues that are conserved among the MAC proteins of the present invention, are predicted to be particularly unamenable to alteration. Such conserved amino acids can be determined, at least for example, from an alignment of the amino acid sequences of the exemplary MAC proteins described herein.

A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

As used interchangeably herein, an "activity" or "functional activity", refers to an activity exerted by a protein of the present invention, (e.g., a MAC protein), as determined *in vivo* or *in vitro*, according to standard techniques. For example, a functional activity of a MAC protein can be the transport of a MAC substrate across an inner mitochondrial membrane or, alternatively, across a reconstituted lipid bilayer vesicle.

III. Indicators of MAC Activity

According to the present invention, the activity of a MAC can be determined by measuring at least one component of MAC transporter activity (e.g., a change in membrane potential or change in intravesicular pH). It is intended that, for example, chromogenic and/or fluorescent indicators, can be utilized in accordance with the present invention. As used herein, the term "chromogenic indicator" refers to a compound

which produces a colorometric readout as an indicator of, for example, pH or membrane potential. A change in the colorometric readout of such a chromogenic indicator is indicative of a change in, for example, pH or membrane potential. In a preferred embodiment, the change in the colorometric readout of such a chromogenic indicator is a quantitative measurement of the change in, for example, pH or membrane potential. Examples of chromogenic indicators which change their color in response to a change in pH include, but are not limited to, litmus, phenolphthalein, and phenol red. In another embodiment, the Mg^{2+} dye, Magfure, is used to measure citrate entry via CIC due to the ability of citrate to chelate Mg^{2+}.

Also, according to the present invention, a fluorescent indicator can be utilized in accordance with the present invention. As used herein, the term "fluorescent indicator" refers to a compound which produces a fluorescent readout as an indicator of, for example, pH or membrane potential. A change in the fluorescent readout of such an indicator is indicative of a change in, for example, pH or membrane potential. In a preferred embodiment, the change in the fluorescent readout of such an indicator is a quantitative measurement of the change in, for example, pH or membrane potential. Examples of fluorescent indicators which produce a change in fluorescence in response to a change in pH include, but are not limited to, fluorescein and fluorescein derivatives (e.g., fluorescein diacetate (FDA), carboxyfluorescein diacetate (CFDA), the polar fluorescein derivative, BCECF, and the AM ester of BCECF, methylated fluorescein and/or fluorescein diacetate, and fluorescein sulfonic acid and/or fluorescein sulfonic acid diacetate); the seminaphthorhodafluors (SNARF dyes) and seminapthofluoresceins (SNAFL dyes); and/or pyranin. Such fluorescent indicators can be further congugated to inert polysaccharides (e.g., dextrans) to prevent leakage from the assay vesicles of the present invention.

IV. Fatty Acids

According to the present invention, it may be desirable to incorporate free fatty acids as a component of the assay vesicles. For example, it may be desirable to incorporate free fatty acids (FFAs) into an assay vesicle of the present invention when assaying for modulators of the MAC, UCP. In one embodiment, fatty acid molecules

can be incorporated into the assay vesicles of the present invention during the process of forming the assay vesicles. In another embodiment, the assay vesicles of the present invention can be contacted with fatty acid molecules subsequent to the formation of the assay vesicles and prior to contacting the assay vesicles with a test compound and/or a prior to contacting the vesicle with a test compound.

V. Test Compounds

The test compounds of the present invention can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

Examples of methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt et al. (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb et al. (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann et al. (1994). *J. Med. Chem.* 37:2678; Cho et al. (1993) *Science* 261:1303; Carrell et al. (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell et al. (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop et al. (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (e.g., Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull et al. (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla et al. (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

Exemplary compounds which can be screened for activity include, but are not limited to, peptides, nucleic acids, carbohydrates, small organic molecules, and peptidomimetics. The term "peptidomimetic" is intended to encompass compounds that

are comprised, at least in part, of molecular structures different from naturally-occurring L-amino acid residues linked by natural peptide bonds. "Peptidomimetics" are intended to include compounds composed, in whole or in part, of structures such as D-amino acids, non-naturally-occurring L-amino acids, modified peptide backbones and the like, as well as compounds that are composed, in whole or in part, of molecular structures unrelated to naturally-occurring L-amino acid residues linked by natural peptide bonds.

VI. Preparation and Administration of Pharmaceutical Compositions of Compounds Which Modulate Mitochondrial Transport Activity

A. Pharmaceutical Preparations of Identified Compounds

After identifying certain test compounds as potential modulators of mitochondrial transport activity, the practitioner of the subject assay will continue to test the efficacy and specificity of the selected compounds both *in vitro* and *in vivo* as described further herein. Whether for subsequent *in vivo* testing, or for administration to an animal as an approved drug, compounds identified in the subject assay, or a pharmaceutically acceptable salt thereof, can be formulated in pharmaceutical preparations for *in vivo* administration to an animal, preferably a human. Such preparations comprise a therapeutically (or prophylactically) effective amount of the mitochondrial transport modulator, and a pharmaceutically acceptable carrier or excipient. The preparations may be formulated for administration with a pharmaceutically acceptable carrier except insofar as any conventional media or agent is incompatible with the activity of the compound. As used herein, "pharmaceutically acceptable carrier" includes any and all solvents or wetting agents, emulsifying or dispersion media, and the like which may be appropriate for the desired route of administration of the pharmaceutical preparation. The composition can be a liquid solution, suspension, emulsion, tablet, pill, capsule, sustained release formulation, or powder. The composition can be formulated as a suppository, with traditional binders and carriers such as triglycerides. Oral formulation can include standard carriers such as pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, *etc.* Other carriers include water, saline,

saline buffered with phosphate or citrate at suitable concentrations so as to obtain at all times isotonic pharmaceutical preparations of the desired pH, (for example, neutral pH), dextrose, ethanol, mannitol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol and the like) or suitable mixtures thereof. The use of other such media for pharmaceutically active substances is known in the art. The optimum concentration of the active ingredient(s) in the chosen medium can be determined empirically, according to procedures well known to medicinal chemists. The carrier and composition can be sterile. The formulation should suit the mode of administration.

B. Effective Doses of Identified Compounds for Administration

Single and multiple (e.g., 5 to 7 days) dose investigative toxicology studies are typically performed in the efficacy test species using the intended route of administration for the efficacy study. These investigative toxicology studies are performed to identify maximum tolerated dose, subjective bioavailability from the intraperitoneal or oral routes of administration , and estimation of an initial safety margin. Initial bioavailability and pharmacokinetics (blood clearance) of the compounds may be determined, with standard cold or radioactive assay methods, to assist in defining appropriate dosing regimens for the compounds in the animal models.

The effective dose of the mitochondrial transport modulator will typically be in the range of about 0.01 to about 50 mg/kgs, preferably about 0.1 to about 10 m g/kg of mammalian body weight, administered in single or multiple doses. Generally, the mitochondrial transport modulator may be administered to patients in need of such treatment in a daily dose range of about 0.5 to about 2000 mg per patient.

The amount of the mitochondrial transport modulator which will be effective in the treatment or prevention of a particular disorder or condition will depend on the nature of the disorder or condition, and can be determined by standard clinical techniques. In addition, *in vitro* or *in vivo* assays may optionally be employed to help identify optimal dosage ranges. Effective doses may be extrapolated from dose-response curves derived from *in vitro* or animal model test systems. The precise dosage level of the mitochondrial transport modulator, as the active component(s), should be determined by the attending physician or other health care provider and will depend upon well

known factors, including route of administration, and the age, body weight, sex and general health of the individual; the nature, severity and clinical stage of the disease; and the use (or not) of concomitant therapies.

C. Administration of Identified Compounds

Various delivery vehicles are known and can be used to administer the mitochondrial transport modulator, *e.g.*, encapsulation in liposomes, microparticles, injectable "deposit formulations", microcapsules, *etc.* Materials and methods for producing the various formulations are well known in the art [see *e.g.* US Patent Nos. 5,182,293 and 4,837,311 (tablets, capsules and other oral formulations as well as intravenous formulations)]. Suitable vehicles and their formulation inclusive of other proteins are described, for example, in the book *Remington's Pharmaceutical Sciences* (Remington's Pharmaceutical Sciences. Mack Publishing Company, Easton, Pa., USA 1985). Administration can be systemic or local. Methods of introduction include but are not limited to pulmonary administration, intradermal, intramuscular, intraperitoneal, intravenous, subcutaneous, intranasal, epidural and oral routes, for example by infusion or bolus injection, by absorption through epithelial or mucocutaneous linings (*e.g.*, oral mucosa, rectal and intestinal mucosa, *etc.*) and may be administered together with other biologically active agents. Typically, compositions for intravenous administration are solutions in sterile isotonic aqueous buffer. Where necessary, the composition may also include a solubilizing agent and a local anesthetic to ease pain at the side of the injection. Generally, the ingredients are supplied either separately or mixed together in unit dosage form, for example, as a lyophilized powder or water free concentrate in a hermetically sealed container such as an ampoule or sachette indicating the quantity of active agent. Where the composition is to be administered by infusion, it can be dispensed with an infusion bottle containing sterile pharmaceutical grade water or saline. Where the composition is administered by injection, an ampoule of sterile water for injection or saline can be provided so that the ingredients may be mixed prior to administration. Topical administration to an individual of an effective amount of the biased agonist can be accomplished by administering the compound(s) directly to the affected area of the skin of the individual. For this purpose, the mitochondrial transport

modulator is administered or applied in a composition including a pharmacologically acceptable topical carrier, such as a gel, an ointment, a lotion, or a cream, which includes, without limitation, such carriers as water, glycerol, alcohol, propylene glycol, fatty alcohols, triglycerides, fatty acid esters, or mineral oils.

Other topical carriers include liquid petroleum, isopropyl palmitate, polyethylene glycol, ethanol (95%), polyoxyethylene monolaurate (5%) in water, or sodium lauryl sulfate (5%) in water. Other materials such as anti-oxidants, humectants, viscosity stabilizers, and similar agents may be added as necessary.

In cases where it is desirable to administer the mitochondrial transport modulator locally to the area in need of treatment this may be achieved by, for example, and not by way of limitation, local infusion during surgery, topical application, by injection, by means of a catheter, by means of a suppository, or by means of a skin patch or implant, said implant being of a porous, non-porous, or gelatinous material, including membranes, such as sialastic membranes, or fibers which release the compound into the skin, by either passive or active release mechanisms.

Exemplification

The invention now being generally described will be more readily understood by reference to the following examples, which are included merely for purposes of illustration of certain aspects and embodiments of the present invention and are not intended to limit the invention.

EXAMPLE 1:

Preparation of lipid assay vesicles loaded with fluorescent pH indicator dye.

A key approach to study the function and regulation of UCPs is to trap a pH-sensitive fluorescent dye inside vesicles including a MAC as a component of the lipid bilayer to monitor the movement of protons across the bilayer. Vesicles can be prepared by a variety of methods. For example, vesicles can be prepared according to the following techniques:

RAPID METHOD

This method involves preparing lipid bilayer vesicles comprising FAA and lysophospholipids. The vesicle preparations can be analyzed by NMR and electron microscopy. Small unilamellar vesicles can be formed spontaneously from equimolar mixtures of lyso 1-acyl phosphatidylcholine (lyso-PC) and oleic acid. A dried lipid film is hydrated in 50 mM Tris buffer, pH 7, 20 °C and vortexed vigorously. The resulting lipid dispersion appears translucent with a slight bluish tinge typical of small unilamellar vesicles. The ^{31}P NMR spectra of this dispersion is also characteristic of such vesicles, showing partially resolved signals for outer and inner monolayer lyso-PC.

METHOD OF GARLID ET AL.

Egg phosphatidylcholine, cardiolipin (optional), and phosphatidic acid are mixed at a weight ratio of 1:2.65:60 with octyl-POE and aqueous buffer. Octyl-POE is used during reconstitution of these vesicles to dissolve the lipids and to achieve the optimal detergent:Bio-Beads ratio. Cardiolipin, a natural component of inner membrane, causes a reproducibly larger liposomal volume. The lipids are dissolved by stirring and heating in a 50° water bath and the resulting clear solution cooled. Liposomes are formed by detergent removal using Bio-Beads SM-2, as follows. The lipid-detergent-buffer mixture is incubated for 90 min at 4° with Bio-Beads SM-2 that have been preequilibrated with internal medium. The mixture is loaded onto a column or centrifuged to collect eluate. This can be followed by a second 30-min incubation with fresh Bio-Beads to remove residual detergent from the formed liposomes. Successful formation of liposomes is readily apparent from the milky, opalescent appearance of the eluate. (Garlid *et al.* (1995) *Methods Enzymol.* 260:331-348).

Note: The composition of the bilayer membrane can be varied from phosphatidylcholine alone to mixtures of phospholipids resembling those in the inner mitochondrial membrane.

SONICATION

Egg phosphatidylcholine is dissolved in 2:1 (vol/vol) $CHCl_3/CH_3OH$, and triolein is dissolved in $CHCl_3$. All triolein used in the vesicle preparations is 90%

isotopically substituted [1-^{13}C]triolein. The desired amounts of lipids are transferred into a 50-ml round-bottom flask, redispersed with 20 ml of $CHCl_3$, and dried as a thin film for 12 hr under reduced pressure. Then 1.6 ml of buffer (0.05 M KBr/0.01 M potassium phosphate/0.1 mM EDTA/0.1% sodium azide, pH 7.4), or 0.5-2.0% (wt/vol) aqueous KCl is added to the flask. In the latter case, distilled, deionized water is boiled to bring the pH to neutrality, and all subsequent steps are done under N_2 to keep the pH above 6.5. The sample is agitated for 1 hr at room temperature on a Vortex mixer and then transferred to a centrifuge tube, using 0.1 ml of buffer and 0.1 ml of ^2H_2O to rinse the flask. The sample compositions are given as % triolein by weight of tltal lipid (i.e., 5% triolein, 95% PtdCho), with the PtdCho concentration ranging between 10 and 100 mg/ml. The sample is sonicated by using a Branson sonifier with a micro-tip, at power level 3 in a pulsing mode with a 30% duty cycle. The temperature, monitored by a thin thermocouple inserted into the sample, is $\leq$35°C. Samples are centrifuged for 30 min at low speed to remove titanium fragments.

Selected samples are fractionated by ultracentrifugationn for 10 hr at 140,000 g at 15°C in 0.53% KCL (p = 1.004 g/ml).

After NMR analysis, samples are analyzed for composition and purity. No (<1%) unesterified fatty acid or lysolecithin is detected by thin-layer chromatography. The phosphatidylcholine concentration of the samples in aqueous KCl is determined by a modified Bartlett method.

INCORPORATION OF INDICATOR DYES INTO ASSAY VESICLES

Furthermore, the methods can be further modified to facilitate the formation of vesicles including an internal pH indicator or potentiometric indicator (e.g., a fluorescent dye).

Vesicles (e.g., small unilamellar vesicles, SUVs) are prepared by sonication, except that 0.5 mM pyranin, for example, is present in 100 mM Hepes/KOH buffer at pH 7.4. [Alternatively, large unilamellar vesicles (LUVs) are prepared by extruding a hydrated egg phosphatidylcholine (Avanti Polar Lipids) suspension 19 times through an extrusion apparatus.] Untrapped pyranin is removed by placing 1.0 ml of the vesicle suspension on a gel filtration column (20 ml of Sephadex G-25, medium grade),

washing with 100 mM Hepes/KOH (pH 7.4), and using a UV lamp to monitor the separation. Accordingly, the fluorophore is removed from the external membrane by passing the vesicles over a column, and the dye remains trapped inside the vesicle, where it reports internal pH.

Alternatively, according to the method of Garlid et al., for example, the fluorescent probe can be added after the lipid solution has been cooled. To remove extravesicular probe, 250-µl aliquots of the proteoliposome suspension are passed twice through 5-ml Sephadex G-25-300 columns, which have been preequilibrated with internal medium lacking probe. Note: This resin contains high amounts of K^+, which is removed with extensive washing in storage buffer (normally identical to internal medium lacking fluorescent probe).

The methods described herein, can also be applied to the inclusion of any of the dyes, for example, described in Table 2.

TABLE 2:

pH Indicators

Parent Flurophore	pH Range	Typical Measurement
SNAFL indicators	7.2-8.2	Excitation Ratio 490/540 nm or emission ratio 540/630 nm
SNARF indicators	7.0-8.0	Emmission ratio 580/640 nm
HPTS (pyranine)	7.0-8.0	Excitation ratio 450/405 nm
BCECF	6.5-7.5	Excitation ratio 490/450 nm
Fluoresceins and carboxyfluorosceins	6.0-7.2	Excitation ratio 490/450 nm

TABLE 3:

Potentiometric Indicators

Dyes	Structure (Charge)	Optical Response
ANEP dyes (aminonaphthyl-ethenylpyridinium	Styryl (cationic or zwitterionic)	FAST; fluorescence excitation ratio 440/505 nm decreases upon membrane depolarization
RH dyes (Rina Hildesheim)	Styryl (cationic or zwitterionic)	FAST; generally similar to ANEP dyes with excitation red shift upon membrane hyperpolarization
Impermeant Oxonols (oxonol dyes with phenylsulfonate substituents	Hybrid oxonol (anionic)	FAST; absorbance changes upon membrane hyperpolarization
Carbocyanines (Indo- DiI), thia (DiS) and oxa- (DiO) carbocyanines with short (n=1-7) alkyl tails	Carbocyanine (cationic)	SLOW; fluorescence generlly decreases upon membrane hyperpolarization
Rhodamine and the methyl and ethyl esters of tetramethylrfodamine	Rhodamine (cationic)	SLOW; used to obtain unbiased images of potential-dependent dye distribution
Oxonols	Oxonol (anionic)	SLOW; flourescence decreases upon membrane hyperpolarization
DiBAC Dyes (bis-barbituric acid oxonols)	Oxonol (anionic)	SLOW; flourescence decreases upon membrane hyperpolarization

ASSAY VESICLE CHARACTERIZATION

Ideal lipid vesicles have a low permeability to ions and the pH gradient returns to equilibrium very slowly. Incorporation of a fluorescent dye into an assay vesicle, can be used as an indicator of the success of generating a "non-leaky" vesicle preparation. For example, the ability of these vesicles to retain trapped contents can be established using pyranine, a membrane impermeant fluorescent dye. In particular, when FAA are added

to non-leaky vesicles, the pH drop is sustained over long time periods (minutes). It returns slowly to equilibrium as K^+ from the external buffer exchanges with ^+H inside the vesicle. This process is greatly accelerated by addition of valinonycin to vesicles, which results in inward transport of K^+ (Kamp & Hamilton (1995) *Biochemistry* 34:11928-11937). Inclusion of 20 mol% of cholesterol has no effect on the spontaneous formation of vesicles.

Success in preparing non-leaky vesicles with trapped fluorescent dye can be further ascertained using exogenous free fatty acid (FFA) which flip-flops across lipid membranes and acidifies the interior volume (decreases fluorescence of the pH indicator dye). A recently published manuscript documents the mechanisms of fatty acid movement across the membrane of intact cells and the ability to monitor this movement in adipocytes using a combination of two different dyes. Civelek, *et al.* (1996) *Proc. Natl. Acad. Sci., USA* 93:10139-10144. The mechanism by which fatty acids move into and out of adipocytes has not been resolved. We found that changes in intracellular pH (pH_i) in adipocytes correlate with the movement of FAA across cellular membranes as predicted by the Kamp and Hamilton model of passive diffusion of FAA . Exposure of adipocytes to the lipolytic agents, norepinephrine and isoproterenol, or external FAA results in rapid intracellular acidification that is reversed by FAA metabolism or its removal by albumin. In contrast, insulin causes a concentration dependent alkalinization of the cell, consistent with its main function to promote esterification. Inhibition of Na^+-H^+ exchange in adipocytes does not prevent the changes in pH_i caused by FAA, lipolytic agents or insulin. A fatty acid dimer, which diffuses into the cell but is not metabolized, causes irreversible acidification while a non-metabolized amine causes irreversible alkalinization. Taken together, the data suggest that changes in pH_i occur in adipocytes in response to the passive diffusion of un-ionized FAA (flip-flop) into and out of the cell and in response to their metabolism and production within the cell. In addition, the use of intracellular pH_i and extracellular FAA probes provide real-time monitoring of FAA movement.

EXAMPLE 2:

Preparation of MACs, in particular, UCP-1, for incorporation into lipid assay vesicles.

This example describes the preparation and purification of the MAC, UCP, for use in the preparation of assay vesicles. In particular, exemplary methods are provided for bacterial expression of UCP-1, overexpression of UCP-1 in yeast, and purification of UCP from hamster mitochondria or, alternatively, from yeast overexpressing UCP-1.

RECOMBINANT PRODUCTION AND PURIFICATION OF UCP-1 FROM BACTERIA

Construction of the Expression Plasmid. The open reading frame (ORF) encoding the MAC (e.g., UCP-1 (SEQ ID NO:1), UCP-2 (SEQ ID NO:3), or UCP-3 (SEQ ID NO:5)) plus additional 3' untranslated sequence is amplified by PCR. In these amplification reactions, a forward primer is utilized which contains an restriction endonuclease site plus additional 5' sequence (approximately 10-20 nucleotides) in order to allow efficient restriction digestion. If necessary, modifications are introduced into the forward primer in order to eliminate existing rare codons at the beginning of the ORF which might minimize the level of express in *E. coli*. However, care must be taken to ensure that sequence modifications are not introduced near the 3' end of the primer, in order for efficient amplification to proceed unperturbed. If necessary, the primer can be extended in the 3' direction. A reverse primer is designed which corresponds to sequence following the end of the ORF. The location of this primer is chosen to correspond to a region of the template that is both near the end of the ORF and will allow specific priming. The reverse primer contains also a restrictionendonuclease site plus additional 5' bases (approximately 10-20 nucleotides). Amplifications are performed using standard conditions that have been previously described in detail (Kaplan *et al.* (1995) *J. Biol. Chem.* 270:4108-4144). Upon obtaining a single predominant amplification product of the predicted size, the product is purified using, for example, the Geneclean II Kit (Bio 101, Inc.). The purified product is sequentially digested with the appropriate restriction endonucleases (depending on the sites added *via* the primers. The resulting digest is extracted and purified, again, for example, with the Geneclean II Kit. The purified fragment is then directionally cloned into either an appropriate expression

plasmid. For example, this exemplary expression procedure is described in detail in Kaplan (1996) *J. Bioenerg. Biomembr.* 28(1):41-47, in which the expression plasmids, pET-21a(+) or pET-28a(+) (Novagen) were selected for expression of the MAC, CIC. These plasmids are prepared by digested with restriction endonucleases to generate ends complementary to those of the purified fragment and the linerized vector purified by "genecleaning." The ligation and subsequent transformation of NovaBlue Competent Cells (Novagen) are performed essentially according to the manufacturer's instructions (Novagen pET System Manual, 1995). Transformants are screened for the presence of the UCP insert via: (i) direct colony PCR (using the same primer combination as above); and/or (ii) restriction analysis of purified plasmid DNA using the appropriate restriction endonucleases. BL21(DE3) competent cells (Novagen), the expression host, are then transformed with 1μl of a 50-fold dilution of plasmid DNA, purified (via the Wizard Minipreps DNA Purification System (Promega)) from NovaBlue cells, the storage host. Transformants are screened for inserts as described above. The cloned UCP fragment is then sequenced in its entirety, using plasmid DNA purified from BL21(DE3) cells as the template, in order to ensure that no mutations have occurred.

Induction of High-Level Expression of the UCP. A single colony of *E. coli* BL21(DE3) cells containing the target plasmid is used to inoculate LB media (1% bactotryptone, 0.5% yeast extract, 1% NaCl, pH 7.5) plus antibiotic (i.e., pET 28a(+) plasmid, 50 μg of carbenicillin/ml; pET 28a(+) plasmid, 30 μg of kanamycin/ml) (Novagen pET System Manual, 1995). The culture is incubated with shaking (350 rpm) at 37°C until an absorbence at 600 nm of 0.6-0.8 is obtained. A 200-ml aliquot is then removed, placed on ice for 5 min, and harvested as described below. Then 1.0 mM IPTG is added to the remainder of the culture in order to induce UCP expression and the incubation is continued for an additional 2 h. At this time 200-ml aliquots are removed and placed on ice for 5 min.

Purification of the Overexpressed UCP. Cells are harvested by centrifugation at 5,000 x g for 5 min at 4°C. The supernatants are discarded and the sample pellets are stored on ice until all tubes are processed. The pellets are then resuspended in 20 ml of Buffer A (50 mM Tris-HCl, 2 mM EDTA, pH 8.0). Cell lysis is accomplished by

incubation of the suspension with 100 μg of lysozyme/ml (freshly prepared in Buffer A) and 0.1% (v/v) Triton X-100 for 15 min at 30°C with occasional mixing (Novagen pET System Manual, 1995). The cell lysate is then sonicated, in order to shear the DNA, until there is a marked decrease in viscosity (i.e., typically 3-7 cycles of 30 sec sonication on ice followed by 30 sec of cooling/cycle; 70% duty cycle; output control = 1.6; Branson sonifier 250; large tip). The suspension is centrifuged at 12,000 X g for 15 min, and the pellet is resuspended in 2 ml of Buffer B (10 mM Tris-HC1, 0.1 mM EDTA, pH 7.0; 1.0 mM dithioerythritol) and recentrifuged. The resulting pellet is resuspended in 2 ml of Buffer B and the inclusion body fraction isolated by centrifugation at 131,000 x g for 4.5 h through a step gradient consisting of 12.4 ml of 40% (w/v) sucrose and 18.6 ml of 53% sucrose (Fiermonte *et al.* (1993) *Biochem. J.* 294:293-299) (the sucrose solutions are prepared in Buffer B). The inclusion body pellet is resuspended in 30 ml of Buffer B and centrifuged at 12,000 x g for 15 min. The UCP is then extracted by resuspension of the inclusion body pellet in 2 ml of 1.2% (w/v) sarkosyl dissolved in Buffer B (Fiermonte *et al.* (1993)). Following centrifugation at 314,000 x g for 30 min, the resulting supernatant contains the solubilized UCP.

RECOMBINANT PRODUCTION AND PURIFICATION OF UCP-1 FROM YEAST

Yeast Expression System. The *S. cerevisiae* strain JB 516 (*MATa, ura3, ade1, leu2, his4, gal*$^+$) is transformed with the *Escherichia coli/Saccharomyces cerevisiae* multicopy shuttle vector pCGS110 (Collaborative Research Inc.) containing the coding region of human UCP cDNA (e.g., the coding region of SEQ ID NO:1, SEQ ID NO:3, or SEQ ID NO:5) inserted at the unique *Bam*HI site beside the inducible *Gal*I promoter. The vector contains the *ura3* gene as the yeast selectable marker. To increase the level of expression of UCP in yeast, 5' sequences surrounding the initiating ATG of UCP cDNA can be altered to resemble the sequence of a highly expressed yeast glyceraldehyde-3 phosphate dehydrogenase gene (G3PDa) (Hamilton *et al.* (1987) *Nucleic Acid Res.* 15:3581-3593) and highly used codons in yeast (Sharp *et al.* (1986) *Nucleic Acid Res.* 14:5125-5143). This procedure, as well as the yeast expression described herein, is described in detail in Murdza-Inglis *et al.* (1991) *J. Biol. Chem.*

260:11871-11875, wherein the authors expressed high levels of the rat uncoupling protein.

The altered cDNA can be constructed in a bacterial plasimd vector (e.g., pGEM5Zf (Promega)) and subsequently subcloned into pCGS110 prior to transformion into yeast. Yeast transformants are grown at 30°C in selective medium containing 0.17% yeast nitrogen base, 0.5% $(NH_4)_2SO_4$, and 0.005% each of adenine, leucine, and histidine. The carbon source is 2% lactate, 0.05% glucose (Daum *et al.* (1982) *J. Biol. Chem.* 257:13028-13033).

Mitochondrial Isolation. Mitochondria are isolated from yeast transformants as described by Gasser (1983) *Methods Enzymol.* 97:329-336 from cells induced with 0.2% galactose for 8-10 h. The nuclear fraction is recovered at 3500 rpm for 5 min, and the mitochondrial fraction, at 9000 rpm for 10 min. Mitochondria are resuspended in a solution containing 0.6 M mannitol, 20 mM Hepes-KOH, pH 7.4, and 0.1% fatty acid-free bovine serum albumin.

Inner Membrane Preparation. Yeast mitochondria are fractionated in a 1.0-ml volume using a 0.4:1.0 ratio of digitonin to mitochondrial protein. The resulting mitoplasts are resuspended in 500 µl of hypotonic medium containing 10 mM Tris, 1 mM EDTA, pH 7.2, and sonicated 3 x 20 s in a bath sonicator to release the matrix contents. Membranes are collected at 150,000 x *g* for 20 min and resuspended in 500 µl of the mitochondrial resuspension buffer described above.

Purification of Uncoupling Protein from Yeast Mitochondria. UCP can purified from yeast mitochondria, for example, using the same protocols that have been described for tissue mitochondria (See Jezek *et al.* (1990) *J. Biol. Chem.* 265:19296-19302).

PURIFICATION OF UCP-1 FROM EUKARYOTIC MITOCHONDRIA

Purification of UCP from eukaryotic (e.g., mammalian or yeast) mitochondria can be achieved using the following exemplary procedure.

Membrane Preparation. Mitochondria from brown adipose tissue or *S. cerevisiae* (containing heterologously expressed UCP) are washed with extraction buffer

(e.g., 250 mM sucrose, 10 mM TEA-TES, 1 mM K-EGTA, pH 6.7, containing 2 mg BSA/ml) and centrifuged.

Protein Solubilization. Extraction buffer contains 25 mM SO1/2. 1.5 mM EGTA (TEA^- salts), and 50 mM phosphate. pH 7.2 (Tris salt). To 36 mg of a dried lipid mixture whose composition is given in Table 1 add 0.550 ml of extraction buffer and 70 µl of Octyl-POE. Lipids are solubilized by heating to 50°, then cooled to 0°. The mitochondrial pellet, containing 10 mg of protein, is gently homogenized with this buffer-lipid-detergent mixture.

Hydroxylapatite Chromatography. The extraction buffer and 0.45g of dry hydroxylapatite (Bio-Gel HTP. Bio-Rad) are placed in a 3-ml polyethylene syringe with silicon fibers in the outlet and centrifuged for 2 min at 800 *g* to remove free buffer. The protein-lipid-detergent mixture is loaded onto the column, mixed with the hydroxylapatite, incubated for 10 min at 20° to inactivate the ATP/ADP exchanger, and then incubated for 25 min at 0°. The column is centrifuged for 2 min at 800*g*. The flow-through eluate from the hydroxylapatite column contains predominately (>95%) UCP and is used directly for reconstitution.

Purification Assays. Fractions are identified by the presence of a 32-kDa protein on SDS-PAGE gels, by Western blot analysis using antibodies raised against the purified uncoupling protein, and by reconstitutive activity.

EXAMPLE 3:

Preparation of Assay Vesicles containing MACs as a Component of the Vesicle Bilayer as well as Containing pH Indicators and/or Potentiometric Probes

INCORPORATION OF MACS INTO ASSAY VESICLES

The method can be further modified to facilitate the formation of vesicles including a particular MAC, for example, UCP-1. For example, purified or recombinant MACs can be included during vesicle formation using the well-established methods of Jezek et al. (1994) *J. Biol. Chem.* 269:26184-190; Jekek et al. (1990) *J. Biol. Chem.*

265:19296-302; Garlid et al. (1996) *J. Biol. Chem.* 271:2615-2620; and Garlid et al. (1995) *Methods Enzymol.* 260:331-348.

ASSAY VESICLE CHARACTERIZATION

The composition of vesicles can be optimized for reproducibility of vesicle formation, ease of preparation and ultimately effective insertion of the MAC into the membrane. For example, the topography of MAC insertion into the bilayer (e.g., the "sidedness" or orientation of the MAC) can be determined using orientation-specific antibodies such as those described in Brandolin *at al.* (1989), *Biochemistry* 28(3):1093-1100; Capobianco *et al.* (1991) *Biochemistry* 30(20): 4963-4969; Ferreira *et al.* (1990) *J. Biol. Chem.* 265(34): 21202-21206; and Miroux *et al.* (1992) *J. Biol. Chem.* 267:13603-13609. Briefly, antibodies directed to portions of the MAC which face the cytoplasm in the intact cell, can be reacted with vesicle preparations containing a MAC as a component of the lipid bilayer and compared, for example, to vesicles containing no MAC. Interaction of the antibody with the vesicle containing the MAC which is detectable when compared to the interaction of the antibody with the vesicle containing no MAC indicated incorporation of the at least some MAC molecules having the correct sidedness or orientation. Likewise, a lack of interaction of antibodies directed to portions of a MAC which face the matrical side of an intact mitochondria, as compared to the interaction of such antibodies with control vesicles containing no MAC, can also provide a positive indicator of the correct sidedness of MAC incorporation.

Alternatively, orientation can be determined using know inhibitors of MAC activity added to the vesicle preparation either extravesicularly or incorporated internally, during the formation of the assay vesicles. For example, the nucleotide inhibitor of UCP-1, GPD, can be added extravesicularly to a vesicle preparation, and the effect of GDP addition on proton conductance determined. External addition of GDP to UCP-containing vesicles should inhibit the activity of correctly-oriented UCP (the inhibitory binding site for GDP occurs on the cytoplasmic face of the mitochondria in an intact cell) but should have no effect on UCP molecules which have incorporated "in-side-out". Likewise, formation of the UCP-containing vesicle with GDP trapped on the inside of the vesicle should result in inhibition of proton conductance, only in the UCP

molecules have adopted the in-side-out orientation. See, for example, Strieleman *et al.* (1985) *Biochem. Biophys. Res. Commun.* 127(2):509-516 and Katiyar and Shrago (1991) *Biochem. Biophys. Res. Commun.* 175(3): 1104-1111.

EXAMPLE 4:

Enhancement of the sensitivity of the assay by monitoring external pH using the increase in fluorescence of a different pH indicator.

Assay sensitivity can be enhanced by determining the ratio of internal to external changes.

The data presented in Example 1 demonstrates the feasibility of combining two dyes with differing fluorescence characteristics that monitor different aspects of a related change. A reasonable balance between the two dyes is necessary to assure adequate sensitivity of the measurement. This can be achieved by lowering the ratio of vesicles to external medium, by placing the higher yield fluorescent indicator on the outside where it will be more dilute and by adjusting the concentration of external dye.

For example, pyranine, with its lower fluorescence yield (excitation 450 nm, emission at 509 nm), can be trapped inside of vesicles and SNARF (Molecular Probes, Eugene, OR) can be added to the outside (excitation 534 nm, emission 640 nm). The rationale for using a different dye on the outside with different spectral characteristics than the internal dye is to permit continuous assessment of vesicular leakiness.

A satisfactory combination of dyes gives a linear rate of internal acidification and external alkalinization in response to FAA addition during the time interval of the measurement. It may be desirable to begin with a more alkaline internal pH in order to prolong the linear phase of acidification.

EXAMPLE 5:

Conversion of the assay into an alternate, multiwell high-throughput fluorescence reader assay.

The vesicles reconstituted with UCPs and containing the trapped pH dye are designed to be used to test the effects of biomolecules or drugs on the transport function of the UCPs. Not only can the influence of drugs on the FAA induced pH gradient

across the bilayer but also the effects of drugs on inhibition of FAA transport by the endogenous regulators of UCPs, nucleotides can be studied. Thus, the assays of Examples 1-3 can be adapted to a multiwell plate and automated plate reader. In such a format, the fluorescence measurements are straightforward and quick, thus allowing rapid testing of many drugs.

What is claimed:

1. A method for identifying a compound which modulates the activity of a mitichondrial anion carrier (MAC) comprising:

(a) contacting a MAC with a test compound and a MAC substrate which is capable of being transported by the MAC;

(b) determining the ability of the test compound to modulate transport of the MAC substrate; and

(c) identifying the test compound as a modulator of MAC activity.

2. The method of claim 1, wherein the MAC is formulated within a barrier separating a first and a second compartment under conditions such that transport of the MAC substrate across the barrier occurs.

3. The method of claim 1, wherein the MAC substrate is transported from the first compartment to the second compartment.

4. The method of claim 1, wherein the first or second compartment comprises an indicator of MAC activity.

5. A method for identifying a compound which modulates the activity of a mitichondrial anion carrier (MAC) comprising:

(a) contacting an assay vesicle which comprises at least one MAC and an indicator of MAC activity with a test compound and an MAC substrate, wherein the MAC substrate is capable of being transported across the lipid bilayer of the assay vesicle;

(b) determining the ability of the test compound to modulate transport of the MAC substrate across the lipid bilayer of the assay vesicle, wherein determining the ability of the test compound to modulate transport of the MAC substrate across the lipid bilayer of the assay vesicle comprises determining a change in the indicator of MAC activity; and

(c) identifying the test compound as a modulator of MAC activity.

6. The method of claim 5, wherein the MAC is selected from the group consisting of an ADP/ATP carrier (AAC), a phosphate carrier (PiC), a pyruvate carrier (PYC), an aspartate/glutamate carrier (AGC), an oxoglutarate carrier (KGC/OGC), a dicarboxylate carrier (DIC), a citrate carrier (CIC), a glutamate carrier (GC), an ATPMg/P_i carrier (APC), and an uncoupling protein (UCP).

7. The method of claim 5, wherein the MAC is a pyruvate carrier (PYC) and the MAC substrate is pyruvate.

8. The method of claim 5, wherein the MAC is a phosphate carrier (PiC) and the MAC substrate is inorganic phosphate (Pi).

9. The method of claim 5, wherein the MAC is a glutamate carrier (GC) and the MAC substrate is glutamate.

10. The method of claim 5, wherein the MAC is an uncoupling protein (UCP) and the MAC substrate is a fatty acid anion (FAA^-).

11. The method of claim 10, wherein the MAC is selected from the group consisting of UCP1, UCP2, or UCP3.

12. A method for identifying a compound which modulates the activity of a mitochondrial anion carrier (MAC) comprising:

(a) contacting an assay vesicle which comprises at least one MAC, a first MAC substrate, and an indicator of MAC activity with a test compound and a second MAC substrate, wherein the first and second MAC substrates are capable of being transported across the lipid bilayer of the assay vesicle;

(b) determining the ability of the test compound to modulate transport of the MAC substrates across the lipid bilayer of the assay vesicle, wherein determining the ability of the test compound to modulate transport of the MAC substrates across the lipid bilayer of the assay vesicle comprises determining a change in the indicator of MAC activity; and

(c) identifying the test compound as a modulator of MAC activity.

13. The method of claim 12, wherein the MAC is an ADP/ATP carrier (AAC), the MAC substrates are ADP and ATP.

14. The method of claim 12, wherein the mitochondrial UCP is selected from the group consisting of uncoupling protein 1 (UCP1), uncoupling protein 2 (UCP2) and uncoupling protein 3 (UCP3).

15. The method of claim 12, wherein the MAC is an aspartate/glutamate carrier (AGC), and the MAC substrates are aspartate and glutamate.

16. The method of claim 12, wherein the MAC is an α-ketoglutarate carrier or oxoglutarate carrier (KGC/OGC) and the MAC substrates are α-ketoglutarate and malate.

17. The method of claim 12, wherein the MAC is a dicarboxylate carrier (DIC) and the MAC substrates are inorganic phosphate (Pi) and a dicarboxylate.

18. The method of claim 12, wherein the MAC is a citrate carrier (CIC) and the MAC substrates are malate and citrate.

19. The method of claim 12, wherein the MAC is an ATPMg/P_i carrier (APC) and the MAC substrates are ATP-Mg^{++} and inorganic phosphate (Pi).

20. The method of claim 12, wherein the MAC is an uncoupling protein (UCP) and the MAC substrate is a fatty acid anion (FAA^-).

21. A method for identifying a compound which modulates the activity of a mitichondrial anion carrier (MAC) comprising:

(a) contacting an assay vesicle which comprises a first MAC, a second MAC, a second MAC substrate, and an indicator of activity of the second MAC with a test compound and a first MAC substrate, wherein the MAC substrates are capable of being transported across the lipid bilayer of the assay vesicle;

(b) determining the ability of the test compound to modulate transport of the first MAC substrate across the lipid bilayer of the assay vesicle, wherein determining the ability of the test compound to modulate transport of the first MAC substrate across the lipid bilayer of the assay vesicle comprises determining a change in the indicator of activity of the second MAC; and

(c) identifying the test compound as a modulator of MAC activity.

22. The method of claim 21, wherein the first MAC is an aspartate/glutamate carrier, the second MAC is a glutamate carrier (GC), the first MAC substrate is aspartate and the second MAC substrate is glutamate.

23. The method of claim 21, wherein the first MAC is an ATPMg/Pi carrier (APC), the second MAC is a phosphate carrier (PiC), the first MAC substrate is $ATPMg^{++}$, and the second MAC substrate is inorganic phosphate (Pi).

24. The method of claim 21, wherein the first MAC is a dicarboxylate and the second MAC substrate is inorganic phosphate (Pi).

25. A method for identifying a compound which modulates the activity of a mitichondrial anion carrier (MAC) comprising:

(a) contacting an assay vesicle which comprises a first MAC, a second MAC, a third MAC, a second MAC substrate, a third MAC substrate, and an indicator of activity of the third MAC with a test compound and a first MAC substrate, wherein the MAC substrates are capable of being transported across the lipid bilayer of the assay vesicle;

(b) determining the ability of the test compound to modulate transport of the first MAC substrate across the lipid bilayer of the assay vesicle, wherein determining the ability of the test compound to modulate transport of the first MAC substrate across the lipid bilayer of the assay vesicle comprises determining a change in the indicator of activity of the third MAC; and

(c) identifying the test compound as a modulator of MAC activity.

26. The method of claim 25, wherein the first MAC is an α-ketoglutarate carrier or oxoglutarate carrier (KGC/OGC), the second MAC is a dicarboxylate carrier, the third MAC is a phosphate carrier (PiC), the first MAC substrate is α-ketoglutarate, the second MAC substrate is malate, and the third MAC substrate is inorganic phosphate (Pi).

27. The method of claim 25, wherein the first MAC is a citrate transporter (CIC), the second MAC is a dicarboxylate transporter (DIC), the third MAC is a phosphate transporter, the first MAC substrate is citrate, the second MAC substrate is malate, and the third MAC substrate is inorganic phosphate (Pi).

28. An assay vesicle comprising a phospholipid and at least one MAC selected from the group consisting an ADP/ATP carrier (AAC), a phosphate carrier (PiC), a pyruvate carrier (PYC), an aspartate/glutamate carrier (AGC), an oxoglutarate carrier (KGC/OGC), a dicarboxylate carrier (DIC), a citrate carrier (CIC), a glutamate carrier (GC), an ATPMg/P_i carrier (APC), and an uncoupling protein (UCP).

FIG.1A

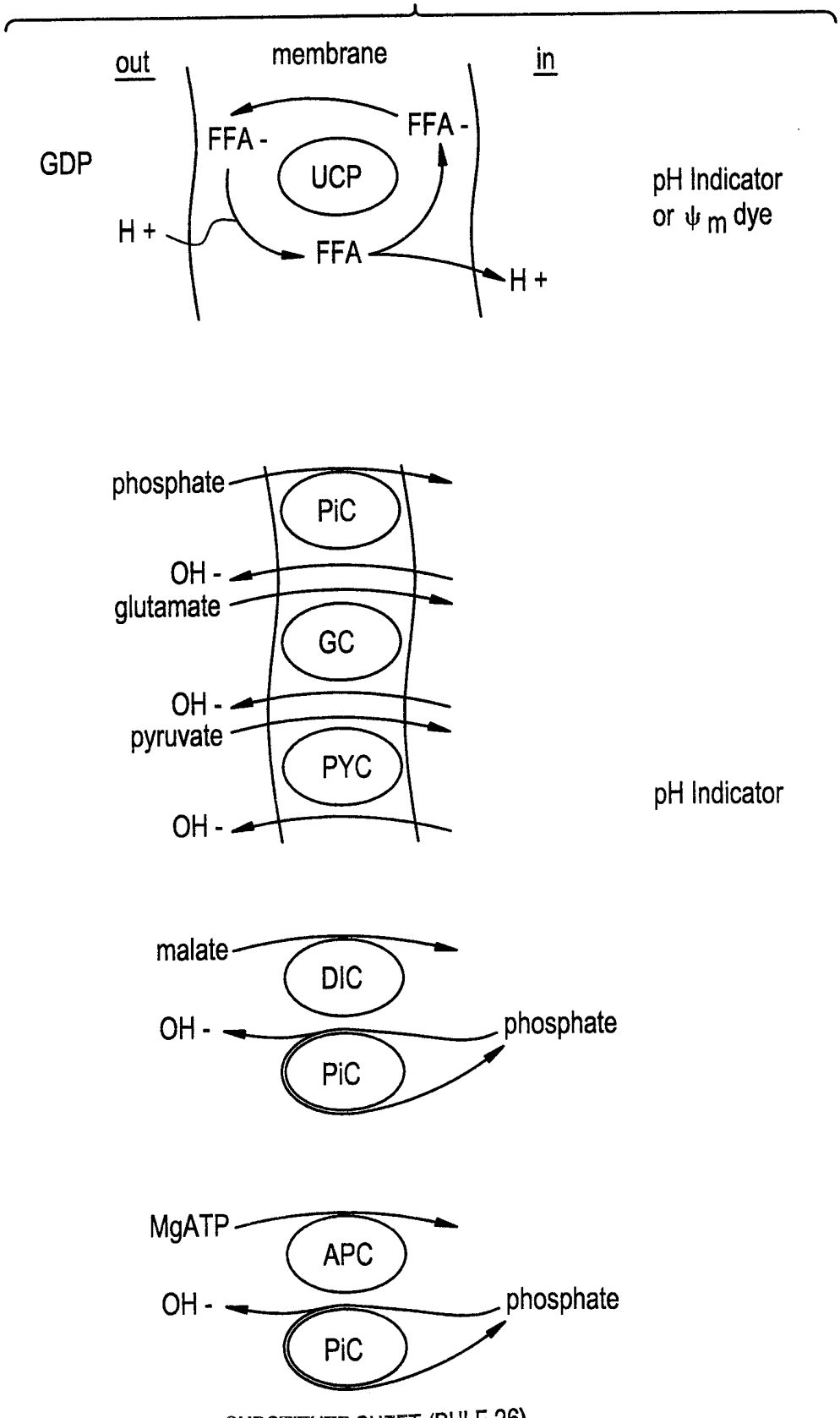

out
membrane
in
FFA -
FFA -
GDP
UCP
pH Indicator
or ψ m dye
H +
FFA
H +
phosphate
PiC
OH -
glutamate
GC
OH -
pyruvate
PYC
pH Indicator
OH -
malate
DIC
OH -
phosphate
PiC
MgATP
APC
OH -
phosphate
PiC

FIG.1B

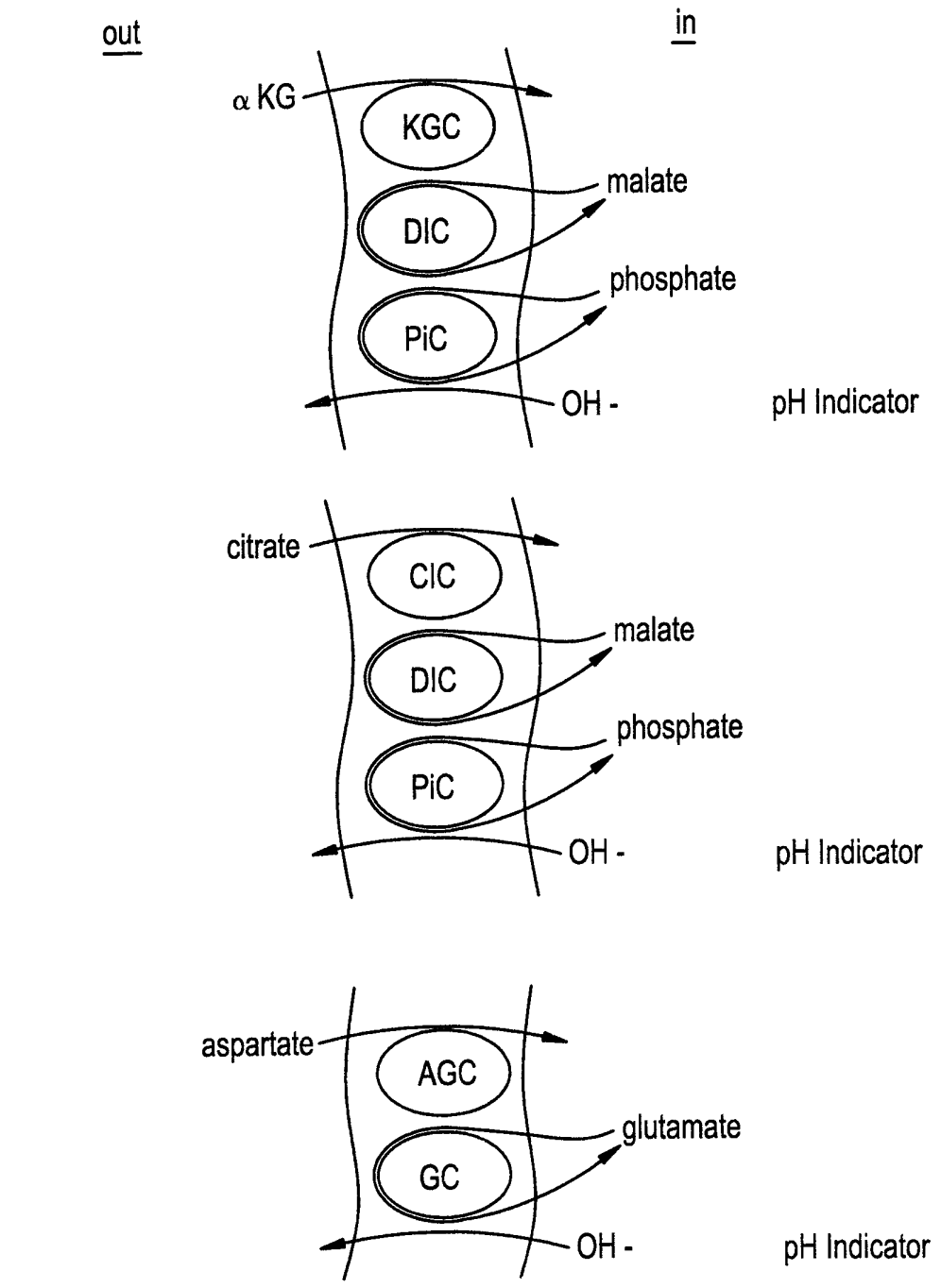

out
in
α KG
KGC
malate
DIC
phosphate
PiC
OH -
pH Indicator
citrate
CIC
malate
DIC
phosphate
PiC
OH -
pH Indicator
aspartate
AGC
glutamate
GC
OH -
pH Indicator

Figure 2A

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                1                                                          60
UCP-1 coding ATGGGGGGCCTGACAGCCTCGGACGTACACCCGACCCTGGGGGTCCAGCTCTTCTCAGCT
UCP-2 coding ATGGTTGGGTTCAAGGCCACAGATGTGCCCCCTACTGCCACTGTGAAGTTTCTTGGGGCT
UCP-3 coding ATGGTTGGACTGAAGCCTTCAGACGTGCCTCCCACCATGGCTGTGAAGTTCCTGGGGGCA

               61                                                         120
UCP-1 coding GGAATAGCGGCGTGCTTGGCGGACGTGATCACCTTCCCGCTGGACACGGCCAAAGTCCGG
UCP-2 coding GGCACAGCTGCCTGCATCGCAGATCTCATCACCTTTCCTCTGGATACTGCTAAAGTCCGG
UCP-3 coding GGCACAGCAGCCTGTTTTGCTGACCTCGTTACCTTTCCACTGGACACAGCCAAGGTCCGC

               121                                                        180
UCP-1 coding CTCCAGGTCCAAGGTGAATGCCCGACGTCCA----GTGTTA---TTAG------GTATAA
UCP-2 coding TTACAGATCCAAGGAGAAAGTCAGG-GGCCAGTGCGCGCTACAGTCAGCGCCCAGTACCG
UCP-3 coding CTGCAGATCCAGGGGGAGAACCAGGCGGTCCAGACG-GCC-CGGCTCGTGC--AGTACCG

               181                                                        240
UCP-1 coding AGGTGTCCTGGGAACAATCACCGCTGTGGTAAAAACAGAAGGGCGGATGAAACTCTACAG
UCP-2 coding CGGTGTGATGGGCACCATTCTGACCATGGTGCGTACTGAGGGCCCCCGAAGCCTCTACAA
UCP-3 coding TGGCGTGCTGGGCACCATCCTGACCATGGTGCGGACTGAGGGTCCCTGCAGCCCCTACAA

               241                                                        300
UCP-1 coding CGGGCTGCCTGCGGGGCTTCAGCGGCAAATCAGCTCCGCCTCTCTCAGGATCGGCCTCTA
UCP-2 coding TGGGCTGGTTGCCGGCCTGCAGCGCCAAATGAGCTTTGCCTCTGTCCGCATCGGCCTGTA
UCP-3 coding TGGGCTGGTGGCCGGCCTGCAGCGCCAGATGAGCTTCGCCTCCATCCGCATCGGCCTCTA

               301                                                        360
UCP-1 coding CGACACGGTCCAGGAGTTCCTCACCGC-AGGGAAAGAAACAGCACCTAGTTTAGGAAGCA
UCP-2 coding TGATTCTGTCAAACAGTTCTACACC---AAGGGCTCTGAGCATGCC-AGCATTGGGAGCC
UCP-3 coding TGACTCCGTCAAGCAGGTGTACACCCCCAAAGGCGCGGACAACTCC-AGCCTCACTACCC

               361                                                        420
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UCP-2 coding GCCTCCTAGCAGGCAGCACCACAGGTGCCCTGGCTGTGGCTGTGGCCCAGCCCACGGATG
UCP-3 coding GGATTTTGGCCGGCTGCACCACAGGAGCCATGGCGGTGACCTGTGCCCAGCCCACAGATG

               421                                                        480
UCP-1 coding TCGTGAAAGTCAGACTTCAAGCACAGAGCCATCTCCA------CGGAATCAAACCTCGCT
UCP-2 coding TGGTAAAGGTCCGATTCCAAGCT--CAGGC---CCGGGCTG----GAGGTGGTCGGAGAT
UCP-3 coding TGGTGAAGGTCCGATTTCAGGCCAGCATACACCTCGGGCCATCCAGGAGCGACAGAAAAT

               481                                                        540
UCP-1 coding ACACGGGGACTTATAATGCGTACAGAATAATAGCAACAACCGAAGGCTTGACGGGTCTTT
UCP-2 coding ACCAAAGCACCGTCAATGCCTACAAGACCATTGCCCGAGAGGAAGGGTTCCGGGGCCTCT
UCP-3 coding ACAGCGGGACTATGGACGCCTACAGAACCATCGCCAGGGAGGAAGGAGTCAGGGGCCTGT

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UCP-1 coding GGAAAGGGACTACTCCCAATCTGATGAGAAGTGTCATCATCAATTGTACAGAGCTAGTAA
UCP-2 coding GGAAAGGGACCTCTCCCAATGTTGCTCGTAATGCCATTGTCAACTGTGCTGAGCTGGTGA
UCP-3 coding GGAAAGGAACTTTGCCCAACATCATGAGGAATGCTATCGTCAACTGTGCTGAGGTGGTGA

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UCP-2 coding CCTATGACCTCATCAAGGATGCCCTCCTGAAAGCCAACCTCATGACAGATGACCTCCCTT
UCP-3 coding CCTACGACATCCTCAAGGAGAAGCTGCTGGACTACCACCTGCTCACTGACAACTTCCCCT
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Figure 2B

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UCP-2 coding GCCACTTCACTTCTGCCTTTGGGGCAGGCTTCTGCACCACTGTCATCGCCTCCCCTGTAG
UCP-3 coding GCCACTTTGTCTCTGCCTTTGGAGCCGGCTTCTGTGCCACAGTGGTGGCCTCCCCGGTGG

            721                                                        780
UCP-1 coding ATGTAGTAAAAACCAGATTTATTAATTCTCCACCAGGACAGTACAAAAGTGTGCCCAACT
UCP-2 coding ACGTGGTCAAGACGAGATACATGAACTCTGCCCTGGGCCAGTACAGTAGCGCTGGCCACT
UCP-3 coding ACGTGGTGAAGACCCGGTATATGAACTCACCTCCAGGCCAGTACTTCAGCCCCCTCGACT

            781                                                        840
UCP-1 coding GTGCAATGAAAGTGTTCACTAACGAAGGACCAACGGCTTTCTTCAAGGGGTTGGTACCTT
UCP-2 coding GTGCCCTTACCATGCTCCAGAAGGAGGGGCCCCGAGCCTTCTACAAAGGGTTCATGCCCT
UCP-3 coding GTATGATAAAGATGGTGGCCCAGGAGGGCCCCACAGCCTTCTACAAGGGATTTACACCCT

            841                                                        900
UCP-1 coding CCTTCTTGCGACTTGGATCCTGGAACGTCATTATGTTTGTGTGCTTTGAACAACTGAAAC
UCP-2 coding CCTTTCTCCGCTTGGGTTCCTGGAACGTGGTGATGTTCGTCACCTATGAGCAGCTGAAAC
UCP-3 coding CCTTTTTGCGTTTGGGATCCTGGAACGTGGTGATGTTCGTAACCTATGAGCAGCTGAAAC

            901                                                945
UCP-1 coding GAGAACT-GTCAAAGTCAAGGCAGACTATGGACTGTGCCACATAA
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ctc ttc tca gct gga ata gcg gcg tgc ttg gcg gac gtg atc acc ttc   96
Leu Phe Ser Ala Gly Ile Ala Ala Cys Leu Ala Asp Val Ile Thr Phe
             20                  25                  30

ccg ctg gac acg gcc aaa gtc cgg ctc cag gtc caa ggt gaa tgc ccg   144
Pro Leu Asp Thr Ala Lys Val Arg Leu Gln Val Gln Gly Glu Cys Pro
         35                  40                  45

acg tcc agt gtt att agg tat aaa ggt gtc ctg gga aca atc acc gct   192
Thr Ser Ser Val Ile Arg Tyr Lys Gly Val Leu Gly Thr Ile Thr Ala
     50                  55                  60

gtg gta aaa aca gaa ggg cgg atg aaa ctc tac agc ggg ctg cct gcg   240
Val Val Lys Thr Glu Gly Arg Met Lys Leu Tyr Ser Gly Leu Pro Ala
 65                  70                  75                  80

ggg ctt cag cgg caa atc agc tcc gcc tct ctc agg atc ggc ctc tac   288
Gly Leu Gln Arg Gln Ile Ser Ser Ala Ser Leu Arg Ile Gly Leu Tyr
                 85                  90                  95

gac acg gtc cag gag ttc ctc acc gca ggg aaa gaa aca gca cct agt   336
Asp Thr Val Gln Glu Phe Leu Thr Ala Gly Lys Glu Thr Ala Pro Ser
            100                 105                 110

tta gga agc aag att tta gct ggt cta acg act gga gga gtg gca gta   384
Leu Gly Ser Lys Ile Leu Ala Gly Leu Thr Thr Gly Gly Val Ala Val
        115                 120                 125

ttc att ggg caa ccc aca gag gtc gtg aaa gtc aga ctt caa gca cag   432
Phe Ile Gly Gln Pro Thr Glu Val Val Lys Val Arg Leu Gln Ala Gln
    130                 135                 140
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145                 150                 155                 160

tac aga ata ata gca aca acc gaa ggc ttg acg ggt ctt tgg aaa ggg   528
Tyr Arg Ile Ile Ala Thr Thr Glu Gly Leu Thr Gly Leu Trp Lys Gly
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act act ccc aat ctg atg aga agt gtc atc atc aat tgt aca gag cta   576
Thr Thr Pro Asn Leu Met Arg Ser Val Ile Ile Asn Cys Thr Glu Leu
            180                 185                 190

gta aca tat gat cta atg aag gag gcc ttt gtg aaa aac aac ata tta   624
Val Thr Tyr Asp Leu Met Lys Glu Ala Phe Val Lys Asn Asn Ile Leu
        195                 200                 205

gca gat gac gtc ccc tgc cac ttg gtg tcg gct ctt atc gct gga ttt   672
Ala Asp Asp Val Pro Cys His Leu Val Ser Ala Leu Ile Ala Gly Phe
    210                 215                 220

tgc gca aca gct atg tcc tcc ccg gtg gat gta gta aaa acc aga ttt   720
Cys Ala Thr Ala Met Ser Ser Pro Val Asp Val Val Lys Thr Arg Phe
225                 230                 235                 240

att aat tct cca cca gga cag tac aaa agt gtg ccc aac tgt gca atg   768
Ile Asn Ser Pro Pro Gly Gln Tyr Lys Ser Val Pro Asn Cys Ala Met
                245                 250                 255

aaa gtg ttc act aac gaa gga cca acg gct ttc ttc aag ggg ttg gta   816
Lys Val Phe Thr Asn Glu Gly Pro Thr Ala Phe Phe Lys Gly Leu Val
            260                 265                 270

cct tcc ttc ttg cga ctt gga tcc tgg aac gtc att atg ttt gtg tgc   864
Pro Ser Phe Leu Arg Leu Gly Ser Trp Asn Val Ile Met Phe Val Cys
        275                 280                 285

ttt gaa caa ctg aaa cga gaa ctg tca aag tca agg cag act atg gac   912
Phe Glu Gln Leu Lys Arg Glu Leu Ser Lys Ser Arg Gln Thr Met Asp
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Cys Ala Thr
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Pro Leu Asp Thr Ala Lys Val Arg Leu Gln Val Gln Gly Glu Cys Pro
        35                  40                  45
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Thr Ser Ser Val Ile Arg Tyr Lys Gly Val Leu Gly Thr Ile Thr Ala
     50                  55                  60

Val Val Lys Thr Glu Gly Arg Met Lys Leu Tyr Ser Gly Leu Pro Ala
 65                  70                  75                  80

Gly Leu Gln Arg Gln Ile Ser Ser Ala Ser Leu Arg Ile Gly Leu Tyr
                 85                  90                  95

Asp Thr Val Gln Glu Phe Leu Thr Ala Gly Lys Glu Thr Ala Pro Ser
            100                 105                 110

Leu Gly Ser Lys Ile Leu Ala Gly Leu Thr Thr Gly Gly Val Ala Val
        115                 120                 125

Phe Ile Gly Gln Pro Thr Glu Val Val Lys Val Arg Leu Gln Ala Gln
    130                 135                 140

Ser His Leu His Gly Ile Lys Pro Arg Tyr Thr Gly Thr Tyr Asn Ala
145                 150                 155                 160

Tyr Arg Ile Ile Ala Thr Thr Glu Gly Leu Thr Gly Leu Trp Lys Gly
                165                 170                 175

Thr Thr Pro Asn Leu Met Arg Ser Val Ile Ile Asn Cys Thr Glu Leu
            180                 185                 190

Val Thr Tyr Asp Leu Met Lys Glu Ala Phe Val Lys Asn Asn Ile Leu
        195                 200                 205

Ala Asp Asp Val Pro Cys His Leu Val Ser Ala Leu Ile Ala Gly Phe
    210                 215                 220

Cys Ala Thr Ala Met Ser Ser Pro Val Asp Val Val Lys Thr Arg Phe
225                 230                 235                 240

Ile Asn Ser Pro Pro Gly Gln Tyr Lys Ser Val Pro Asn Cys Ala Met
                245                 250                 255

Lys Val Phe Thr Asn Glu Gly Pro Thr Ala Phe Phe Lys Gly Leu Val
            260                 265                 270

Pro Ser Phe Leu Arg Leu Gly Ser Trp Asn Val Ile Met Phe Val Cys
        275                 280                 285

Phe Glu Gln Leu Lys Arg Glu Leu Ser Lys Ser Arg Gln Thr Met Asp
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Cys Ala Thr
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atcgggccat gatagccact ggcagctttg aagaacggga caccttaga gaagcttgat 180

cttggaggcc tcaccgtgag accttacaag gccggattcc ggcagagttc ctctatctcg 240

tcttgttgct gattaaaggt gcccctgtct ccagtttttc tccatctcct gggacgtagc 300

aggaaatcag catc atg gtt ggg ttc aag gcc aca gat gtg ccc cct act 350
Met Val Gly Phe Lys Ala Thr Asp Val Pro Pro Thr
1 5 10

gcc act gtg aag ttt ctt ggg gct ggc aca gct gcc tgc atc gca gat 398
Ala Thr Val Lys Phe Leu Gly Ala Gly Thr Ala Ala Cys Ile Ala Asp
15 20 25

ctc atc acc ttt cct ctg gat act gct aaa gtc cgg tta cag atc caa 446
Leu Ile Thr Phe Pro Leu Asp Thr Ala Lys Val Arg Leu Gln Ile Gln
30 35 40

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Gly Glu Ser Gln Gly Pro Val Arg Ala Thr Val Ser Ala Gln Tyr Arg
45 50 55 60

ggt gtg atg ggc acc att ctg acc atg gtg cgt act gag ggc ccc cga 542
Gly Val Met Gly Thr Ile Leu Thr Met Val Arg Thr Glu Gly Pro Arg
65 70 75

agc ctc tac aat ggg ctg gtt gcc ggc ctg cag cgc caa atg agc ttt 590
Ser Leu Tyr Asn Gly Leu Val Ala Gly Leu Gln Arg Gln Met Ser Phe
80 85 90

gcc tct gtc cgc atc ggc ctg tat gat tct gtc aaa cag ttc tac acc 638
Ala Ser Val Arg Ile Gly Leu Tyr Asp Ser Val Lys Gln Phe Tyr Thr
95 100 105

aag ggc tct gag cat gcc agc att ggg agc cgc ctc cta gca ggc agc 686
Lys Gly Ser Glu His Ala Ser Ile Gly Ser Arg Leu Leu Ala Gly Ser
110 115 120

acc aca ggt gcc ctg gct gtg gct gtg gcc cag ccc acg gat gtg gta 734
Thr Thr Gly Ala Leu Ala Val Ala Val Ala Gln Pro Thr Asp Val Val
125 130 135 140

aag gtc cga ttc caa gct cag gcc cgg gct gga ggt ggt cgg aga tac 782
Lys Val Arg Phe Gln Ala Gln Ala Arg Ala Gly Gly Gly Arg Arg Tyr
145 150 155

caa agc acc gtc aat gcc tac aag acc att gcc cga gag gaa ggg ttc 830
Gln Ser Thr Val Asn Ala Tyr Lys Thr Ile Ala Arg Glu Glu Gly Phe
160 165 170

cgg ggc ctc tgg aaa ggg acc tct ccc aat gtt gct cgt aat gcc att 878
Arg Gly Leu Trp Lys Gly Thr Ser Pro Asn Val Ala Arg Asn Ala Ile
175 180 185

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gtc aac tgt gct gag ctg gtg acc tat gac ctc atc aag gat gcc ctc   926
Val Asn Cys Ala Glu Leu Val Thr Tyr Asp Leu Ile Lys Asp Ala Leu
    190                 195                 200

ctg aaa gcc aac ctc atg aca gat gac ctc cct tgc cac ttc act tct   974
Leu Lys Ala Asn Leu Met Thr Asp Asp Leu Pro Cys His Phe Thr Ser
205                 210                 215                 220

gcc ttt ggg gca ggc ttc tgc acc act gtc atc gcc tcc cct gta gac   1022
Ala Phe Gly Ala Gly Phe Cys Thr Thr Val Ile Ala Ser Pro Val Asp
                225                 230                 235

gtg gtc aag acg aga tac atg aac tct gcc ctg ggc cag tac agt agc   1070
Val Val Lys Thr Arg Tyr Met Asn Ser Ala Leu Gly Gln Tyr Ser Ser
            240                 245                 250

gct ggc cac tgt gcc ctt acc atg ctc cag aag gag ggg ccc cga gcc   1118
Ala Gly His Cys Ala Leu Thr Met Leu Gln Lys Glu Gly Pro Arg Ala
        255                 260                 265

ttc tac aaa ggg ttc atg ccc tcc ttt ctc cgc ttg ggt tcc tgg aac   1166
Phe Tyr Lys Gly Phe Met Pro Ser Phe Leu Arg Leu Gly Ser Trp Asn
    270                 275                 280

gtg gtg atg ttc gtc acc tat gag cag ctg aaa cga gcc ctc atg gct   1214
Val Val Met Phe Val Thr Tyr Glu Gln Leu Lys Arg Ala Leu Met Ala
285                 290                 295                 300

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Ala Cys Thr Ser Arg Glu Ala Pro Phe
                305

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        35                  40                  45

Gly Pro Val Arg Ala Thr Val Ser Ala Gln Tyr Arg Gly Val Met Gly
    50                  55                  60

Thr Ile Leu Thr Met Val Arg Thr Glu Gly Pro Arg Ser Leu Tyr Asn
65                  70                  75                  80

Gly Leu Val Ala Gly Leu Gln Arg Gln Met Ser Phe Ala Ser Val Arg
                85                  90                  95

Ile Gly Leu Tyr Asp Ser Val Lys Gln Phe Tyr Thr Lys Gly Ser Glu
            100                 105                 110

His Ala Ser Ile Gly Ser Arg Leu Leu Ala Gly Ser Thr Thr Gly Ala
        115                 120                 125

Leu Ala Val Ala Val Ala Gln Pro Thr Asp Val Val Lys Val Arg Phe
    130                 135                 140

Gln Ala Gln Ala Arg Ala Gly Gly Gly Arg Arg Tyr Gln Ser Thr Val
145                 150                 155                 160

Asn Ala Tyr Lys Thr Ile Ala Arg Glu Glu Gly Phe Arg Gly Leu Trp
                165                 170                 175

Lys Gly Thr Ser Pro Asn Val Ala Arg Asn Ala Ile Val Asn Cys Ala
            180                 185                 190

Glu Leu Val Thr Tyr Asp Leu Ile Lys Asp Ala Leu Leu Lys Ala Asn
        195                 200                 205

Leu Met Thr Asp Asp Leu Pro Cys His Phe Thr Ser Ala Phe Gly Ala
    210                 215                 220

Gly Phe Cys Thr Thr Val Ile Ala Ser Pro Val Asp Val Val Lys Thr
225                 230                 235                 240

Arg Tyr Met Asn Ser Ala Leu Gly Gln Tyr Ser Ser Ala Gly His Cys
                245                 250                 255

Ala Leu Thr Met Leu Gln Lys Glu Gly Pro Arg Ala Phe Tyr Lys Gly
            260                 265                 270

Phe Met Pro Ser Phe Leu Arg Leu Gly Ser Trp Asn Val Val Met Phe
        275                 280                 285

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Lys Phe Leu Gly Ala Gly Thr Ala Ala Cys Phe Ala Asp Leu Val Thr
20 25 30

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Phe Pro Leu Asp Thr Ala Lys Val Arg Leu Gln Ile Gln Gly Glu Asn
35 40 45

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Gln Ala Val Gln Thr Ala Arg Leu Val Gln Tyr Arg Gly Val Leu Gly
50 55 60

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Thr Ile Leu Thr Met Val Arg Thr Glu Gly Pro Cys Ser Pro Tyr Asn
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Gly Leu Val Ala Gly Leu Gln Arg Gln Met Ser Phe Ala Ser Ile Arg
80 85 90 95

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Ile Gly Leu Tyr Asp Ser Val Lys Gln Val Tyr Thr Pro Lys Gly Ala
100 105 110

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115 120 125

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130 135 140

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Phe Gln Ala Ser Ile His Leu Gly Pro Ser Arg Ser Asp Arg Lys Tyr
145 150 155

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Ser Gly Thr Met Asp Ala Tyr Arg Thr Ile Ala Arg Glu Glu Gly Val
160 165 170 175

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                180                 185                 190

gtc aac tgt gct gag gtg gtg acc tac gac atc ctc aag gag aag ctg   804
Val Asn Cys Ala Glu Val Val Thr Tyr Asp Ile Leu Lys Glu Lys Leu
            195                 200                 205

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Leu Asp Tyr His Leu Leu Thr Asp Asn Phe Pro Cys His Phe Val Ser
        210                 215                 220

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Ala Phe Gly Ala Gly Phe Cys Ala Thr Val Val Ala Ser Pro Val Asp
    225                 230                 235

gtg gtg aag acc cgg tat atg aac tca cct cca ggc cag tac ttc agc   948
Val Val Lys Thr Arg Tyr Met Asn Ser Pro Pro Gly Gln Tyr Phe Ser
240                 245                 250                 255

ccc ctc gac tgt atg ata aag atg gtg gcc cag gag ggc ccc aca gcc   996
Pro Leu Asp Cys Met Ile Lys Met Val Ala Gln Glu Gly Pro Thr Ala
                260                 265                 270

ttc tac aag gga ttt aca ccc tcc ttt ttg cgt ttg gga tcc tgg aac   1044
Phe Tyr Lys Gly Phe Thr Pro Ser Phe Leu Arg Leu Gly Ser Trp Asn
            275                 280                 285

gtg gtg atg ttc gta acc tat gag cag ctg aaa cgg gcc ctg atg aaa   1092
Val Val Met Phe Val Thr Tyr Glu Gln Leu Lys Arg Ala Leu Met Lys
        290                 295                 300

gtc cag atg tta cgg gaa tca ccg ttt tgaacaagac aagaaggcca         1139
Val Gln Met Leu Arg Glu Ser Pro Phe
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Phe Leu Gly Ala Gly Thr Ala Ala Cys Phe Ala Asp Leu Val Thr Phe
             20                  25                  30

Pro Leu Asp Thr Ala Lys Val Arg Leu Gln Ile Gln Gly Glu Asn Gln
         35                  40                  45

Ala Val Gln Thr Ala Arg Leu Val Gln Tyr Arg Gly Val Leu Gly Thr
     50                  55                  60

Ile Leu Thr Met Val Arg Thr Glu Gly Pro Cys Ser Pro Tyr Asn Gly
 65                  70                  75                  80
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Leu Val Ala Gly Leu Gln Arg Gln Met Ser Phe Ala Ser Ile Arg Ile
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Gly Leu Tyr Asp Ser Val Lys Gln Val Tyr Thr Pro Lys Gly Ala Asp
100 105 110

Asn Ser Ser Leu Thr Thr Arg Ile Leu Ala Gly Cys Thr Thr Gly Ala
115 120 125

Met Ala Val Thr Cys Ala Gln Pro Thr Asp Val Val Lys Val Arg Phe
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Gln Ala Ser Ile His Leu Gly Pro Ser Arg Ser Asp Arg Lys Tyr Ser
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Gly Leu Trp Lys Gly Thr Leu Pro Asn Ile Met Arg Asn Ala Ile Val
180 185 190

Asn Cys Ala Glu Val Val Thr Tyr Asp Ile Leu Lys Glu Lys Leu Leu
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Asp Tyr His Leu Leu Thr Asp Asn Phe Pro Cys His Phe Val Ser Ala
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Phe Gly Ala Gly Phe Cys Ala Thr Val Val Ala Ser Pro Val Asp Val
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Val Lys Thr Arg Tyr Met Asn Ser Pro Pro Gly Gln Tyr Phe Ser Pro
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Tyr Lys Gly Phe Thr Pro Ser Phe Leu Arg Leu Gly Ser Trp Asn Val
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Val Met Phe Val Thr Tyr Glu Gln Leu Lys Arg Ala Leu Met Lys Val
290 295 300

Gln Met Leu Arg Glu Ser Pro Phe
305 310

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/12623

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C07K 14/705; C12N 15/12; G01N 33/53
US CL : 435/4, 69.1; 530/350, 300; 536/23.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/4, 69.1; 530/350, 300; 536/23.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, E	JABUREK, M. et al. Transport Function and Regulation of Mitochondrial Uncoupling Proteins 2 and 3. J. Biol. Chem. 10 September 1999, Vol. 274, No. 37, pages 26003-26007, see entire document.	1-6 and 10-11
Y	JEZEK, P. et al. A Structure-Activity Study of Fatty Acid Interaction with Mitochondrial Uncoupling Protein. 1997, Vol. 408, pages 166-170, see entire document.	1-6 and 10-11
Y	JEZEK, P. et al. Transport of Anions and Protons by the Mitochondrial Uncoupling Protein and its Regulation by Nucleotides and Fatty Acids. A New Look at Old Hypotheses. 21 October 1994, Vol. 269, No. 42, pages 26184-26190, see entire document.	1-6 and 10-11

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
09 SEPTEMBER 1999

Date of mailing of the international search report
25 OCT 1999

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Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/12623

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	HALESTRAP, A.P. Pyruvate and Ketone-Body Transport Across the Mitochondrial Membrane. Exchange Properties, pH-Dependence and Mechanism of the Carrier. June 1978, Vol. 172, No. 3, pages 377-387, see entire document.	1-6 and 10-11
Y	NALECZ, K. A. et al. The Activity of Pyruvate Carrier in a Reconstituted System:Substrate Specificity and Inhibitor Sensitivity. 15 August 1992, Vol. 297, No. 1, pages 162-168, see entire document.	1-6 and 10-11
Y	GARLID, K. S. et al. On the Mechanism of Fatty Acid-Induced Proton Transport by Mitochondrial Uncoupling Protein. 02 February 1996, Vol. 271, No. 5, pages 2615-2620, see entire document.	1-6 and 10-11
Y	ZEZEK, P. et al. Reconstituted Plant Uncoupling Mitochondrial Protein Allows for Proton Translocation via Fatty Acid Cycling Mechanism. 26 September 1997, Vol. 272, No. 39, pages 42472-24278, see entire document.	1-6 and 10-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/12623

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-6 and 10-11

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/12623

B. FIELDS SEARCHED
Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, SCISEARCH, EMBASE, JAPIO, WPIDS, EMBL58, N-GENSEQ35, N-ISSUED, EMBL-EST58, GENBANK-EST111, PIR60, SWISS-PROT37,\
search terms:mitochondrial anion carrier, MAC, pyruvate carrier, transport, fatty acid, UCP, anion carrier, lipid bilayer, lipid vesicle, symport, antiport, uncoupling protein, mitochondria

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1. In order for all inventions to be searched, the appropriate additional search fees must be paid.

Group I, claim(s)1-6 and 7, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, an indicator of MAC activity and MAC substrate where MAC is PYC and MAC substrate is pyruvate.
Group II, claim(s)1-6 and 8, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, an indicator of MAC activity and MAC substrate where MAC is PiC and MAC substrate is Pi.
Group III, claim(s)1-6 and 9, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, an indicator of MAC activity and MAC substrate where MAC is GC and MAC substrate is glutamate.
Group IV, claim(s)1-6 and 10-11, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, an indicator of MAC activity and MAC substrate where MAC is UCP and MAC substrate is FAA^-.
Group V, claim(s)1-6, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, an indicator of MAC activity and MAC substrate where MAC is AAC.
Group VI, claim(s)1-6, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, an indicator of MAC activity and MAC substrate where MAC is KGC/OGC.
Group VII, claim(s)1-6, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, an indicator of MAC activity and MAC substrate where MAC is DIC.
Group VIII, claim(s)1-6, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, an indicator of MAC activity and MAC substrate where MAC is CIC.
Group IX, claim(s)1-6, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, an indicator of MAC activity and MAC substrate where MAC is APC.
Group X, claim(s)12 and 13, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, a first MAC substrate, an indicator of MAC activity and a second MAC substrate where MAC is ACC and MAC substrates are ADP and ATP.
Group XI, claim(s)12 and 15, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, a first MAC substrate, an indicator of MAC activity and a second MAC substrate where MAC is AGC and MAC substrates are aspartate and glutamate.
Group XII, claim(s)12 and 16, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, a first MAC substrate, an indicator of MAC activity and a second MAC substrate where MAC is KGC/OGC and MAC substrates are alpha-ketoglutarate and malate.
Group XIII, claim(s)12 and 17, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, a first MAC substrate, an indicator of MAC activity and a second MAC substrate where MAC is DIC and MAC substrates are Pi and dicarboxylate.
Group XIV, claim(s)12 and 18, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, a first MAC substrate, an indicator of MAC activity and a second MAC substrate where MAC is CIC and MAC substrates are malate and citrate.
Group XV, claim(s)12 and 19, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, a first MAC substrate, an indicator of MAC activity and a second MAC substrate where MAC is APC and MAC substrates are $ATP\text{-}Mg^+$ and Pi.
Group XVI, claim(s)12 and 20, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises at least one MAC, a first MAC substrate, an indicator of MAC activity and a second MAC substrate where MAC is UCP and MAC substrate is FAA^-.
Group XVII, claim(s)21 and 22, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises a first MAC, a second MAC substrate, an indicator of second MAC activity and first MAC substrate, where first MAC is aspartate/glutamate carrier, second MAC is GC, first MAC substrate is aspartate and

the second MAC substrate is glutamate.
Group XVIII, claim(s)21 and 23, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises a first MAC, a second MAC, a second MAC substrate, an indicator of second MAC activity and first MAC substrate, where first MAC is APC,second MAC is PiC, first MAC substrate is ATP-Mg^{+} and the second MAC substrate is Pi.
Group XIX, claim(s)21 and 24, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises a first MAC, a second MAC, a second MAC substrate, an indicator of second MAC activity and first MAC substrate, where first MAC is dicarboxylate, second MAC substrate is Pi.
Group XX, claim(s)25 and 26, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises a first MAC, a second MAC, a third MAC, a second MAC substrate, a third MAC substrate, an indicator of third MAC activity and first MAC substrate, where first MAC is KGC/OGC,second MAC is dicarboxylate carrier and third MAC is PiC, first MAC substrate is alpha-ketoglutarate, second MAC substrate is malate and third MAC substrate is Pi.
Group XXI, claim(s)25 and 27, drawn to a method for identifying a compound which modulates the activity of a MAC which comprises a first MAC, a second MAC, a third MAC, a second MAC substrate, a third Mac substrate, an indicator of third MAC activity and first MAC substrate, where first MAC is CIC,
second MAC is DIC and third MAC is phosphate transporter, first MAC substrate is citrate, second MAC substrate is malate and third MAC substrate is Pi.
Group XXII, claim(s)28, drawn to an assay vehicle comprising at least ACC.
Group XXIII, claim(s)28, drawn to an assay vehicle comprising at least PiC.
Group XXIV, claim(s)28, drawn to an assay vehicle comprising at least PYC.
Group XXV, claim(s)28, drawn to an assay vehicle comprising at least AGC.
Group XXVI, claim(s)28, drawn to an assay vehicle comprising at least KGC/OGC.
Group XXVII, claim(s)28, drawn to an assay vehicle comprising at least DIC.
Group XXVIII, claim(s)28, drawn to an assay vehicle comprising at least CIC.
Group XXIX, claim(s)28, drawn to an assay vehicle comprising at least GC.
Group XXX, claim(s)28, drawn to an assay vehicle comprising at least APC.
Group XXXI, claim(s)28, drawn to an assay vehicle comprising at least AUC.

The inventions listed as Groups I-XXX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I is drawn a method of identifying a compound which modulates the activity of MAC, where MAC is PYC and MAC substrate is pyruvate. Pursuant 37 CFR 1.475(d), these claims are considered by the ISA/US to constitute the main invention, and none of the related groups II-XXXI correspond to the main invention. The special technical feature of Group I is a method for identifying compounds that modulate the activity of PYC using an assay system comprising at least one MAC. The methods of Groups II-XXI do not share a special technical feature in any pairing because the methods have materially different process steps using different MACs and each defines a separate invention over the art. The vesicles of Groups XXII-XXXI do not share a special technical feature in any pairing because each is drawn to a different MAC capable of separate use and manufacture.
Since no technical feature of any group other than the main invention is shared by any other invention, unity of invention is lacking.