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(57) Abrégé/Abstract:
A method is provided for promoting the folding of a polypeptide comprising at least one Ig domain which method comprises contacting the polypeptide with a molecular chaperone and a foldase.

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**(57) Abstract**

A method is provided for promoting the folding of a polypeptide comprising at least one Ig domain which method comprises contacting the polypeptide with a molecular chaperone and a foldase.

# METHOD FOR REFOLDING MOLECULES OF POLYPEPTIDES CONTAINING IG DOMAINS

## Field of the invention

5 The present invention relates to a method for refolding recombinant polypeptides comprising Ig domains. such as immunoglobulins.

## Background to the invention

10 Recombinantly produced immunoglobulins and other Ig fold-containing proteins find a wide variety of applications in research and medicine. However. yields of active immunoglobulins from a variety of expression systems tends to be low. Indeed. there is a general problem in expressing Ig-fold containing proteins in many expression systems. This is because in high yield expression systems such as *E. coli*. the proteins have a 15 tendency to aggregate and to form insoluble inclusion bodies. It is then very difficult to refold the insoluble proteins the result being that only very low efficiency of refolding is achieved, if at all. The alternative is to use a mammalian or other higher eukaryotic expression system, but this is often expensive and the protein yield much lower than in *E. coli*. Thus there is a need in the art for an improved method for producing recombinant 20 immunoglobulins in large quantities whilst maintaining high levels of activity.

## Summary of the Invention

Using oxidative refolding chromatography, as previously described by Altamirano *et al.* 25 (1999), we have shown that it is possible to refold denatured CD1A heavy chains and denatured β2M light chains in the absence of ligand to form a corectly folded, ligand-free CD1A/β2M heterodimer complex that is conformationally intact and which has full activity. Thus we have shown that it is possible to refold efficiently *in vitro* a polypeptide comprising an Ig domain.

30

Accordingly the present invention provides a method for promoting the folding of a polypeptide comprising at least one Ig domain. which method comprises contacting the

polypeptide with a molecular chaperone and at least one foldase. It is especially preferred that the contacting takes place under reducing conditions.

Preferably the molecular chaperone and/or foldase are immobilised to a solid phase, more preferably both the chaperone and foldase(s) are immobilised to a solid phase. Preferably the solid phase is a matrix. More preferably the matrix is present in a chromatography column.

Preferably the molecular chaperone is an hsp60 chaperonin or fragment thereof having refolding activity, more preferably a molecular chaperone fragment comprising a region consisting of fragments 191-376, 191-345 or 191-335 of the sequence of *E. coli* GroEL or a homologue thereof.

Preferably the foldase is selected from a thiol/disulphide oxidoreductase and a peptidyl-prolyl isomerase. Preferably the thiol/disulphide oxidoreductase is selected from *E. coli* DsbA and mammalian protein disulphide isomerase and the peptidyl prolyl isomerase is independently selected from cyclophilin, parbulen, SurA and FK506 binding proteins.

In a preferred embodiment the method of the invention comprises contacting the polypeptide with a molecular chaperone and both a thiol/disulphide oxidoreductase and a peptidyl-prolyl isomerase. Preferably, the thiol/disulphide oxidoreductase is DsbA and the peptidyl-prolyl isomerase is cyclophilin A.

The present invention also provides the use of a molecular chaperone and one or more foldases for promoting the folding of a polypeptide comprising at least one Ig domain.

In another aspect the invention provides a polypeptide comprising at least one Ig domain which polypeptide is obtainable by the method of the invention. Said polypeptide is typically obtained at higher yields and having a higher specific activity than a polypeptide obtained using normal methods of protein expression in, for example, non-mammalian expression systems such as *E. coli*. A polypeptide of the invention may be used in therapy.

Detailed description of the invention

Although in general the techniques mentioned herein are well known in the art, reference may be made in particular to Sambrook *et al.*, Molecular Cloning, A Laboratory Manual (1989) and Ausubel *et al.*, Current Protocols in Molecular Biology (1999 – 4th edition), John Wiley & Sons, Inc.

A. Molecular chaperones and Foldases

Molecular chaperones

Chaperones, including chaperonins, are polypeptides which promote protein folding by non-enzymatic means, in that they do not catalyse the chemical modification of any structures in folding polypeptides, by promote the correct folding of polypeptides by facilitating correct structural alignment thereof. Molecular chaperones are well known in the art, several families thereof being characterised. The invention is applicable to any molecular chaperone molecule, which term includes, for example, the molecular chaperones selected from the following non-exhaustive group:

p90 Calnexin, HSP family, HSP70 family, DNA K, DNAJ, HSP60 family (GroEL), ER-associated chaperones, HSP90, Hsc70, sHsps; SecA; SecB, Trigger factor, zebrafish hsp 47, 70 and 90, HSP 47, GRP 94, Cpn 10, BiP, GRP 78, C1p, FtsH, Ig invariant chain, mitochondrial hsp70, EBP, mitochondrial m-AAA, Yeast Ydj1, Hsp104, ApoE, Syc, Hip, TriC family, CCT, PapD and calmodulin (see WO99/05163 for references).

Two major families of protein folding chaperones which have been identified, the heat shock protein 60 (hsp60) class and the heat shock protein 70 (hsp70) class, are especially preferred for use herein. Chaperones of the hsp60 class are structurally distinct from chaperones of the hsp70 class. In particular, hsp60 chaperones appear to form a stable scaffold of two heptamer rings stacked one atop another which interacts with partially folded elements of secondary structure. On the other hand, hsp70 chaperones are monomers of dimers and appear to interact with short extended regions of a polypeptide.

Hsp70 chaperones are well conserved in sequence and function. Analogues of hsp70 include the eukaryotic hsp70 homologue originally identified as the IgG heavy chain binding protein (BiP). BiP is located in all eukaryotic cells within the lumen of the
5 endoplasmic reticulum (ER). The prokaryotic DnaK hsp70 protein chaperone in *Escherichia coli* shares about 50% sequence homology with an hsp70 KAR2 chaperone in yeast. Moreover, the presence of mouse BiP in yeast can functionally replace a lost yeast KAR2 gene.

10 Hsp60 chaperones are universally conserved and include hsp60 homologues from large number of species, including man. They include, for example, the *E. coli* GroEL polypeptide; *Ehrlichia sennetsu* GroEL; *Trichomonas vaginalis* hsp60; rat hsp60; and yeast hsp60.

15 In a preferred aspect, the present invention relates to fragments of polypeptides of the hsp60 family. These proteins being universally conserved, any member of the family may be used; however, in a particularly advantageous embodiment, fragments of GroEL, such as *E. coli* GroEL, are employed, especially those fragments termed minichaperones which are substantially monomeric in solution (see WO98/13496). Particularly preferred
20 fragments of *E. coli* GroEL described in WO98/13496 are discussed below.

The molecular chaperone may moreover be a circular permutation of a chaperone polyopeptide sequence. Circular permutation is described in Graf and Schachman, PNAS(USA) 1996, 93:11591; the strategy set forth may be applied generally to most of
25 the polypeptides with close N and C termini. The circularly permutated sequence chaperones or chaperone fragments maintain their protein folding activity. Essentially, the polypeptide is circularised by fusion of the existing N and C termini, and cleavage of the polypeptide chain elsewhere to create novel N and C termini.

30 Chaperone activity may be determined in practice by an ability to refold cyclophilin A but other suitable proteins such as glucosamine-6-phosphate deaminase or a mutant form of indoleglycerol phosphate synthase (IGPS) (amino acid residues 49-252) may be used. A

rhodanese refolding assay may also be used. Details of a suitable refolding assay are given below.

Preferred chaperone polypeptides of the present invention have protein refolding activity in the absence of adenosine triphosphate of more than 50%, preferably 60%, even more preferably 75%, said refolding activity being determined by contacting the chaperone polypeptide with an inactivated protein of known specific activity prior to inactivation, and then determining the specific activity of the said protein after contact with the polypeptide, the % refolding activity being:

$$\frac{\text{specific activity of protein after contact with polypeptide}}{\text{specific activity of protein prior to inactivation}} \times \frac{100}{1}$$

Preferably, the chaperone activity is determined by the refolding of cyclophilin A. More preferably, 8 M urea denatured cyclophilin A (100 μM) is diluted into 100 mM potassium phosphate buffer pH 7.0, 10 mM DTT to a final concentration of 1 μM and then contacted with at least 1 μM of said polypeptide at 25°C for at least 5 min, the resultant cyclophilin A activity being assayed by the method of Fischer *et al.* (1984).

It is preferred that chaperone polypeptides of the present invention are monomeric in solution and incapable of multimerisation in solution. Monomeric GroEL minichaperones are disclosed in WO98/13496. Typically, multimerisation is prevented by using chaperone polypeptides that lack the interacting domains found outside the apical domain, although it could be achieved by suitable mutations.

## Foldases

In general terms, a foldase is an enzyme which participates in the promotion of protein folding through its enzymatic activity to catalyse the rearrangement or isomerisation of bonds in the folding polypeptide. They are thus distinct from a molecular chaperone, which bind to polypeptides in unstable or non-native structural states and promote correct folding without enzymatic catalysis of bond rearrangement. Many classes of foldase are

known, and they are common to animals, plants and bacteria. They include peptidyl prolyl isomerases and thiol/disulphide oxidoreductases. The invention comprises the use of all foldases which are capable of promoting protein folding through covalent bond rearrangement.

5

Moreover, as used herein, the term "a foldase" includes one or more foldases. In general, in the present specification the use of the singular does not preclude the presence of a plurality of the entities referred to, unless the context specifically requires otherwise.

10 *Thiol/disulphide oxidoreductase.* As the name implies, thiol/disulphide oxidoreductases catalyse the formation of disulphide bonds and can thus dictate the folding rate of disulphide-containing polypeptides. The invention accordingly comprises the use of any polypeptide possessing such an activity. This includes chaperone polypeptides, or fragments thereof, which may possess protein disulphide isomerase activity. In
15 eukaryotes, thiol/disulphide oxidoreductases are generally referred to as protein disulphide isomerases (PDIs). PDI interacts directly with newly synthesised secretory proteins and is required for the folding of nascent polypeptides in the endoplasmic reticulum (ER) of eukaryotic cells.

20 Enzymes found in the ER with PDI activity include mammalian PDI, yeast PDI, mammalian ERp59, mammalian prolyl-4-hydroxylase, yeast GSBP and mammalian T3BP, *A. niger* PdiA and yeast EUGI (see WO99/05163 for references). In prokaryotes, equivalent proteins exist, such as the DsbA protein of *E. coli*. Other peptides with similar activity include, for example, p52 from *T. cruzi*. These polypeptides, and other
25 functionally equivalent polypeptides, are included with the scope of the present invention, as are derivatives of the polypeptides which share the relevant activity (see below). Preferably, the thiol/disulphide oxidoreductase according to the invention is selected from mammalian PDI or *E. coli* DsbA.

30 *Peptidyl-prolyl isomerase.* Peptidyl-prolyl isomerases (PPIs) are present in a wide variety of cells. Known examples include cyclophilin, parbulen, SurA and FK506 binding proteins FKBP51 and FKBP52 (see WO99/05163 for references). PPI is

responsible for the *cis-trans* isomerisation of peptidyl-prolyl bonds in polypeptides, thus promoting correct folding. The invention includes any polypeptide having PPI activity. This includes chaperone polypeptides, or fragments thereof, which may possess PPI activity.

5

*Derivatives, variants and fragments.* The present invention relates to derivatives of molecular chaperones and foldases (such as peptidyl-prolyl isomerases and thiol/disulphide oxidoreductases). In a preferred aspect, therefore, the terms "molecular chaperone", "peptidyl-prolyl isomerase" and "thiol-disulphide oxidoreductase" include

10 derivatives thereof which retain the stated activity. The derivatives which may be used according to the present invention include splice variants encoded by mRNA generated by alternative splicing of a primary transcript, amino acid mutants, glycosylation variants and other covalent derivatives of molecular chaperones or foldases which retain the functional properties of molecular chaperones, peptidyl-prolyl isomerases and/or thiol/disulphide

15 oxidoreductases.

Exemplary derivatives include molecules which are covalently modified by substitution, chemical, enzymatic, or other appropriate means with a moiety other than a naturally occurring amino acid. Such a moiety may be a detectable moiety such as an enzyme or a

20 radioisotope. Further included are naturally occurring variants of molecular chaperones or foldases found within a particular species, whether mammalian, other vertebrate, yeast, prokaryotic or otherwise. Such a variant may be encoded by a related gene of the same gene family, by an allelic variant of a particular gene, or represent an alternative splicing variant of a molecular chaperone or foldase.

25

Derivatives also include variants of naturally occurring chaperones/foldases, which may, for example, have been engineered or selected from libraries of mutagenised libraries derived from naturally occurring forms.

30 As noted above, the components of the combination according to the invention may comprise derivatives of molecular chaperones or foldases, including variants of such polypeptides which retain common structural features thereof. Variants which retain

common structural features can be fragments of molecular chaperones or foldases. Fragments of molecular chaperones or foldases comprise smaller polypeptides derived from therefrom. Preferably, smaller polypeptides derived from the molecular chaperones or foldases according to the invention define a single feature which is characteristic of the
5 molecular chaperones or foldases. Fragments may in theory be almost any size, as long as they retain the activity of the molecular chaperones or foldases described herein.

When applied to chaperone molecules, a fragment is anything other that the entire native molecular chaperone molecule which nevertheless retains chaperonin activity.
10 Advantageously, a fragment of a chaperonin molecule remains monomeric in solution. Preferred fragments are described below. Advantageously, chaperone fragments are between 50 and 200 amino acids in length, preferably between 100 and 200 amino acids in length and most preferably about 150 amino acids in length.

15 With respect to molecular chaperones of the GroEL/hsp-60 family, a preferred set of fragments have been identified which possess the desired activity. These fragments are set forth in our copending international patent application WO98/13496 and in essence comprise any fragment comprising at least amino acid residues 230-271 of intact GroEL, or their equivalent in another hsp60 chaperone. Preferably, the fragments should not
20 extend beyond residues 150-455 or 151-456 of GroEL or their equivalent in another hsp60 chaperones.

Advantageously, the fragments comprise the apical domain of GroEL, or its equivalent in other molecular chaperones, or a region homologous thereto as defined herein. The apical
25 domain spans amino acids 191-376 of intact GroEL. This domain is found to be homologous amongst a wide number of species and chaperone types. In a highly preferred embodiment, the fragments are selected from fragments consisting essentially of residues 191-376, 191-345, 191-335 or 193-335 of the sequence of intact GroEL.

30 Derivatives of the molecular chaperones or foldases also comprise mutants thereof, including mutants of fragments and other derivatives, which may contain amino acid deletions, additions or substitutions, subject to the requirement to maintain the activity of

the molecular chaperones or foldases described herein. Thus, conservative amino acid substitutions may be made substantially without altering the nature of the molecular chaperones or foldases, as may truncations from the 5' or 3' ends. Deletions and substitutions may moreover be made to the fragments of the molecular chaperones or
5 foldases comprised by the invention. Mutants may be produced from a DNA encoding a molecular chaperone or foldase which has been subjected to in vitro mutagenesis resulting e.g. in an addition, exchange and/or deletion of one or more amino acids. For example, substitutional, deletional or insertional variants of molecular chaperones or foldases can be prepared by recombinant methods and screened for immuno-crossreactivity with the
10 native forms of the relevant molecular chaperone or foldase.

The fragments, mutants and other derivative of the molecular chaperones or foldases preferably retain substantial homology with the native molecular chaperones or foldases. As used herein, "homology" means that the two entities share sufficient characteristics for
15 the skilled person to determine that they are similar in origin and function. Preferably, homology is used to refer to sequence identity. Thus, the derivatives of molecular chaperones or foldases preferably retain substantial sequence identity with native forms of the relevant molecular chaperone or foldase.

20 "Substantial homology", where homology indicates sequence identity, means more than 40% sequence identity, preferably more than 45% sequence identity and most preferably a sequence identity of at least 50%, 60% or more, as judged by direct sequence alignment and comparison.

25 Homology comparisons can be conducted by eye, or more usually, with the aid of readily available sequence comparison programs. These commercially available computer programs can calculate % homology between two or more sequences.

% homology may be calculated over contiguous sequences, i.e. one sequence is aligned with
30 the other sequence and each amino acid in one sequence directly compared with the corresponding amino acid in the other sequence, one residue at a time. This is called an

"ungapped" alignment. Typically, such ungapped alignments are performed only over a relatively short number of residues (for example less than 50 contiguous amino acids).

Although this is a very simple and consistent method, it fails to take into consideration that, for example, in an otherwise identical pair of sequences, one insertion or deletion will cause the following amino acid residues to be put out of alignment, thus potentially resulting in a large reduction in % homology when a global alignment is performed. Consequently, most sequence comparison methods are designed to produce optimal alignments that take into consideration possible insertions and deletions without penalising unduly the overall homology score. This is achieved by inserting "gaps" in the sequence alignment to try to maximise local homology.

However, these more complex methods assign "gap penalties" to each gap that occurs in the alignment so that, for the same number of identical amino acids, a sequence alignment with as few gaps as possible - reflecting higher relatedness between the two compared sequences - will achieve a higher score than one with many gaps. "Affine gap costs" are typically used that charge a relatively high cost for the existence of a gap and a smaller penalty for each subsequent residue in the gap. This is the most commonly used gap scoring system. High gap penalties will of course produce optimised alignments with fewer gaps. Most alignment programs allow the gap penalties to be modified. However, it is preferred to use the default values when using such software for sequence comparisons. For example when using the GCG Wisconsin Bestfit package (see below) the default gap penalty for amino acid sequences is -12 for a gap and -4 for each extension.

Calculation of maximum % homology therefore firstly requires the production of an optimal alignment, taking into consideration gap penalties. A suitable computer program for carrying out such an alignment is the GCG Wisconsin Bestfit package (University of Wisconsin, U.S.A.; Devereux *et al.*, 1984, Nucleic Acids Research 12:387). Examples of other software that can perform sequence comparisons include, but are not limited to, the BLAST package (see http://www.ncbi.nih.gov/BLAST/), FASTA (Atschul *et al.*, 1990, J. Mol. Biol., 403-410; FASTA is available for online searching at, for example,

http://www.2.ebi.ac.uk.fasta3) and the GENEWORKS suite of comparison tools. However it is preferred to use the GCG Bestfit program.

Although the final % homology can be measured in terms of identity, the alignment
5 process itself is typically not based on an all-or-nothing pair comparison. Instead, a scaled similarity score matrix is generally used that assigns scores to each pairwise comparison based on chemical similarity or evolutionary distance. An example of such a matrix commonly used is the BLOSUM62 matrix - the default matrix for the BLAST suite of programs. GCG Wisconsin programs generally use either the public default values or a
10 custom symbol comparison table if supplied (see user manual for further details). It is preferred to use the public default values for the GCG package, or in the case of other software, the default matrix, such as BLOSUM62.

Once the software has produced an optimal alignment, it is possible to calculate %
15 homology, preferably % sequence identity. The software typically does this as part of the sequence comparison and generates a numerical result.

The skilled person can identify suitable homologues by, for example, carrying out a search of online databases using all or part of a molecular chaperone/foldase sequence as a query
20 sequence. For example, a search of the Swissprot database using the BlastP program Ver 2.0.8 (default settings) (Jinghui Zhang *et al.*, 1997, Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", Nucleic Acids Res. 25:3389-3402) and amino acids 191 to 376 of *E. coli* GroEL as the query sequence identified well over a hundred homologous sequences, many of which gave homology scores of at least 50%
25 identity. Homologues identified include members of the hsp60 chaperonin family which includes the eubacterial GroEL, mitochondrial hsp60 and chloroplast cpn60. Other specific homologues together with their database accession numbers are detailed in WO98/13496.

Alternatively, sequence similarity may be defined according to the ability to hybridise to a
30 complementary strand of a nucleotide sequence encoding any of the chaperone or foldases mentioned above, such as *E. coli* GroEL, *E. coli* DsbA or mammalian cyclophilin A.

Preferably, the sequences are able to hybridise with high stringency. Stringency of hybridisation refers to conditions under which polynucleic acids hybrids are stable. Such conditions are evident to those of ordinary skill in the field. As known to those of skill in the art, the stability of hybrids is reflected in the melting temperature (Tm) of the hybrid which decreases approximately 1 to 1.5°C with every 1% decrease in sequence homology. In general, the stability of a hybrid is a function of sodium ion concentration and temperature. Typically, the hybridisation reaction is performed under conditions of higher stringency, followed by washes of varying stringency.

As used herein, high stringency refers to conditions that permit hybridisation of only those nucleic acid sequences that form stable hybrids in 1 M $Na^+$ at 65-68°C. High stringency conditions can be provided, for example, by hybridisation in an aqueous solution containing 6x SSC, 5x Denhardt's, 1 % SDS (sodium dodecyl sulphate), 0.1 $Na^+$ pyrophosphate and 0.1 mg/ml denatured salmon sperm DNA as non specific competitor. Following hybridisation, high stringency washing may be done in several steps, with a final wash (about 30 min) at the hybridisation temperature in 0.2 - 0.1x SSC, 0.1 % SDS.

Moderate stringency refers to conditions equivalent to hybridisation in the above described solution but at about 60-62°C. In that case the final wash is performed at the hybridisation temperature in 1x SSC, 0.1 % SDS.

Low stringency refers to conditions equivalent to hybridisation in the above described solution at about 50-52°C. In that case, the final wash is performed at the hybridisation temperature in 2x SSC, 0.1 % SDS.

It is understood that these conditions may be adapted and duplicated using a variety of buffers, e.g. formamide-based buffers, and temperatures. Denhardt's solution and SSC are well known to those of skill in the art as are other suitable hybridisation buffers (see, e.g. Sambrook, *et al.* (1989) *ibid* or Ausubel, *et al.* (1999) *ibid.* Optimal hybridisation conditions have to be determined empirically, as the length and the GC content of the probe also play a role.

B. Immobilisation of molecular chaperones/foldases on solid phase supports

In a preferred aspect, the contact between the Ig domain-containing polypeptides and the molecular chaperone and foldase occurs with the molecular chaperone and/or foldase immobilised on a solid support. Examples of commonly used solid supports include beads, "chips", resins, matrices, gels, and the material forming the walls of a vessel. Matrices, and in particular gels, such as agarose gels, may conveniently be packed into columns. A particular advantage of solid phase immobilisation is that the reagents may be removed from contact with the polypeptide(s) with facility.

Solid phase materials for use in batch or to be packed into columns are widely available – see for example Sigma's 1999 reagent catalogue entitled "Biochemicals, organic compoounds and diagnostic reagents" which includes a range of activated matrices suitable for coupling polypeptides such as cyanogen bromide activated matrices based on sepharose/agarose.

Molecular chaperones/foldases may be immobilised to a solid phase support such as by covalent means or otherwise. A variety of methods for coupling polypeptides to solid phase supports are known in the art. In a preferred aspect of the present invention molecular chaperones and/or preferably foldase polypeptides may be attached to a solid phase support using a method which comprises a reversible thiol blocking step. This is important where the peptide contains a disulphide. An example of such a method is described below.

Preferably, before protection the disulphides are reduced using a reducing agent such as DTT (dithiothreitol), under for example an inert gas, such as argon, to prevent reoxidation. Subsequently, the polypeptide is cyanylated, for example using NCTB (2-nitro, 5-thiocyanobenzoic acid) preferably in stoichiometric amounts, and subjected to controlled hydrolysis at high (non-acidic) pH, for example using $NaHCO_3$. In the case of DsbA, the pH of the hydrolysis reaction is preferably between 6.5 and 10.5 (the pK of DsbA is 4.0), more preferably between 7.5 and 9.5, and most preferably around about 8.5. The thiols are thus reversibly protected.

The polypeptide is then brought into contact with the solid phase component, for example at between 2.0 and 20.0 mg polypeptide/ml of solid component, preferably between 5.0 and 10.0 and most preferably around about 6.5 mg. The coupling is again carried out at a high (non-acidic) pH, for example using an $NaHCO_3$ coupling buffer. In the case of DsbA, the pH of the coupling reaction is preferably between 6.5 and 10.5, more preferably between 7.5 and 9.5, and most preferably around about 8.5.

Preferably, after coupling the remaining active groups may be blocked, such as with ethanolamine, and the uncoupled polypeptide removed by washing. Thiol groups may finally be regenerated on the coupled polypeptide by removal of the cyano groups, for example by treatment with DTE or DTT.

C. Methods of refolding polypeptides

The present invention provides a method for promoting the correct folding/refolding of a polypeptide comprising at least one Ig domain which method involves the use of a combination of a molecular chaperone and a foldase. The combination of a molecular chaperone and a foldase provides a synergistic effect on protein folding which results in a greater quantity of active, correctly folded protein being produced than would be expected from a merely additive relationship.

Preferably, one or more of the components used to promote protein folding in accordance with the present invention is immobilised on a solid support. However, both molecular chaperones and foldases may be used in solution. They may be used in free solution, but also in suspension, for example bound to a matrix such as beads, for example sepharose beads, or bound to solid surfaces which are in contact with solutions, such as the inside surfaces of bottles containing solutions, test tubes and the like.

Typically the method of the present invention is used to assist in refolding recombinantly produced Ig domain-containing polypeptides, which are obtained in an unfolded or misfolded form. Thus, recombinantly produced polypeptides may be contacted with a

molecular chaperone and a foldase to unfold, refold and/or reactivate recombinant polypeptides which are inactive due to misfolding and/or are unfolded as a result of there extraction from the host cells in which they were expressed (such as from bacterial inclusion bodies). Such a process may also be termed "reconditioning".

5

The method of the invention may be employed to maintain the folded conformation of Ig domain-containing polypeptides, for example during storage, in order to increase shelf life. Under storage conditions, many proteins lose their activity, as a result of disruption of correct folding. The presence of molecular chaperones, in combination with foldases, 10 reduces or reverses the tendency of polypeptides to become unfolded and thus greatly increases the shelf life thereof.

The method of the invention may be used to promote the correct folding of Ig domain-containing polypeptides which, through storage, exposure to denaturing conditions or 15 otherwise, have become misfolded. Thus, the invention may be used to recondition Ig domain-containing polypeptides. For example, immunoglobulins in need of reconditioning may be passed down a column to which is immobilised a combination of a molecular chaperone and a foldase in accordance with the invention. Alternatively, beads having immobilised thereon such a combination may be suspended in a solution 20 comprising the immunoglobulins in need of reconditioning. Moreover, the components of the combination according to the invention may be added in solution to the immunoglobulins in need of reconditioning.

The present invention also provides a method for altering the structure of an Ig domain-25 containing polypeptide. Structural alterations include folding, unfolding and refolding. The effect of the alterations is preferably to improve the yield, specific activity and/or quality of the molecule. This may typically be achieved by resolubilising, reconditioning and/or reactivating incorrectly folded molecules post-synthesis.

30 The terms "reconditioning" and "reactivating" thus encompass *in vitro* procedures. Particular examples of *in vitro* procedures may include processing polypeptides that have

been solubilised from cell extracts (such as inclusion bodies) using strong denaturants such as urea or guanidium chloride.

The terms "refold", "reactivate" and "recondition" are not intended as being mutually exclusive. For example, an inactive protein, perhaps denatured using urea, may have an unfolded structure. This inactive protein may then be refolded with a polypeptide of the invention thereby reactivating it. In some circumstances there may be an increase in the specific activity of the refolded/reactivated protein compared to the protein prior to inactivation/denaturation: this is termed "reconditioning".

The molecule is typically an unfolded or misfolded polypeptide which is in need of folding. Alternatively, however, it may be a folded polypeptide which is to be maintained in a folded state. Preferably, the polypeptide contains at least one disulphide linkage (or two cysteine residues capable of forming such as linkage under suitable conditions).

The invention envisages at least two situations. A first situation is one in which the polypeptide to be folded is in an unfolded or misfolded state, or both. In this case, its correct folding is promoted by the method of the invention. A second situation is one in which the polypeptide is substantially already in its correctly folded state, that is all or most of it is folded correctly or nearly correctly. In this case, the method of the invention serves to maintain the folded state of the polypeptide by affecting the folded/unfolded equilibrium so as to favour the folded state. This prevents loss of activity of an already substantially correctly folded polypeptide. These, and other, eventualities are covered by the reference to "promoting" the folding of the polypeptide.

As used herein, a polypeptide may be unfolded when at least part of it has not yet acquired is correct or desired secondary or tertiary structure. A polypeptide is misfolded when it has acquired an at least partially incorrect or undesired secondary or tertiary structure. Techniques are known in the art for assessing polypeptide structure – such as circular dichroism.

Contacting of the Ig domain-containing polypeptides with the chaperone/foldase combination preferably occurs under reducing conditions, such as in the presence of a combination of oxidised glutathione (GSSG) and glutathione (GSH) which act as a redox buffer system and prevent formation of disulphide bonds present in the oxidised state.

5

A particularly convenient method for contacting the molecular chaperone/foldase combination with the Ig domain-containing polypeptides involves incubating the Ig domain-containing polypeptides with the molecular chaperone/foldase combination, whereby the chaperone and foldase are immobilised to sepharose/agarose beads, in a tube,

10 such as an eppendorf tube, in a procedure known as a batch incubation. The tube contents are gently mixed for typically at least 5 minutes, preferably at least 1 to 3 hrs, before allowing the beads to settle by, for example, gravity or low speed centrifugation. The Ig domain-containing polypeptides in aqueous solution are then simply decanted off.

15 Another convenient method involves placing a solid phase matrix such as sepharose beads, to which the chaperone and foldase are immobilised, in a chromatography column, applying a sample comprising the polypeptide to be refolded to the top of the column and eluting the polypeptide through the column using a suitable buffer at a suitable rate. Such methods are well known in the art.

20

Polypeptides comprising at least one Ig domain

The term "Ig domain" is well known in the art and also includes Ig-like domains. An Ig domain comprises a motif termed an immunoglobulin fold which typically contains two

25 broad sheets of antiparallel β strands, generally bridged by a conserved disulphide bond (see Biochemistry. 1995. L. Stryer. Freeman. p370). Polypeptides comprising at least one Ig domain or Ig-like domain are generally members of the so-called immunoglobulin superfamily (for a review see Hunkapiller and Hood, 1989). Many members of the immunoglobulin superfamily are present as cell surface molecules where they may be

30 monomeric, dimers or higher oligomers.

In addition to naturally occurring polypeptides, variants or engineered polypeptides (such as polypeptides designed from first principles) comprising one or more Ig domains may also be used in the method of the invention. Variants and engineered polypeptides include alternatively spliced isoforms and differentially post-translationally modified forms (e.g. having different glycosylation patterns). Furthermore, polypeptides may be part of multi-protein complexes (such as hetero- or homodimers or other multimeric forms), the precise stoichiometry of which may be varied. Variants and engineered polypeptides may comprise mutations such as insertions and/or deletions. They may also comprise chemically modifications.

Polypeptides suitable for refolding using the method of the invention may also include polypeptides comprising core structural motifs similar to those seen in Ig folds such that they fold using a similar pathway to an Ig domain. A suggested pathway for Ig domain folding is described in Clarke *et al.*, 1999.

Examples of polypeptides comprising at least one Ig domain include immunoglobulins such as antibodies, or fragments thereof, MHC polypeptides such as MHC class I and class II polypeptides, T cell receptors (including CD3 ε, δ, γ), CD4, CD8 and CD28 co-receptors and the various Fc receptors present on lymphocytes and other white blood cells. Members of the immunoglobulin superfamily also include cell-adhesion molecules such as N-CAM and receptors for growth factors such as the NGF receptor, PDGF receptor, FGF receptor and VEGF receptor.

Other examples of polypeptides comprising one or more Ig-like domains are listed in Halaby and Mornon, 1998 (see also references therein). These include antibiotic proteins such as actinoxantin, bacterial proteins including chaperones and enzymes, carcinoembryonic antigen and related antigens, cytokine receptors, mammalian enzymes such as phosphatases and tyrosine kinases, proto-oncogenes, extracellular matrix proteins such as collagen, superoxide dismutase, immunoglobulins, immunoglobulin receptors, cell surface antigens of the immune system such as B7, CD2, CD3., CD7, CD22, ICAM-1, ICAM-2, ICAM-2, p58, V7 and CTLA4, invertebrate proteins such as hemocyanin, fascicilin, lachesin, neural/ectodermal development factor, molluscan

defence molecule and sevenless, muscle proteins such as connectin and myosin light-chain kinase, neural proteins such as axonal surface protein, tumour markers such as DCC, B-CAM, IAP (CD47) and viral proteins such as vaccinia virus hemaglutinin, herpes simplex virus glycoprotein C and cowpox virus interleukin 1 binding protein.

5

Polypeptides comprising at least one Ig domain have been identified for a variety of species of organisms and thus polypeptides comprising at least one Ig domain may originate from vertebrates such as mammals, invertebrates, such as insects, nematodes and marine sponges, plants, prokaryotes such as eubacteria, and viruses.

10

It is particularly preferred that the polypeptide comprising at least one Ig domain is an antigen presenting molecule, more preferably an antigen presenting molecule involved in T cell recognition of microbial antigens such as lipid and glycolipid antigens. In a preferred embodiment, the polypeptide is a member of the CD1 family, non-polymorphic, 15 MHC class 1-like molecules that associate non-covalently with β2-microglobulin.

The Ig domain-containing polypeptides to be processed by the method of the invention is typically obtained from cell extracts of host cells expressing recombinant Ig domain-containing polypeptides or their precursors. However, recombinant proteins may be 20 obtained from any source such as *in vitro* translation systems. Host cells include prokaryotes such as *E. coli*, yeast and insect cells (the baculovirus system is capable of very high level protein expression). Expression of the polypeptide comprising at least one Ig domain in the host cell is preferably at high levels to maximise yield. However, as discussed above, it is likely that a substantial proportion of the polypeptide comprising at 25 least one Ig domain will be insoluble and consequently techniques to solubilise normally insoluble components of the cell extracts (such as inclusion bodies) to maximise extraction of the polypeptide comprising at least one Ig domain will typically be employed. Such techniques include sonication of cells in the presence of strong denaturants such as urea or guanidium chloride.

30

Solubilised cell extracts may optionally be partially purified by, for example, a variety of affinity chromatography techniques prior to contacting with the chaperone/foldase combination according to the method of the invention.

5 Thus the starting material for the refolding/reconditioning method of the invention is typically denatured polypeptides in solutions of agents such as urea/guanidium chloride. Alternatively, or in addition, soluble polypeptide samples may be specifically denatured by the addition of appropriate denaturing agents prior to refolding. The untreated polypeptides samples may be dialysed against a suitable refolding buffer prior to contact 10 with the chaperone/foldase combination if required.

At the end of the refolding/reconditioning process, the refolded polypeptides are typically desalted by dialysis against a suitable storage buffer and/or the use of a desalting column into a suitable storage buffer. Suitable buffers include 25 mM sodium phosphate, 150 15 mM NaCl and 0.1% PEG 6000 (pH 7.4).

The activity of the refolded/reconditioned polypeptide comprising at least one Ig domain preferably has at least 30% activity relative to wild type polypeptide (for example an equivalent antibody secreted by a hybridoma), which has been treated in the same way, 20 more preferably at least 40, 45 or 50% activity. Activity may conveniently be assessed using, for example, an ELISA.

D. Uses of refolded/reconditioned Ig domain-containing polypeptides

25 Polypeptides comprising at least one Ig domain which polypeptides are produced by the method of the present invention may be used in a variety of applications both therapeutic and non-therapeutic. For example, they may be used in scientific research, such as assay systems including antibody-antigen assays and proteomic chip systems. They may also be used in therapeutic applications requiring the use of Ig-domain containing polypeptides, 30 include the treatment or amelioration of infectious, neoplastic, immunological, autoimmune, degenerative, vascular, inflammatory, endocrine, drug or toxin related or other disease or acute, subacute or chronic conditions. As a specific example, they may

be used in immunoregulation, via the administration of soluble Ig-domain containing polypeptides to individuals which interact with specific ligands or their soluble or cell membrane bound receptors, or have an indirect effect on cellular function, for example in immunosuppression, cell adhesion (such as in the prevention of tumour cell metastasis), 5 or on isolated body organs (such as in organ transplantation or artificial tissue such as skin, renal or hepatic substitutes). Another specific use is in cancer therapy using therapeutic compounds linked to antibodies or fragments thereof (such as ADEPT). Further therapeutic uses include prophylaxis, either directly or indirectly, for example in the prevention of infectious, neoplastic, immunological (immunomodulation such as the 10 manipulation of B and T cells and their subsets), autoimmune, degenerative, vascular, inflammatory, endocrine, drug or toxin related or other disease or acute, subacute or chronic conditions.

Polypeptides obtainable by the method of the invention may also be used in 15 immunotherapy and/or to treat a disease associated with a defect in an Ig domain containing polypeptide. Other applications include medical diagnosis, for example in protein array "chip" devices which assay levels of soluble Ig-domain containing and other types of soluble molecules in liquid biological samples such as serum, urine, saliva, cerebrospinal fluid and ascitic fluid.

20

Further applications include industrial processing of enzymes, use in biological computing devices or as biosensors. Polypeptides produced by the method of the invention may also be used in self-assembling molecular system devices such as when they are linked by covalent or other means to other molecules such as DNA tags to pattern 25 the Ig-domain containing polypeptides onto surfaces at the molecular scale.

## E. Administration

Polypeptides and/or antibodies obtained by the method of the invention may preferably be 30 combined with various components to produce compositions of the invention. Preferably the compositions are combined with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition (which may be for human or animal use). Suitable

carriers and diluents include isotonic saline solutions, for example phosphate-buffered saline. The composition of the invention may be administered by direct injection. The composition may be formulated for parenteral, intramuscular, intravenous, subcutaneous, intraocular or transdermal administration. Typically, each protein may be administered at 5 a dose of from 0.01 to 30 mg/kg body weight, preferably from 0.1 to 10 mg/kg, more preferably from 0.1 to 1 mg/kg body weight.

The routes of administration and dosages described are intended only as a guide since a skilled practitioner will be able to determine readily the optimum route of administration 10 and dosage for any particular patient and condition.

The invention will now be further described by way of Examples, which are meant to serve to assist one of ordinary skill in the art in carrying out the invention and are not intended in any way to limit the scope of the invention.

15

## EXAMPLES

The CD1 antigens are non-polymorphic, MHC class 1-like molecules that associate non-covalently with β2-microglobulin. The CD1 proteins are a family of antigen-presenting 20 molecules that allow T cells to recognise a range of antigens including lipids, glycolipids, peptides, and other types of antigens derived from microbial and viral pathogens. These include *mycobacterium tuberculosis*, a pathogen that is of global significance and which is responsible for considerable morbidity and mortality.

25 **Materials and Methods**

**Mixed bed mini-chaperone/DsbA/PPI gels**

*Expression, purification and immobilisation of the mini-chaperone.*

The mini-chaperone (191-345 peptide fragment from *E. coli* GroEL), is cloned and 30 expressed in *E. coli* as a fusion protein containing a 17-residue N-terminal histidine tail (Zahn *et al.*, 1996). The mini-chaperone is immobilised on agarose gel beads as previously reported (Altamirano *et al.*, 1997) except that NHS-activated Sepharose-4 Fast

Flow (Pharmacia Biotech, Sweden) is used. This activated gel, which has a longer spacer arm than that used in our former preparation, is more efficient and stable. Leakage is reduced to zero and the capacity to refold cyclophilin A, is increased to 6 mg of substrate per mL of wet gel, that is 1.5 times the value for the previously reported refolding gel.

5

*Expression, purification and Immobilisation of Human PPI.*

Human PPI (peptidyl-prolyl *cis-trans*-isomerase) is expressed and purified as described (Jasanoff *et al.*, 1994) with some minor modifications. Briefly a plasmid carrying the gene of fusion protein GST-PPI is used to transform the *E. coli C41* D3 strain (Miroux
10 and Walker, 1996). The cells are grown in 2xTY medium at 34°C. Innoculae are grown up to $A_{600}$ = 0.5 before induction with 0.7 mM isopropyl β-D-thiogalactoside and the cultures are allowed to grow for 16 h at 25 °C before being harvested. The cell pellet is resuspended in buffer (50 mM sodium phosphate, pH 7.5, 100 mM NaCl, 1% Triton X100 and 0.2 mM PMSF), sonicated to release proteins, and the protein is purified by
15 affinity chromatography using glutathione agarose. The bound fusion protein is then treated with thrombin on the column to obtain free PPI. The thrombin also present in the eluate is removed by affinity chromatography on benzamidine agarose. The purity of the PPI is verified by SDS-PAGE and FPLC using a Superdex 75 column (Pharmacia Biotech). PPI is assayed as previously described and bound to NHS-Sepharose 4 fast flow
20 as described above for mini-chaperone immobilisation.

*Cloning, expression, and purification of DsbA.*

The *E. coli dsbA* gene is amplified by PCR using *dsbA*-Fo and *dsbA*-Ba primers, based on its known sequence. The amplified whole expressed gene, including its signal peptide is
25 digested with *NcoI* and *BamHI* and cloned into the high expression plasmid pCE820 (Lewis *et al.*, 1993). The pMA14 (pCE820-*DsbA*) is purified and the sequence is confirmed by standard sequencing techniques. The *dsbA* gene product is overproduced in the *E. coli C41 D3* strain (Miroux and Walker, 1996) and appears almost exclusively in the periplasmic fraction. The cells are grown in 2XTY medium at 37°C. Innoculae are
30 grown up to $A_{600}$= 0.2 before induction with 0.7 mM isopropyl β-D-thiogalactoside and the cultures are allowed to grow for 12-14 h at 30 °C before being harvested. Cell proteins are fractionated in spheroplasts and the resulting soluble periplasm content is

prepared by using the lysozyme/EDTA method. The suspension containing the spheroplasts is centrifuged (48.000 X g, 30 min. at 4 °C). Proteins are desalted in 10 mM MOPS/NaOH, pH 7.0 by diafiltration using 10 kDa cut-off membranes in a tangential flow system (Minisette, Filtron). DsbA protein is purified by ion-exchange chromatography using a Mono-Q HR 10/10 FPLC column (Pharmacia, Biotech) which is eluted with a shallow KCl gradient (0-250 mM). DsbA emerges at about 70 mM KCl and is > 95% pure as shown by SDS-PAGE (20% gels) and also by gel filtration chromatography (Superdex 75, Pharmacia Biotech). The concentration of DsbA protein is calculated from its absorption at 280 nm, using the absorption coefficient $A_{280}$, 1mg/mL/cm = 1.10 for the native oxidised protein. The activity of the soluble DsbA protein is determined by using the spectrofluorometric method described by Wunderlich (1993).

*Reversible blocking of Cys-30 in DsbA protein in an inert atmosphere.*

All the experiments are performed in a glove box in an argon (Ar) atmosphere and the solution reagents are pre-saturated with Ar. The disulphide group at the active site of DsbA is reduced with 5 mM DTT, in 25 mM MES-$K^+$ buffer pH 6.0 for 1 h; DTT is then removed by dialysis under Ar to avoid reoxidation. DsbA is then cyanylated under Ar with NTCB (2-nitro-5-thiocyanate benzoate) (Altamirano, *et al.*, 1989; Altamirano *et al.*, 1992) at a final concentration of 5 mM. The reaction is practically instantaneous and it is apparent from the appearance of a yellow colour from the departing group, the anion 2-nitro-5-thiobenzoate. After 30 min the extent of the reaction is evaluated by measuring its absorption at 412 nm ($\varepsilon_{412}$= 14,140 $M^{-1}$ $cm^{-1}$) and it is found to be stoichiometric (Altamirano *et al.*, 1992). The protein is chromatographically desalted (desalt 10/10 column, Pharmacia Biotech) in 50 mM $NaHCO_3$ buffer, pH 8.3/0.5 M KCl.

*Attachment to NHS-activated Sepharose-4 Fast Flow Gel.*

5 mL of wet gel (NHS-activated sepharose-4 fast flow from Pharmacia Biotech, Sweden) is washed with 15 volumes of cold 1 mM HCl and then suspended in 50 mM $NaHCO_3$ at pH 8.3/0.5 M KCl, mixed in an end-over-end shaker for 1 min at room temperature. DsbA protein, with its thiols reversibly blocked, is added to the gel suspension (7 mg protein/mL gel) and mixed in an end-over-end shaker for 2 h at room temperature. It is

then washed with the coupling buffer. The remaining active groups are blocked by adding 2.5 M ethanolamine at pH 8 and mixing at room temperature for 4 h. Uncoupled DsbA is removed by washing with five cycles of alternately high and low pH buffer solution (Tris-HCl 0.1M pH 7.8 containing 0.5 M NaCl followed by acetate buffer, 0.1M, pH 4 plus 5 0.5 M NaCl). The gel is finally washed with 5-10 gel volumes of refolding buffer (see below) and SH groups regenerated by treatment with DTT. The gel is washed with ten times gel volume of refolding buffer. After this, the immobilised DsbA protein is oxidised as detailed under experimental protocol. The coupling efficiency of this procedure is higher than 95 %.

10

All the refolding experiments are performed in a batch mode. After use, the gel is regenerated by washing with 5 volumes of stored buffer (100 mM sodium phosphate pH 8 + 2 mM EDTA + 0.5 M KCl). The gel is stable for at least one year when stored at $4^{o}C$ in 100 mM sodium phosphate pH 7.0, containing 2 mM EDTA.

15

**Batchwise renaturation of CD1A/β2M.**

*Preparation of Denatured and reduced CD1A/β2M.*

CD1A/β2M (250 mg) is dissolved (with the help of a douncer) in a minimum volume of freshly made 6 M guanidinium chloride prepared in 0.1M potassium phosphate buffer (pH 20 8) and adjusted to pH8 with an appropriate volume of 4N NaOH. It is then, reduced with 0.1 M DTT and left for 2 h in light-free conditions at $25^{o}C$ to ensure the completeness of the reaction. The fluorescence and CD spectrum of reduced and denatured CD1A/β2M are the typical ones for a denatured protein.

25 **Refolding matrix and folding of CD1A/β2M toxin**

The ternary refolding matrix is obtained by mixing equal concentrations of mini-chaperone, DsbA protein and PPI. The matrix buffer is equilibrated with a freshly made pH 8 buffer prepared with 50 mM Tris-HCl (pH 8), 0.3 M L-arginine hydrochloride, 8 mM oxidised glutathione, 1 mM sodium EDTA and 0.2 M PMSF (refolding buffer). 30 The denatured and reduced human CD1A heavy chain and β2M light chain are then mixed together in a molar ratio of 1:4 (CD1A:β2M). The mixture is then added very slowly (dropwise), mixed and immediately diluted 100-fold with a resuspension of the

ternary refolding matrix which as been equilibriated with the refolding buffer described above. A maximum of 4 mg protein was added for each 1 ml of packed ternary matrix material. The mixture is then kept mixing slowly at 4°C. After between 10 mins to 12 h, the gel suspension is then centrifuged to separate the supernatant. The gel pellet is
5 washed with refolding buffer containing 0.5 M KCl. The preparations are eventually concentrated, chromatographically desalted by replacing the refolding buffer by water or 50 mM ammonium acetate buffer (pH 5.5) and then lyophilised.

Each of the three refolding proteins (mini-chaperone or DsbA or PPI) used is also
10 individually tested and a control experiment is also made using refolding buffer alone.

**Results**

**Refolding and reconstitution of recombinant human CD1A/β2M complex.**

15 The refolding matrix miniGroEL/DsbA/PPI (oxidative refolding chromatography) (Altimirano *et al.*, 1999) proved to be efficient in restoring the native structure and biological properties of the ligand-free CD1A/β2 microglobin heterodimer complex (Table I and Table II).

20 Samples from different refolding protocols were analysed by gel filtration chromatography and by measurement of their nephelometric turbidity (Table I). Each of the three refolding proteins (miniGroEL-agarose, DsbA-agarose, PPI-agarose, (as defined by Altamirano *et al.*, 1999)) was also individually tested using the same refolding buffer described for the ternary matrix (Table II). Control experiments were performed in which
25 the denatured and reduced protein were added either to the refolding buffer alone or to the refolding buffer plus a mixture of miniGroEL and foldases in solution (Table I). HPLC analysis demonstrated the presence of three peaks. One of these corresponded to the expected molecular weight (42 kDa) of the refolded ligand-free CD1A/β2 microglobin heterodimer complex (Table II). This peak was absent if refolding was performed with
30 either the chaperone alone, or the foldases alone or a buffer only control.

**Recovery of solubilised protein product.**

The refolding buffer by itself produced less than 5% of soluble protein, the majority of which was aggregated. PPI-agarose gave a yield of about 10% of soluble protein, but it was mainly aggregated. DsbA-agarose gave a 10-15% yield of soluble protein, only 1-5% of which was monodisperse (Table I). The binary refolding matrix of miniGroEL and DsbA gave a high yield of protein, of which 74% was monodisperse. The ternary matrix (miniGroEL/DsbA/PPI-agarose) solubilises essentially all the sample (98%), of which 87% appears as a single symmetrical chromatographic peak at the retention time corresponding to its expected molecular mass (Table I). Its profile in reverse-phase HPLC using analytical C-18 column is the same as the native protein (data not shown). The nephelometric turbidity of the samples was measured at 350 nm (Table I), which indicated its extent of aggregation (Altamirano *et al.*, 1999).

**Immunogenicity of refolded CD1A**

Refolded CD1A was assayed for activity using the CD1A-specific monoclonal antibody NA1/34 in a specially configured inhibition assay. The refolded molecule was found to have recovered the full conformational antigenicity as compared with a control sample of CD1A produced by the human CD1A mouse myeloma transfectant 10B3.

**Structural analysis**

Fluorescence and CD spectrum analysis revealed profiles that were typical for that of a refolded protein.

**Discussion**

This result constitutes the first example of the *in vitro* refolding of this class of molecule in the absence of a ligand. The result strongly indicates that it will be possible to use oxidative refolding chromatography both for the production of other empty CD1 molecules including human CDb, -c, -d and -e and for the production of empty and ligand-full MHC class I and II molecules *in vitro*. Further, the result also indicates that oxidative refolding chromatography will enable the *in vitro* refolding of many if not all the other members of the immunoglobulin supergene family and other Ig-like fold

containing proteins, including molecules of significant biological and potential immunotherapeutic importance, such as CD8, CD4 and immunoglobulins. Oxidative refolding chromatography should also enable the production *in vitro* of other immunoglobulin supergene family complexes, both natural and artificial. Empty and
5 ligand-full soluble soluble CD1, MHC and other Ig superfamily molecules, their engineered and naturally occurring alternative forms and their complexes, are themselves likely to be of potential therapeutic, prophylactic, general industrial, diagnostic, scientific and biotechnological importance.

10 All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the described methods and system of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed
15 should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or related fields are intended to be within the scope of the following claims.

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CLAIMS

1. A method for promoting the folding of a polypeptide comprising at least one Ig domain which method comprises contacting the polypeptide with a molecular chaperone and a foldase.

2. A method according to claim 1 wherein said molecular chaperone and/or foldase are immobilised to a solid phase.

3. A method according to claim 2 wherein the solid phase is a matrix.

4. A method according to claim 3 wherein the matrix is present in a chromatography column.

5. A method according to any one of the preceding claims wherein the polypeptide is an unfolded or misfolded polypeptide.

6. A method according to any one of the preceding claims wherein the molecular chaperone is an hsp-60 chaperonin or fragment thereof having refolding activity.

7. A method according to claim 6, wherein the molecular chaperone fragment comprises a region consisting of fragments 191-376, 191-345, 191-335 or 193-335 of the sequence of *E. coli* GroEL or a homologue thereof.

8. A method according to any preceding claim, wherein the foldase is selected from a thiol/disulphide oxidoreductase and a peptidyl-prolyl isomerase.

9. A method according to claim 8, wherein the thiol/disulphide oxidoreductase is selected from *E. coli* DsbA and mammalian protein disulphide isomerase.

10. A method according to claim 8, wherein the peptidyl prolyl isomerase is selected from cyclophilin, parbulen, SurA and FK506 binding proteins.

11. A method according to any one of the preceding claims comprising contacting the polypeptide with a molecular chaperone and both a thiol/disulphide oxidoreductase and a peptidyl-prolyl isomerase.

12. A method according to claim 11 wherein the thiol/disulphide oxidoreductase is DsbA and the peptidyl-prolyl isomerase is cyclophilin A.

13. A method according to any one of the preceding claims wherein the contacting takes place under reducing conditions.

14. A method according to any one of the preceding claims wherein the polypeptide has been expressed in a host cell selected from a prokaryote, a yeast and an insect cell.

15. Use of a molecular chaperone and one or more foldases for promoting the folding of a polypeptide comprising an Ig domain.

16. A polypeptide comprising at least one Ig domain, which polypeptide is obtainable by the method of any one of claims 1 to 14.

17. Use of a polypeptide according to claim 16 in therapy.

18. Use of a polypeptide according to claim 16 in an industrial process.