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(54) Titre : PROCEDE DE PRODUCTION DE CAROTENOIDE
(54) Title: CAROTENOID PRODUCTION METHOD

(57) Abrégé/Abstract:
A mutant carotenoid-producing bacterium that includes a gene set forth in any of (a) to (c). (a) A gene of a carotenoid-producing bacterium, which codes for a protein that includes a mutant amino acid sequence in which at least the 225th amino acid residue of an amino acid sequence of 1-deoxy-D-xylulose 5-phosphate synthase is substituted with another amino acid residue; (b) a gene of a carotenoid-producing bacterium, which codes for a protein that includes a mutant amino acid sequence in which at least the 305th amino acid residue of an amino acid sequence of decaprenyl diphosphate synthase is substituted with another amino acid residue; and (c) both of the genes in (a) and (b).

ABSTRACT

A mutant carotenoidogenic bacterium, comprising any of genes (a)-(c) below:

(a) a gene encoding a protein comprising a mutant amino acid sequence in which at least the 225th amino acid residue in the amino acid sequence of 1-deoxy-D-xylulose 5-phosphate synthase of a carotenoidogenic bacterium has been substituted with other amino acid residue;

(b) a gene encoding a protein comprising a mutant amino acid sequence in which at least the 305th amino acid residue in the amino acid sequence of decaprenyl diphosphate synthase of a carotenoidogenic bacterium has been substituted with other amino acid residue; and

(c) both of the genes (a) and (b) above.

DESCRIPTION

CAROTENOID PRODUCTION METHOD

FIELD OF THE INVENTION

[0001] The present invention relates to a method for producing a carotenoid using a mutant strain of a carotenoidogenic bacterium.

BACKGROUND ART

[0002] Carotenoids are useful natural pigments that can be used as feed additives, food additives, pharmaceutical products and the like. Examples of carotenoids include astaxanthin, canthaxanthin, zeaxanthin, β-cryptoxanthin, lycopene, β-carotene, adonirubin, adonixanthin, echinenone, asteroidenone and 3-hydroxyechinenone. Among them, astaxanthin is useful as a feed additive such as a body color improving agent for farmed fish such as salmon, trout and red sea bream, and an egg-yolk color improving agent for poultry and the like. Natural astaxanthins are also highly valuable in industries as safe food additives and health food ingredients. Similar to astaxanthin, adonixanthin and adonirubin are expected of their use as feed additives, food additives, pharmaceutical products and the like.

[0003] In addition, β-carotene is used as a feed additive, a food additive, a pharmaceutical product and a like, canthaxanthin is used as a feed additive, a food additive, a cosmetic product and the like, and zeaxanthin is used as a food additive, a feed additive and the like. Furthermore, lycopene, echinenone, β-cryptoxanthin, 3-hydroxyechinenone and asteroidenone are also expected of their use as feed additives, food ingredients and the like. As a method for producing such carotenoids, a chemical synthesis method, an extraction method from a natural product, a production method by culturing microorganisms, and the like are known.

[0004] As the chemical synthesis method for astaxanthin, a method by β-carotene conversion (Pure Appl. Chem., 57, 741, 1985 (Non-patent document 1)) and a method by synthesizing from a C15 phosphonium salt (Helv. Chim. Acta, 64, 2436, 1981 (Non-patent

document 2)) are known. As the extraction method from a natural product, astaxanthin may be extracted and collected from fish like salmon or red sea bream, or a crustacean like shrimp, crab or krill in which astaxanthin is present.

[0005] Examples of the method for producing a carotenoid using microorganisms include a culture method using a green alga *Haematococcus pluvialis* (Japanese Patent Application Publication No. 2007-97584 (Patent document 1)), a fermentation method using red yeast *Phaffia rhodozyma* (Japanese Patent Application Publication No. H11(1999)-69969 (Patent document 2)), a fermentation method using a bacterium belonging to the genus *Paracoccus* (hereinafter, also referred to as a "bacterium of the genus *Paracoccus*"), a fermentation method using a bacterium belonging to the genus *Brevundimonas* (Japanese Patent Application Publication No. 2006-340676 (Patent document 3)), and a fermentation method using a bacterium belonging to the genus *Erythrobacter* (Japanese Patent Application Publication No. 2008-259449 (Patent document 4)). Examples of the bacteria of the genus *Paracoccus* that produce carotenoids include strains E-396 and A-581-1 (Japanese Patent Application Publication No. H7(1995)-79796 (Patent document 5) and International Journal of Systematic Bacteriology (1999), 49, 277-282 (Non-patent document 3)). Examples of other carotenoid-producing bacteria belonging to the genus *Paracoccus* include *Paracoccus marcusii* strain MH1 (Japanese Patent Application Publication No. 2001-512030 (Patent document 6)), *Paracoccus haeundaensis* strain BC74171 (International Journal of Systematic and Evolutionary Microbiology (2004), 54, 1699-1702 (Non-patent document 4)), bacterium *Paracoccus* strain sp. N-81106 (Japanese Patent Application Publication No. 2007-244205 (Patent document 7)), *Paracoccus zeaxanthinifaciens* (International Journal of Systematic and Evolutionary Microbiology (2003), 53, 231-238 (Non-patent document 5)) and *Paracoccus* sp. strain PC-1 (pamphlet of WO2005/118812 (Patent document 8)).

[0006] The above-mentioned methods for producing carotenoids, however, have several problems. For example, a carotenoid produced by a chemical synthesis method may be safe but gives unfavorable impression to the consumers. The production cost of carotenoids extracted from natural products are much more expensive than the chemical synthesis method. Among the productions using microorganisms, production by culturing a gree alga or a yeast is low in productivity and difficult in extracting carotenoids from the cultured products since these microorganisms have strong cell walls.

[0007] Meanwhile, several culture methods and production methods have been reported for carotenoid productions using bacteria belonging to the genus *Paracoccus* because they are advantageous in terms of fast bacterial growth rate, high carotenoid productivity, and easy carotenoid extraction from the cultured product.

[0008] For example, Japanese Patent Application Publication No. 2007-143492 (Patent document 9) discloses a method of adding an iron salt during culturing, pamphlet of WO2010/044469 (Patent document 10) discloses a method of adding amino acids to the medium, Japanese Patent Application Publication No. 2011-188795 (Patent document 11) discloses a method of adding biotin to the medium, and Japanese Patent Application Publication No. 2012-139164 (Patent document 12) discloses a method of adding a calcium compound to the medium to 3.6 mM or more.

However, details as to which gene of the carotenoid-producing bacteria contributes to the increase in the production efficiency have been unclear.

PRIOR ART DOCUMENTS

[0009] Patent documents

Patent document 1: Japanese Patent Application Publication No. 2007-97584

Patent document 2: Japanese Patent Application Publication No. H11(1999)-69969

Patent document 3: Japanese Patent Application Publication No. 2006-340676

Patent document 4: Japanese Patent Application Publication No. 2008-259449

Patent document 5: Japanese Patent Application Publication No. H7(1995)-79796

Patent document 6: Japanese Patent Application Publication No. 2001-512030

Patent document 7: Japanese Patent Application Publication No. 2007-244205

Patent document 8: Pamphlet of WO2005/118812

Patent document 9: Japanese Patent Application Publication No. 2007-143492

Patent document 10: Pamphlet of WO2010/044469

Patent document 11: Japanese Patent Application Publication No. 2011-188795

Patent document 12: Japanese Patent Application Publication No. 2012-139164

[0010] Non-patent documents

Non-patent document 1: Pure Appl. Chem., 57, 741, 1985

Non-patent document 2: Helv. Chim. Acta, 64, 2436, 1981

Non-patent document 3: International Journal of Systematic Bacteriology (1999), 49, 277-282

Non-patent document 4: International Journal of Systematic and Evolutionary Microbiology (2004), 54, 1699-1702

Non-patent document 5: International Journal of Systematic and Evolutionary Microbiology (2003), 53, 231-238

SUMMARY OF INVENTION

Problem to be Solved by Invention

[0011] The present invention has an objective of providing a mutant carotenoidogenic bacterium, and a method for producing a carotenoid using said bacterium.

[0012] The present inventors have gone through extensive investigation to solve the above-described problems, and as a result of which succeeded in acquiring a bacterium having high astaxanthin productivity among the bacteria subjected to a mutation treatment, thereby accomplishing the present invention.

Means for Solving Problem

[0013] (1) A mutant carotenoidogenic bacterium, comprising any of genes (a)-(c) below:

(a) a gene encoding a protein comprising a mutant amino acid sequence in which at least the 225th amino acid residue in the amino acid sequence of 1-deoxy-D-xylulose 5-phosphate synthase of a carotenoidogenic bacterium has been substituted with other amino acid residue;

(b) a gene encoding a protein comprising a mutant amino acid sequence in which at least the 305th amino acid residue in the amino acid sequence of decaprenyl diphosphate synthase of a carotenoidogenic bacterium has been substituted with other amino acid residue; and

(c) both of the genes (a) and (b) above.

(2) The bacterium according to (1), wherein the amino acid sequence of 1-deoxy-D-xylulose 5-phosphate synthase is the sequence represented by SEQ ID NO:2.

(3) The bacterium according to either one of (1) and (2), wherein the 225th amino acid residue has been substituted from glycine to aspartic acid.

(4) The bacterium according to any one of (1)-(3), wherein the amino acid sequence of decaprenyl diphosphate synthase is the sequence represented by SEQ ID NO:4.

(5) The bacterium according to any one of (1)-(4), wherein the 305th amino acid residue has been substituted from alanine to valine.

(6) The bacterium according to any one of (1)-(5), which has acquired carotenogenic capacity that is higher than the carotenogenic capacity of a carotenoidogenic bacterium without the gene encoding the protein comprising the mutant amino acid sequence.

(7) The bacterium according to (6), which has acquired carotenogenic capacity that is at least 5 times or more the carotenoid production amount of a carotenoidogenic bacterium without the gene encoding the protein comprising the mutant amino acid sequence.

(8) The bacterium according to any one of (1)-(7), wherein the carotenoidogenic bacterium belongs to the genus *Paracoccus*.

(9) The bacterium according to (8), wherein the bacterium belonging to the genus *Paracoccus* is strain E-396.

(10) The bacterium according to any one of (1)-(9), wherein the carotenoid is astaxanthin.

(11) A method for producing a carotenoid, comprising culturing the bacterium according to any one of (1)-(10), and collecting the carotenoid from the resulting cultured product.

(12) The method according to (11), wherein the carotenoid production amount is at least 5 times or more the carotenoid production amount of a carotenoidogenic bacterium without the gene encoding the protein comprising the mutant amino acid sequence.

(13) The method according to either one of (11) and (12), wherein the carotenoid is astaxanthin.

(14) A method for screening for a carotenoidogenic bacterium, comprising subjecting a carotenoidogenic bacterium to a mutation treatment, and selecting a bacterium having any of characteristics (a)-(c) below from the bacteria subjected to the mutation treatment:

(a) a characteristic where the activity of 1-deoxy-D-xylulose 5-phosphate synthase is increased compared to said activity in the bacterium before the mutation treatment;

(b) a characteristic where the activity of decaprenyl diphosphate synthase is decreased compared to said activity in the bacterium before the mutation treatment; and

(c) both of the characteristics (a) and (b) above.

(15) A method for producing a carotenoid, comprising culturing the bacterium selected by the method according to (14), and collecting a carotenoid from the resulting cultured product.

(16) A gene encoding a protein comprising a mutant amino acid sequence in which at least the 225th amino acid residue in the amino acid sequence of 1-deoxy-D-xylulose 5-phosphate synthase has been substituted with other amino acid residue.

(17) A gene comprising either of DNA (a) or (b) below:

(a) DNA comprising the nucleotide sequence represented by SEQ ID NO:5; or

(b) DNA that hybridizes with DNA having a nucleotide sequence complementary to said DNA (a) under stringent conditions, and that encodes a protein having 1-deoxy-D-xylulose 5-phosphate synthase activity.

(18) A gene encoding a protein comprising a mutant amino acid sequence in which at least the 305th amino acid residue in the amino acid sequence of decaprenyl diphosphate synthase has been substituted with other amino acid residue.

(19) A gene comprising either of DNA (a) or (b) below:

(a) DNA comprising the nucleotide sequence represented by SEQ ID NO:7; or

(b) DNA that hybridizes with DNA having a nucleotide sequence complementary to said DNA (a) under stringent conditions, and that encodes a protein with decreased decaprenyl diphosphate synthase activity.

(20) A recombinant vector comprising any of genes (a)-(c) below:

(a) the gene according to (16) or (17);

(b) the gene according to (18) or (19); and

(c) the genes (a) and (b) above.

(21) A transformant comprising the recombinant vector according to (20).

(22) A method for producing a carotenoid, comprising culturing the transformant according to (21), and collecting a carotenoid from the resulting cultured product.

EFFECT OF THE INVENTION

[0014] The present invention provides a high carotenoid producing bacterium. Carotenoids can efficiently be produced by using the bacterium of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0015] [Figure 1] A diagram showing specific productivities of total carotenoid and astaxanthin in strain E-396 and strain ASB-57.

[Figure 2] An image showing a template structure of Enzyme A.

[Figure 3A] A depiction showing the alignment between Enzyme A and the template structure (2O1X). The deduced active sites are depicted in green. The disordered region is represented by a blue bar.

[Figure 3B] A depiction showing the alignment between Enzyme A and the template structure (2O1X). The deduced active sites are depicted in green. The disordered region is represented by a blue bar.

[Figure 4] Images showing the template structure and a model structure of a complex of Enzyme A with TPP coenzyme. The structure of the complex of *D. radiodurans*-derived 1-deoxy-D-xylulose 5-phosphate synthase (DXS) with TPP (2O1X) as the template (left) and the model structure of constructed Enzyme A (right). In the formed homodimer, each subunit binds TPP and Mg. TPP and Mg are shown in space-filling representation.

[Figure 5] Images showing the model structure of the complex of Enzyme A with a TPP intermediate and a substrate. A complex model in which the constructed Enzyme A is bonded with a hydroxyethyl-TPP intermediate having pyruvic acid attached to TPP coenzyme and glyceraldehyde 3-phosphate (GAP) is shown.

[Figure 6] A depiction showing the interaction between Enzyme A and TPP coenzyme.

[Figure 7] Images showing the amino acid residues of Enzyme A that were predicted responsible for the interaction with TPP. The residues predicted to be responsible for the interaction with TPP are represented by sticks.

[Figure 8] Images showing the amino acid residues that were predicted responsible for the interaction between Enzyme A and the hydroxyethyl group of the hydroxyethyl-TPP intermediate. The residues predicted to be responsible for the interaction with the hydroxyethyl group are represented by sticks.

[Figure 9] Images showing the amino acid residues that were predicted

responsible for the interaction between Enzyme A and glyceraldehyde 3-phosphate (GAP).

[Figure 10] A depiction showing the interaction between Enzyme A and the substrate pyruvic acid.

[Figure 11] A depiction showing the interaction between Enzyme A and the substrate glyceraldehyde 3-phosphate.

[Figure 12] A depiction showing the alignment between Enzyme A and other types of DXS. EnzymeA: Enzyme A, DXS_ECOLI: DXS (*E.coli*), DXS_VITVI: DXS (*Vitis vinifera*), DXS_DEIRA: (*Deinococcus radiodurans*). The disordered region in the template structure (DXS_DEIRA) is represented by a blue bar. The active sites are shown in green boxes while mutations that showed activity enhancement in DXS_ECOLI and DXS_VITVI are marked with red triangles.

[Figure 13] Images showing the location of the disordered region in Enzyme A. Asn180 and Met182 in the loop on the N-terminal side of the disordered region (dotted blue line) bind Mg while Ile184 binds TPP.

[Figure 14] Images showing a model structure of the disordered region of Enzyme A. The model structure of the disordered region (light blue) in Enzyme A (left) and the electrostatic potential maps of the model structures of the disordered regions (right).

[Figure 15] A diagram showing the deduced effects of mutant enzyme A in the astaxanthin synthesis pathway.

[Figure 16] An image showing a template structure of Enzyme C. *Rhodobacter capsulatus*-derived decaprenyl diphosphate synthase (PDB ID: 3MZV) is shown.

[Figure 17] A depiction showing the alignment between Enzyme C and the template structure (3MZV). The deduced active sites are depicted in green.

[Figure 18] Images showing the template structure and a model structure of a complex of Enzyme C with IPP and FPP. *R. capsulatus*-derived decaprenyl diphosphate synthase (3MZV) as the template (left) and the model structure of constructed Enzyme C (right). FPP and IPP are shown in space-filling representation.

[Figure 19] Images showing the template structure, and the model structure of the complex of Enzyme C with IPP and FPP. FPP and IPP bind each other in the head-to-tail direction while the condensation reaction occurs between an isopentenyl group of IPP and a phosphate group of FPP (left image, arrow). In the long-chain prenyl diphosphate synthase,

the reaction product further binds IPP and extends deep into the substrate binding site (right image, arrow).

[Figure 20] Images showing comparison between the template structure and the model structure of Enzyme C. The complex models of template *R. capsulatus*-derived decaprenyl diphosphate synthase (left) and Enzyme C (right) with the substrate. The structures with matching amino acid residues are depicted in green. The substrate binding region and its surrounding structure match entirely.

[Figure 21] A depiction showing the alignment between Enzyme C and decaprenyl diphosphate synthase (*Paracoccus zeaxanthinifaciens*).

[Figure 22] Images showing a complex model of Enzyme C with FPP and IPP. The ribbon representation (left) and the surface profile (right). Chain A (light red) and chain B (light blue) are shown. FPP and IPP are shown in space-filling representation.

[Figure 23] Images showing the amino acid residues of Enzyme C predicted to be responsible for the interactions with FPP (top), IPP (center) and Mg (bottom).

[Figure 24] A depiction showing the interaction of Enzyme A with substrates FPP and IPP.

[Figure 25] A depiction showing the interaction of Enzyme A with Mg.

[Figure 26] Images showing conformational models of wild-type and mutant A305V Enzyme C. Ala305 (green) and Val305 (magenta) are shown in space-filling representation.

[Figure 27] Images showing the surrounding structures of Ala305 of the wild type (left) and Val305 of the mutant (right). Ala305 makes contact with the peripheral amino acid residues. Mutation Val305 causes steric hindrance with the peripheral structure. Ala305 is depicted in green while Val305 is depicted in magenta.

[Figure 28] A diagram showing comparison of intramolecular energies between the wild type (blue) and mutant A305V (red).

[Figure 29] Images showing comparison of the structures between the wild type and mutant A305V. Mutation A305V causes change in the structure of the amino acid residues around Ala305 (green) and Val305 (magenta) (left). This structural change also affects the adjacent α-helix (right).

[Figure 30] Images showing the effect of A305V in Enzyme C.

[Figure 31] Images showing the effect of A305V in Enzyme C.

[Figure 32] Images showing the effect of A305V in Enzyme C.

[Figure 33] A diagram showing the deduced effect of mutant Enzyme C in the astaxanthin synthesis pathway.

MODE FOR CARRYING OUT THE INVENTION

[0016] Hereinafter, the present invention will be described in detail.

1. General

The present invention relates to a high carotenoid producing bacterium, which is a bacterium comprising either or both of genes (a) and (b) below.

(a) a gene encoding a protein comprising a mutant amino acid sequence in which at least the 225th amino acid residue in the amino acid sequence of 1-deoxy-D-xylulose 5-phosphate synthase of a carotenoidogenic bacterium has been substituted with other amino acid residue; and

(b) a gene encoding a protein comprising a mutant amino acid sequence in which at least the 305th amino acid residue in the amino acid sequence of decaprenyl diphosphate synthase of a carotenoidogenic bacterium has been substituted with other amino acid residue.

[0017] In order to develop a bacterium having high carotenogenic capacity, the present inventors have examined the carotenogenic capacity in strain E-396 and strains thereof that had been subjected to a mutation treatment, and analyzed the mutation of a gene encoding an enzyme involved in the carotenoid synthesis pathway in these strains.

As a result, a strain (referred to as "strain ASB-57") that had higher carotenogenic capacity than the parent strain E-396 was acquired. Genome analysis for strain ASB-57 confirmed mutations in the amino acid sequence of 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and the amino acid sequence of decaprenyl diphosphate synthase (DPS). Thus, a functional analysis was conducted by prediction based on a conformational analysis of the amino acids, by which the mutation of at least the 225th amino acid residue of DXS and/or the 305th amino acid residue of DPS was considered to contribute to the high carotenoid production.

The present invention was accomplished based on the above-described findings.

[0018] 2. Mutant carotenoidogenic bacterium

A carotenoidogenic bacterium of the present invention is a mutant bacterium that can produce a carotenoid with high efficiency, which can be obtained by subjecting a parent strain to a mutation treatment and using the mutations at the 225th amino acid residue of DXS and/or the 305th amino acid residue of DPS as indicators . A carotenoidogenic bacterium of the present invention is herein referred to as a "mutant carotenoidogenic bacterium".

(1) Parent strain

According to the present invention, a bacterium used as the parent strain for obtaining a mutant carotenoidogenic bacterium is not limited at all as long as it produces a carotenoid, and may be, for example, a bacterium belonging to the genus *Paracoccus*, the genus *Brevundimonas* or the genus *Erythrobacter*.

A bacterium belonging to the genus *Paracoccus*, a bacterium belonging to the genus *Brevundimonas* or a bacterium belonging to the genus *Erythrobacter* can preferably be used, and a bacterium belonging to the genus *Paracoccus* can more preferably be used. Since all of the genus *Paracoccus*, the genus *Erythrobacter* and the genus *Brevundimonas* belong to the class *Alphaproteobacteria* in the phylum *Proteobacteria*, and are common in bacterial taxonomy, bacteria belonging to these genera can be used for the present invention.

[0019] Among the bacteria belonging to the genus *Paracoccus*, *Paracoccus carotinifaciens*, *Paracoccus marcusii*, *Paracoccus haeundaensis* and *Paracoccus zeaxanthinifaciens* can preferably be used, and *Paracoccus carotinifaciens* can particularly preferably be used. Specific examples of the strains of the bacteria belonging to the genus *Paracoccus* include strain E-396 of *Paracoccus carotinifaciens* (FERM BP-4283) and strain A-581-1 of the bacteria of the genus *Paracoccus* (FERM BP-4671), whose mutants can also preferably be used for the present invention.

Examples of the carotenoidogenic bacteria belonging to the genus *Erythrobacter* include *Erythrobacter* JPCC M sp. (Japanese Patent Application Publication No. 2008-259452) and *Erythrobacter* JPCC O sp. (Japanese Patent Application Publication No. 2008-259449).

Examples of carotenoidogenic bacteria belonging to the genus *Brevundimonas* include *Brevundimonas* sp. strain SD212 (Japanese Patent Application Publication No.

2009-27995), *Brevundimonas* sp. strains FERM P-20515 and 20516 (Japanese Patent Application Publication No. 2006-340676), and *Brevundimonas vesicularis* (Gene, Vol.379, p.101-108, 1 Sep 2006).

[0020] In addition, a bacterium whose nucleotide sequence of DNA corresponding to 16S ribosomal RNA is highly homologous with the nucleotide sequence of strain E-396 represented by SEQ ID NO:9 is preferably used as the carotenoidogenic bacterium. Herein, the homology of the nucleotide sequence is preferably 95% or more, more preferably 96% or more, still more preferably 97% or more, particularly preferably 98% or more, and most preferably 99% or more.

The nucleotide sequence of DNA corresponding to 16S ribosomal RNA refers to a nucleotide sequence obtained by substituting U (uracil) of the nucleotide sequence of 16S ribosomal RNA with T (thymine).

[0021] Classification of microorganisms based on the homology of this nucleotide sequence of 16S ribosomal RNA has been the recent mainstream. Since conventional classification of microorganisms is based on conventional microbiological characteristics such as motility, auxotrophy, sugar assimilation and the like, a microorganism may be classified incorrectly when morphological change or the like is caused due to naturally occurring mutation. On the other hand, the nucleotide sequence of 16S ribosomal RNA is genetically very stable and thus reliability of the classification technique based on that homology is considerably enhanced compared to that of the conventional classification technique.

[0022] Homologies of the nucleotide sequence of 16S ribosomal RNA of *Paracoccus carotinifaciens* strain E-396 with the nucleotide sequences of 16S ribosomal RNA of other carotenoidogenic bacteria, i.e., *Paracoccus marcusii* strain DSM 11574, strain N-81106 of the bacterium of the genus *Paracoccus*, *Paracoccus haeundaensis* strain BC 74171, strain A-581-1 of the bacterium of the genus *Paracoccus*, *Paracoccus zeaxanthinifaciens* strain ATCC 21588, and *Paracoccus* sp. strain PC-1 are 99.7%, 99.7%, 99.6%, 99.4%, 95.7% and 95.4%, respectively, which represent that these strains are closely related taxonomically. Hence, these strains are found to form a group of bacteria that produce carotenoids. Therefore, these strains can preferably be used for the present invention, and are capable of efficiently producing carotenoids.

[0023] According to the present invention, known mutants having improved carotenoid productivity can also be used. Examples of such known mutants include a strain having high astaxanthin production capacity (Japanese Patent Application Publication No. 2001-95500), a strain that selectively produce an increased amount of canthaxanthin (Japanese Patent Application Publication No. 2003-304875), a strain that selectively produce increased amounts of zeaxanthin and β-cryptoxanthin (Japanese Patent Application Publication No. 2005-87097), and a strain that selectively produce an increased amount of lycopene (Japanese Patent Application Publication No. 2005-87100).

[0024] Strain E-396 as an exemplarly carotenoidogenic bacterium used as the parent strain for the present invention has been internationally deposited to the International Patent Organism Depositary (NITE-IPOD), the National Institute of Technology and Evaluation (NITE) as follows.

International depositary authority: International Patent Organism Depositary, National Institute of Technology and Evaluation (NITE)

2-5-8, Kazusakamatari, Kisarazu-shi, Chiba, 292-0818, JAPAN

Identification reference: E-396

Accession number: FERM BP-4283

Date of the original deposit: April 27, 1993

[0025] In addition, strain A-581-1 as another exemplarly carotenoidogenic bacterium used as the parent strain in the present invention has been internationally deposited to the above-mentioned authority as follows.

Identification reference: A-581-1

Accession number: FERM BP-4671

Date of the original deposit: May 20, 1994

[0026] (2) Mutation treatment and screening

A mutant carotenoidogenic bacterium of the present invention can be obtained by subjecting the above-described parent strain to a mutation treatment and using the mutations at the 225th amino acid residue of DXS and/or the 305th amino acid residue of DPS as indicators.

A method of the mutation treatment is not particularly limited as long as it can induce mutation. For example, a chemical method using a mutation agent such as

N-methyl-N'-nitro-N-nitrosoguanidine (NTG) or ethyl methanesulfonate (EMS), a physical method such as ultraviolet irradiation or X-ray irradiation, a biological method using gene recombination or transposon, or the like may be employed. Although the bacterium subjected to the mutation treatment is not particularly limited, it is preferably a carotenoidogenic bacterium.

Moreover, according to the present invention, a protein having the above-described mutation can be prepared by introducing point mutation into a gene (DNA) encoding said protein. For such a mutagenesis method, a mutagenesis kit utilizing a site-directed mutagenesis method such as Kunkel method or Gapped duplex method, for example, QuikChange™ Site-Directed Mutagenesis Kit (from Stratagene), GeneTailor™ Site-Directed Mutagenesis System (from Invitrogen), TaKaRa Site-Directed Mutagenesis System (Mutan-K, Mutan-Super Express Km, etc: from Takara Bio) or the like can be used. In addition, a method such as site-directed mutagenesis described in "Molecular Cloning, A Laboratory Manual (4th edition)" (Cold Spring Harbor Laboratory Press (2012)) or the like can be used.

[0027] While a method for screening a mutant is not particularly limited, a known genome analysis tool such as PacBio *RS* II (from Pacific Biosciences) or MiSeq (from Illumina) can be used for gene analysis to confirm the presence of a mutation of the nucleotide sequence corresponding to the 225th amino acid residue of DXS and/or the 305th amino acid residue of DPS.

Furthermore, along with the above-described genome analysis, the mutant of interest can, for example, be selected by the color tone of the colony on the agar medium, or selected by culturing the mutants in a test tube, a flask, a fermentation tank or the like and using the production amount of the carotenoid as an indicator in an analysis of carotenoid pigment utilizing absorbance, high performance liquid chromatography, thin-layer chromatography or the like.

The mutation and screening steps can be carried out once, or the mutation and screening steps can be repeated twice or more, for example, by obtaining mutants by a mutation treatment and screening and subjecting the resultant to another mutation treatment and screening to acquire a mutant with improved productivity.

[0028] The mutant carotenoidogenic bacterium screened as such has a gene encoding an

amino acid sequence in which the 225th amino acid residue of DXS is altered to other amino acid and/or the 305th amino acid residue of DPS is altered to other amino acid residue.

Mutation of the 225th amino acid residue of DXS to other amino acid contributes to the increase in the the enzymatic activity of DXS. Thus, synthesis from pyruvic acid to 1-deoxy-D xylulose-5-phosphate is promoted, which leads to the increase in the production of isopentenyl diphosphate (IPP) that serves as a substrate for astaxanthin synthesis.

Mutation of the 305th amino acid residue of DPS to other amino acid residue contributes to the reduction in the enzymatic activity of DPS. This mutation suppresses synthesis from farnesyl diphosphate (FPP) to decaprenyl diphosphate (DPP). Since IPP is used for the synthesis of DPP from FPP, the above-described mutation will reduce the amount of IPP used for the DPP synthesis and said IPP will be utilized as a substrate for the above-described astaxanthin synthesis.

[0029] Here, according to the present invention, as long as an amino acid sequence has the 225th amino acid residue of DXS altered to other amino acid and/or the 305th amino acid residue of DPS altered to other amino acid residue, and has a gene coding for a protein comprising an amino acid sequence having such DXS activity and/or a protein comprising an amino acid sequence with reduced (suppressed) DPS activity, one or more amino acid residues in an amino acid sequence of other region of the amino acid sequence of DXS and/or DPS may be altered by substitution, deletion, addition or the like.

Accordingly, a mutant carotenoidogenic bacterium of the present invention may comprise the gene (a) below, the gene (b) below, or both of the genes (a) and (b) below.

[0030] (a) A gene encoding a protein comprising a mutant amino acid sequence in which at least the 225th amino acid residue has been substituted with other amino acid residue in the amino acid sequence of DXS of a carotenoidogenic bacterium.

Examples of such mutant DXS genes include the followings.

(i) A gene encoding a protein that comprises a mutant amino acid sequence in which the 225th amino acid residue in the amino acid sequence of DXS (for example, SEQ ID NO:2) has been substituted with other amino acid residue, and that has DXS activity.

An example of such a mutant amino acid sequence includes one represented by SEQ ID NO:6, while an example of the above-mentioned gene includes one represented by SEQ ID NO:5. According to the present invention, it is preferably an amino acid sequence

in which glycine as the 225th amino acid residue in the amino acid sequence represented by SEQ ID NO:2 is substituted with aspartic acid.

(ii) A gene encoding a protein that comprises a mutant amino acid sequence in which the 225th amino acid residue in the amino acid sequence of DXS (for example, SEQ ID NO:2) is substituted with other amino acid residue and in which one or more (for example, one to several) amino acid residues other than the 225th amino acid residue have been deleted, substituted or added, and that has DXS activity.

(iii) A gene consisting of DNA comprising the nucleotide sequence represented by SEQ ID NO:5.

(iv) A gene comprising DNA that hybridizes with DNA consisting of a nucleotide sequence complementary to DNA comprising the nucleotide sequence represented by SEQ ID NO:5 under stringent conditions, and that encodes a protein having DXS activity.

Among the DNA encoding the amino acid sequence of DXS (SEQ ID NO:1) of a carotenoidogenic bacterium, the above-described nucleotide sequence represented by SEQ ID NO:5 is one that codes for a protein comprising an amino acid sequence in which the 225th amino acid residue has been substituted with other amino acid residue.

[0031] (b) A gene encoding a protein comprising a mutant amino acid sequence in which at least the 305th amino acid residue in the amino acid sequence of DPS of a carotenoidogenic bacterium has been substituted with other amino acid residue.

Examples of such genes include the followings.

(i) A gene encoding a protein that comprises a mutant amino acid sequence in which the 305th amino acid residue in the amino acid sequence of DPS (for example, SEQ ID NO:4) has been substituted with other amino acid residue, and that has reduced DPS activity.

An example of such a mutant amino acid sequence includes one represented by SEQ ID NO:8, while an example of the above-described gene includes one represented by SEQ ID NO:7. According to the present invention, it is preferably an amino acid sequence in which alanine as the 305th amino acid residue in the amino acid sequence represented by SEQ ID NO:4 is substituted with valine.

(ii) A gene encoding a protein that comprises a mutant amino acid sequence in which the 305th amino acid residue in the amino acid sequence of DPS (for example, SEQ

ID NO:4) is substituted with other amino acid residue and in which one or more (for example, one to several) amino acid residues other than the 305th amino acid residue have been deleted, substituted or added, and that has reduced DPC activity.

(iii) A gene consisting of DNA comprising the nucleotide sequence represented by SEQ ID NO:7.

(iv) A gene comprising DNA that hybridizes with DNA consisting of a nucleotide sequence complementary to DNA comprising the nucleotide sequence represented by SEQ ID NO:7 under stringent conditions, and that encodes a protein having reduced DPS activity.

Among the DNA encoding the amino acid sequence of DPS (SEQ ID NO:3) of a carotenoidogenic bacterium, the above-described nucleotide sequence represented by SEQ ID NO:7 is one that codes for a protein comprising an amino acid sequence in which the 305th amino acid residue has been substituted with other amino acid residue.

[0032] Here, the hybridization can be performed according to a known method (for example, Sambrook J. et al., Molecular Cloning, A Laboratory Manual (4th edition) (Cold Spring Harbor Laboratory Press (2012)). Highly stringent conditions refer to conditions under which so-called specific hybrids, but not non-specific hybrids are formed, which are, for example, a sodium concentration of 10 mM-300 mM, preferably 20 mM-100 mM and a temperature of 25°C-70°C, preferably 42°C-55°C.

[0033] Examples of such a mutant carotenoidogenic bacterium include strain ASB-57, strain ASK-8 and strain ASH-66.

Strain ASB-57 has a gene encoding a protein comprising an amino acid sequence in which glycine as the 225th amino acid residue of DXS is altered to aspartic acid, and alanine as the 305th amino acid residue of DPS is altered to valine. The amino acid sequence of DXS in strain ASB-57 and the nucleotide sequence of the gene thereof are represented by SEQ ID NOS:6 and 5, respectively. Moreover, the amino acid sequence of DPS in strain ASB-57 and the nucleotide sequence of the gene thereof are represented by SEQ ID NOS:8 and 7, respectively

[0034] (4) Preparation of gene recombinant

According to the present invention, the gene encoding the above-described mutant DXS and/or the gene encoding the above-described mutant DPS is introduced into a host for transfomation, thereby obtaining a gene recombinant type mutant carotenoidogenic

bacterium.

A recombinant vector can be obtained by introducing the mutant DXS gene and/or the mutant DPS gene into a vector, and a transformant can be obtained by introducing said recombinant vector into a host by employing any known method such as Sambrook J. et al., Molecular Cloning, A Laboratory Manual (4th edition) (Cold Spring Harbor Laboratory Press (2012).

When synthesizing the above-described DXS gene and DPS gene by genetic engineering, DNA coding for said enzyme is first designed and synthesized. The design and synthesis of DNA can be conducted, for example, by a PCR method using a vector containing the full-length gene or the like as a template and primers designed to synthesize the DNA region desired. Then, the above-described DNA is linked with a suitable vector to obtain a recombinant vector for protein expression, and this recombinant vector is introduced into a host such that the gene of interest is expressed, thereby obtaining a transformant (Sambrook J. et al., Molecular Cloning, A Laboratory Manual (4th edition) (Cold Spring Harbor Laboratory Press (2012)).

As the vector, a phage or a plasmid that can autonomously grow in a host microorganism is used. Alternatively, an animal virus or an insect virus vector can also be used. A recombinant vector can be prepared by cleaving purified DNA with a suitable restriction enzyme, and inserting the resultant at a restriction enzyme site of suitable vector DNA or the like to link with the vector. The host used for transformation is not particularly limited as long as it is capable of expressing the gene of interest. For example, it may be a bacterium (*Bacillus subtilis*, bacteria of the genus *Paracoccus*, etc.), a yeast, animal cells (COS cells, CHO cells, etc.), plant cells, insect cells or an insect. The method for introducing a recombinant vector into a host is known.

Moreover, the method for introducing a mutation into gene is the same as described above.

[0035] (3) Production of carotenoid

According to the present invention, the above-described carotenoidogenic bacterium or transformant can be cultured in a predetermined medium to stably produce a carotenoid at a high concentration.

While the produced carotenoid is not particularly limited, it may be, for example,

astaxanthin, canthaxanthin, zeaxanthin, β-cryptoxanthin, lycopene, β-carotene, adonirubin, adonixanthin, echinenone, asteroidenone or 3-hydroxyechinenone, preferably astaxanthin, canthaxanthin, zeaxanthin or β-cryptoxanthin, and more preferably astaxanthin or zeaxanthin. The carotenoids produced by the present invention may be of a single type or a combination of multiple types.

[0036] Hereinafter, a method for culturing the mutant carotenoidogenic bacterium or transformant of the present invention will be described.

[0037] A carotenoid-producing medium used for culture of the present invention may be added with any component as long as it allows growth of a carotenoidogenic bacterium or transformant and production of a carotenoid. Although any medium containing such an additive can be used, it is preferably a medium containing a carbon source, a nitrogen source, an inorganic salt and if necessary a vitamin or the like.

[0038] Examples of the carbon source include sugars such as glucose, sucrose, lactose, fluctose, trehalose, mannose, mannitol and maltose, organic acids such as acetic acid, fumaric acid, citric acid, propionic acid, malic acid, malonic acid and pyruvic acid, alcohols such as ethanol, propanol, butanol, pentanol, hexanol, isobutanol and glycerol, fats and oils such as soybean oil, rice bran oil, olive oil, corn oil, sesame oil and linseed oil, Among them, glucose or sucrose is preferably used. Among these carbon sources, one or more types can be used. While the amount added to the medium before culture (starting medium) varies according to the type of the carbon source and can appropriately be adjusted, it is usually 1-100 g, preferably 2-50 g per 1L of the medium. Furthermore, the carbon source may be added not only to the starting medium but also preferably additionally supplied during culture successively or continuously.

[0039] As an inorganic salt as the nitrogen source, one or more types among ammonium salts such as ammonium nitrate, ammonium sulfate, ammonium chloride and ammonium phosphate, nitrates such as potassium nitrate, ammonia and urea can be used. While the amount added varies and can appropriately be adjusted according to the type of the nitrogen source, it is usually 0.1 g-20 g and preferably 0.2-10 g per 1L of the medium.

Furthermore, as an organic nitrogen source, for example, one or more types among corn steep liquor (including filtrated product), Pharmamedia, soybean pulp, soybean powder, peanut meal, soy peptone, Distillers' solbules, dry yeast, yeast extract, casamino acid,

glutamic acid and aspartic acid can be used. While the concentration added varies and can appropriately be adjusted according to the type of the nitrogen source, it is usually 0-80 g/L and preferably 1-30 g/L.

[0040] The inorganic nitrogen source and the organic nitrogen source are usually added to the starting medium, but they may also preferably be additionally supplied successively or continuously.

As the inorganic salt, for example, one or more types among phosphates such as potassium dihydrogen phosphate, dipotassium hydrogen phosphate and disodium hydrogen phosphate, magnesium salts such as magnesium sulfate and magnesium chloride, iron salts such as iron sulfate and iron chloride, calcium salts such as calcium chloride and calcium carbonate, sodium salts such as sodium carbonate and sodium chloride, manganese salts such as manganese sulfate, copper salts such as copper sulfate, zinc salts such as zinc sulfate, molybdenum salts such as sodium molybdate, nickel salts such as nickel sulfate, selenium salts such as sodium selenate, tungsten salts such as sodium tungstate, aluminum salts such as aluminum chloride, chromium salts such as chromium chloride, and potassium borate and iodide can be used. While the amount added varies and can appropriately be adjusted according to the type of the inorganic salt, it is usually 0.0001-15 g per 1L of the medium. The concentration is preferably 0.02-15 g/L in a case of a phosphate, a magnesium salt, a calcium salt, a sodium salt or an iron salt, and preferably 0.1-15 mg/L when a manganese salt, a copper salt, a zinc salt, a molybdenum salt, a nickel salt, a selenium salt, a tungsten salt, an aluminum salt, a chromium salt, or a potassium borate or iodide is added. An inorganic salt is usually added to the starting medium, but it may also be additionally supplied successively or continuously.

[0041] As a vitamin, for example, cyanocobalamin, riboflavin, pantothenic acid, pyridoxine, thiamine, ascorbic acid, folic acid, niacin, p-aminobenzoic acid, biotin, inositol, choline or the like can be used. While the amount added varies and can appropriately be adjusted according to the type of the vitamin, it is usually 0.001-1000 mg and preferably 0.01-100 mg per 1L of the medium. A vitamin is usually added to the starting medium, but it may also be additionally supplied successively or continuously.

[0042] According to the present invention, an antifoaming agent is preferably used in order to suppress foaming of the culture solution. Any type of antifoaming agent can be

used as long as it serves to suppress generation of foam or eliminate generated foam, and has little inhibitory action against the produced bacterium. For example, an alcohol-based antifoaming agent, a polyether-based antifoaming agent, an ester-based antifoaming agent, a fatty acid-based antifoaming agent, a silicon-based antifoaming agent, a sulfonic acid-based antifoaming agent, and the like can be exemplified. While the amount added varies and can appropriately be adjusted according to the type of the antifoaming agent, it is usually 0.01 g-10 g per 1L of the medium.

[0043] The antifoaming agent is usually added to the starting medium prior to sterilization. In addition, it may continuously or intermittently be added during the culture. As a method of adding an antifoaming agent during the culture, a method in which the antifoaming agent is automatically added once a sensor senses foam generation, a method in which the antifoaming agent is added constantly using a programmable timer, a method in which the antifoaming agent is added as a mixture with a feeding carbon source, nitrogen source or pH regulator or the like so as to link with the growth rate, or the like can be exemplified. An antifoaming agent added to the starting medium and an antifoaming agent added during the culture may be of the same type or they may be different according to their actions.

[0044] According to the present invention, the early pH of the medium is adjusted to 2-12, preferably 6-9 and more preferably 6.5-8.0. The pH in the above-mentioned range is preferably maintained during the culture as well. As the pH regulator, an aqueous sodium hydroxide solution, an aqueous potassium hydroxide solution, an aqueous sodium carbonate solution, ammonia water, ammonia gas, an aqueous sulfate solution or a mixture thereof can be exemplified.

According to the present invention, the medium is subjected to a sterilization treatment and thereafter used for culturing a bacterium. The sterilization treatment can appropriately be carried out by those skilled in the art. For example, the medium in a suitable vessel may be heat sterilized with an autoclave. Alternatively, it may be sterilized by filtration with a sterilizing filter.

[0045] A mutant carotenoidogenic bacterium or transformant of the present invention is inoculated onto a medium prepared as described above and cultured under predetermined conditions. Inoculation is carried out by appropriately growing the bacterial strain by seed

culture using a test tube, a flask, a fermentation tank or the like, and adding the resulting culture solution to a carotenoid-producing medium. The medium used for the seed culture is not particularly limited as long as it is a medium that allows the carotenoidogenic bacterium to grow well.

[0046] Culture is carried out in a suitable culture vessel. While the culture vessel may appropriately be selected according to the culture volume, it may be, for example, a test tube, a flask and a fermentation tank.

The culture temperature is 15-40°C, preferably 20-35°C and more preferably 25°C-32°C and culture is usually conducted for 1-18 days, preferably 2-12 days and more preferably 3-8 days under an aerobic condition. The aerobic condition, for example, may refer to shaking culture, aeration-agitation culture or the like, where the dissolved oxygen concentration is preferably controlled to lie within a certain range. The dissolved oxygen concentration can be controlled, for example, by varying the rotation speed for agitation, the ventilation volume, the internal pressure or the like. The dissolved oxygen concentration is preferably controlled to 0.3-10 ppm, more preferably 0.5-7 ppm and still more preferably 1-5 ppm.

[0047] The number of bacterial cells or the number of transformants of a carotenoidogenic bacterium after culturing the mutant carotenoidogenic bacterium or transformant of the present invention can be measured by OD. In addition, the carotenoid contained in the resulting cultured product or the carotenoid collected from the cultured product after culturing the carotenoidogenic bacterium or transformant can be quantified by high performance liquid chromatography. After culturing the carotenoidogenic bacterium or transformant, the carotenoid can be collected from the resulting cultured product as described above.

Examples of the cultured product include a culture solution, a culture supernatant, a concentrated bacterial cell solution, wet bacterial cells, dry bacterial cells and a bacterial cell lysate. The culture supernatant can be prepared by subjecting the culture solution to a centrifugal treatment or a filtration treatment to remove the bacterial cells from the culture solution. The concentrated bacterial cell solution can be obtained by subjecting the culture solution to centrifugation or membrane filtration concentration. The wet bacterial cells can be obtained by centrifuging or filtrating the culture solution. The dry bacterial cells can be

obtained by drying wet bacterial cells or a concentrated bacterial cell solution by a general drying method. The resulting carotenoid-containing dry bacterial cells can directly be used as feed additives.

[0048] The yield by fermentation culture is at least 150 mg/L, for example, 150 mg/L, 400 mg/L, 2000 mg/L or 4000 mg/L of carotenoid. While the amount of the carotenoid contained in the culture solution varies depending on the bacterial cells used, it may contain, for example, 400 mg/L-4000 mg/L and still more preferably 500 mg/L-3500 mg/L of carotenoid.

The bacterium of the present invention has production capacity of an amount at least 5 times, preferably 10 times or more the carotenoid production amount of a carotenoidogenic bacterium that does not have the gene encoding the protein comprising the mutant amino acid sequence of DXS and/or DPS.

[0049] According to the present invention, the method for collecting the carotenoid from the above-described cultured product is not particularly limited, and any method that allows stable and efficient carotenoid collection can be employed. These methods can appropriately be selected from known extraction and purification techniques and carried out by those skilled in the art. Alternatively, according to the present invention, the above-described cultured product can also be used as a carotenoid-containing composition.

Prior to extraction, the cultured product may be subjected to one or more treatments among a chemical treatment using an alkali reagent or a surfactant, a biochemical treatment using a lytic enzyme, a lipolytic enzyme or a proteolytic enzyme, or a physical treatment like ultrasonic waves or pulverization.

For example, in a case where a carotenoid is extracted from the cultured product, a solvent used for extraction and washing is not particularly limited, and the it may be a lower alcohol such as methanol, ethanol and isopropanol, acetone, tetrahydrofuran, methyl ethyl ketone, methyl isobutyl ketone, dichloromethane, chloroform, dimethyl formamide or dimethyl sulfoxide.

[0050] If oxidization of the carotenoid needs to be minimized during the extraction operation, it may be treated in an inert gas atmosphere such as nitrogen gas. Alternatively, an antioxidant used for pharmaceutical products and food can be selected and added to the extraction solvent. Alternatively, these treatments can be combined. Moreover, in order to

minimize photolysis of the carotenoid, the treatment can be conducted under a condition without exposure to light.

The thus-resulting extract can directly be used as the carotenoid, or it may be purifired before use.

[0051] While a method for separating the bacteria or the like remaining in the extract after the extraction operation is not particularly limited, membrane filtration, centrifugation, decantation or the like can be employed.

A method for obtaining the carotenoid precipitate from the extracted liquid generally includes heating and/or concentration under reduced pressure, or crystallization. Alternatively, the carotenoid pigments can be separated without concentration by precipication of carotenoid pigments at a low temperature, or precipitation with an acidic/alkaline agent or a salt. For an industrial use, crystallization is favorable.

[0052] The resulting carotenoid precipitate may be suspended and agitated using a solvent such as a small amount of a lower alcohol for washing if necessary. While the washing procedure is not particularly limited, examples of a practically preferable method include a method employing filtration following the suspension/agitation, and a method in which a liquid is passed from top of the precipitate.

The resulting cultured product, extract or purified product may be used as a carotenoid alone or as a mixture at arbitrary proportions.

[0053] 2. Conformational analysis of enzyme involved in astaxanthin synthesis pathway

Since the mutations of the 225th amino acid residue of DXS and the 305th amino acid residue of DPS are found to play an important role in carotenoid synthesis, conformational analyses of these enzymes can be conducted.

According to the present invention, point mutations in two types of enzymes (referred to as Enzymes A and C) on the astaxanthin synthesis pathway were identified. Since the increase in the astaxanthin production was considered to result from the mutations caused in these enzymes, conformational models were constructed for the two types of enzymes having the identified mutations to predict the effects of the amino acid substitutions due to the mutations.

[0054] Enzyme A was deduced to be 1-deoxy-D-xylulose 5-phosphate synthase (DXS). The identified amino acid mutation G225D was in the disordered region near the active site.

From the conformational model, mutation G225D in Enzyme A was deduced to cause a structural change in Enzyme A that was similar to that caused by the mutation in the disordered region that was known to enhance DXS activity, thereby predicting enhanced enzymatic activity in the mutant G225D enzyme. Considering that DXS is under the control of feedback inhibition of IPP, the amount of IPP produced is suggested to increase because feedback inhibition does not work in the mutant G225D enzyme. The increase in the supply of IPP, i.e., a raw material of astaxanthin, due to mutation G225D in Enzyme A seems to cause the increase in the astaxanthin production amount.

[0055] Enzyme C was deduced to be decaprenyl diphosphate synthase. From the conformational model, the identified mutation A305V was deduced to cause steric hindrance with the surrounding amino acid residues and destabilizes the conformation of Enzyme C. FPP and IPP as the raw materials of decaprenyl diphosphate as well as the substrates of Enzyme C are also raw materials for the astaxanthin synthesis. A decrease in the Enzyme C activity due to destabilization is suggested to decrease the amounts of FPP and IPP digested by Enzyme C. As a result, the amounts of FPP and IPP that can be used for the astaxanthin synthesis are increased, which is considered to increase the astaxanthin production amount.

[0056] Accordingly, two effects, namely, the increase in the IPP production amount due to the enhancement of the Enzyme A activity and the increase in the IPP supply to the astaxanthin synthesis pathway due to the decrease in the Enzyme C activity, were deduced to be the effects of the mutations identified this time on the astaxanthin synthesis pathway. These effects appear to result in the increase in the astaxanthin production amount.

[0057] EXAMPLES

Hereinafter, the present invention will be described more specifically by way of examples. The scope of the present invention, however, should not be limited to these examples.

[0058] Example 1

(1) Mutation treatment and genome analysis of bacteria of genus *Paracoccus*

Method of mutation treatment

Screening was carried out for several times by using UV, NTG (nitrosoguanidine) and the like as mutagens on the parent strain (strain E-396) and adopting various selection

pressures. Screening was conducted using the astaxanthin yield as an indicator.

Method of genome analysis

Following genome sequencing using PacBio *RS* II (from Pacific Biosciences) or MiSeq (Illumina) sequencer, a genome analysis was carried out by using an analysis software such as SMART Cell 8 Pac V3 (from Pacific Biosciences), MiSeq Control Software (MCS) v2.4.1.3, Real Time Analysis (RTA) v1.18.54, or bcl2fastq v 1.8.4 (Illumina).

[0059] Results from genome analysis (identification of mutation site)

The mutation site was identified by sorting out those belonging to the genus *Paracoccus* among the amino acid sequences of enzyme genes recorded in the Kyoto Encyclopedia of Genes and Genomes (KEGG) which have high homology with the amino acid sequence of the region that was considered to be the protein having the mutation point from the genome analysis, and further conducting a conformational analysis of the enzyme based on these information to find out a template having a common sequence, thereby determining the final enzyme name of the amino acid sequence of the protein having the mutation site.

[0060] (2) Astaxanthin yield

(i) Culture conditions

Strokes of test tubes: 330 rpm, 28°C, pH 7.2, amount of medium 8 ml/tube

Culture time: 72 hours

Medium:

8 ml of a medium with the following composition was placed in a test tube provided with a cotton plug and having an inner diameter of 18 mm and sterilized in an autoclave at 121°C for 15 minutes to prepare a test tube medium for seeding. The raw materials used for the test tube medium for seeding were those from lots that were confirmed to allow sufficient growth of bacterial cells.

Sucrose: 30 g/L

Corn steep liquor: 30 g/L

Potassium dihydrogen phosphate: 1.5 g/L

Disodium hydrogen phosphate dodecahydrate: 3.8 g/L

Calcium chloride dihydrate: 5.0 g/L

Magnesium sulfate heptahydrate: 0.7 g/L

Iron sulfate heptahydrate: 1.0 g/L

pH7.2

[0061] Next, 7.2 ml of a medium with the following composition was placed into each of test tubes provided with a cotton plug and having an inner diameter of 18 mm to prepare five test tube media for production. The raw materials used for the test tube media for production were those from lots that were confirmed to allow insufficient growth of bacterial cells.

Glucose: 30 g/L

Filtrated corn steep liquor product: 30 g/L

Ammonium sulfate: 1.5 g/L

Potassium dihydrogen phosphate: 1.5 g/L

Disodium hydrogen phosphate dodecahydrate: 3.8 g/L

Calcium chloride dihydrate: 5.0 g/L

Magnesium sulfate heptahydrate: 0.7 g/L

Iron sulfate heptahydrate: 1.0 g/L

Silicon-based antifoaming agent: 0.2 g/L

[0062] (ii) Results

The specific productivity of each strain is shown in Figure 1.

According to this example, strain ASB-57 that had production capacity 10 times or more that of strain E-396 carotenoid was acquired.

[0063] Example 2

Conformational analysis of enzyme involved in astaxanthin synthesis pathway

1. Conformational data and procedure

The conformational models of Enzymes A and C were constructed by homology modeling. For the modeling, software Swiss-Pdb viewer and SWISS-MODEL were used [1, 2]. Mutant models were prepared with Swiss-Pdb viewer. The command "mutate" was used to substitute the amino acid residues, the command "compute energy" was used to compute intramolecular energy, and the command "energy minimization" was used to calculate for energy minimization. Preparation of a complex model with the substrate or the like, detection of the residues in the vicinity of the substrate, measurement of interatomic distance, and display of the conformation were conducted using software Waals (Altif Labs. Inc.). A

conformational model of a low-molecular compound was prepared with MarvinSketch (ChemAxon Ltd.).

[0064] The coordinate data of the conformation of the template structure was acquired from the protein conformational database, Protein DataBase (PDB) (http://www.rcsb.org/pdb/). The template structures that had the highest amino acid matching degree with each of Enzymes A and C were used among the data registered with PDB. The conformational data used as the templates for the homology modeling is shown in Table 1.

Table 1: Conformational data used for homology modeling

Enzyme	PDB ID	Protein	Document
Enzyme A	2O1X	1-Deoxy-D-xylurose-5-phophate synthase	[3]
Enzyme C	3MZV	Decaprenyl diphosphate synthase	[4]

[0065] 2. Construction of conformational model of Enzyme A and analysis of mutant

Enzyme A is 1-deoxy-D-xylulose 5-phosphate synthase (DXS) that synthesizes 1-deoxy-D xylulose 5-phosphate from pyruvic acid and D-glyceraldehyde 3-phosphate in the deoxyxylulose pathway, i.e., one of isoprenoid biosynthesis pathways that biosynthesize isoprenyl diphosphate (IPP) that serves as a raw material for astaxanthin synthesis.

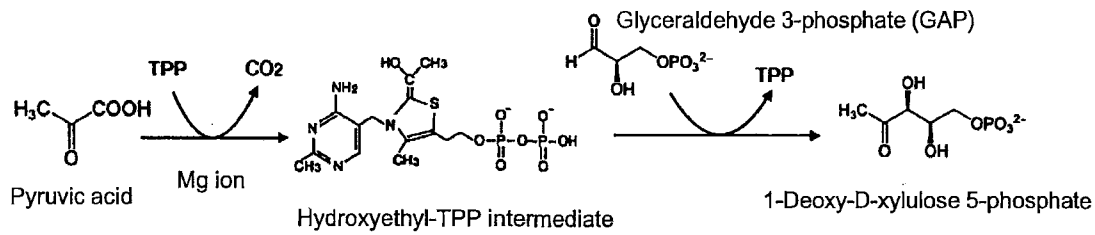
[0066] Mutation G225D identified from the constructed conformational model of Enzyme A by the genome analysis was found to exist in the disordered region in the vicinity of the active site, where the mutation had been reported to enhance the enzymatic activity of DXS in several cases. From the results of the analysis of the mutant model, mutation G225D in Enzyme A was deduced to induce a structural change similar to that caused by the mutation known to enhance the DXS activity, and thus the mutant G225D enzyme was also predicted to have enhanced DXS enzymatic activity similar to the known mutation. Enhancement of the DXS activity increases IPP supply as the raw material of astaxanthin, which is considered to increase the amount of astaxanthin produced.

[0067] 2.1. Enzymatic reaction of 1-deoxy-D-xylulose 5-phosphate synthase

Enzyme A, 1-deoxy-D-xylurose-5-phosphate synthase (DXS), synthesizes 1-deoxy-D-xylulose 5-phosphate from pyruvic acid and D-glyceraldehyde 3-phosphate in

the presence of a magnesium ion (Mg). The catalytic reaction requires thiamine pyrophosphate (TPP) as a coenzyme. First, TPP coenzyme attaches to the substrate pyruvic acid to give a hydroxyethyl-TPP intermediate. Reaction between this intermediate and glyceraldehyde 3-phosphate generates 1-deoxy-D-xylulose 5-phosphate. The enzymatic reaction of Enzyme A is shown below.

Chemical formula 1



[0068] 2.2. Construction of conformational model of Enzyme A

(1) Construction of conformational model of Enzyme A by homology modeling

The conformational model of Enzyme A was constructed by homology modeling based on the conformation (PDB ID:2O1X) [3] of *Deinococcus radiodurans* (*D. radiodurans*)-derived 1-deoxy-D-xylurose-5-phosphate synthase (DXS, template) (Figure 2) whose conformation of the complex with TPP coenzyme had been determined by X-ray crystallography.

[0069] Homology modeling was carried out based on the conformation alignment between Enzyme A and the template structure (Figures 3A and 3B).

The amino acid matching degree between Enzyme A and *D. radiodurans*-derived DXS was 44.1%. In the conformation of the template DXS, the region of the amino acid residues 199-242 (44 residues) was a disordered region and thus the positions of the atoms were unspecified by X-ray crystallography. Therefore, a conformational model of the amino acid residues 7-630 excluding the residues 196-238 (43 residues) corresponding to the disordered region of Enzyme A was constructed. Then, TPP and Mg were embedded by superimposing the conformational model of Enzyme A and the template structure to prepare a complex model of Enzyme A and TPP. Figure 4 shows the template structure and the constructed model structure.

Similar to the template structure, Enzyme A formed a homodimer and had a TPP binding site and a substrate binding site in each of the subunits. The monomer of Enzyme A consists of three domains, namely, domain I (residues 1-319), domain II (residues 320-495) and domain III (residues 496-629).

[0070] (2) Preparation of Enzyme A-substrate complex models

In order to deduce the amino acid residue responsible for the bond between Enzyme A and the substrates, complex models having the substrates bonded to Enzyme A were constructed. Since the coordinates of pyruvic acid and glyceraldehyde 3-phosphate as the substrates were not determined in the template structure 2O1X, first, a model of a complex with the hydroxyethyl-TPP intermediate that had TPP coenzyme attached to pyruvic acid was prepared by embedding the hydroxyethyl-TPP intermediate by superimposition based on a conformation in which a related *Saccharomyces cerevisiae* (*S. cerevisiae*)-derived transketolase (TK) was bound with the hydroxyethyl-TPP intermediate (PDB ID: 1GPU) [6], to detect the pyruvic acid binding site. Similarly, a model of an Enzyme A-glyceraldehyde 3-phosphate complex was prepared by embedding erythrose-4-phosphate based on a conformation of *S. cerevisiae*-derived TK with erythrose-4-phosphate (PDB ID: 1NGS) [7] and further preparing a glyceraldehyde 3-phosphate model from erythrose-4-phosphate.

Figure 5 shows the model structure of the Enzyme A-substrate complex.

[0071] 2.3. Deduction of active site of Enzyme A

In order to deduce the amino acid residue binding the coenzyme and the substrate, interactions between the TPP intermediate, GAP and Mg were examined in the complex model of Enzyme A and the coenzyme.

[0072] (1) Binding site of TPP coenzyme

Similar to the template structure, TPP was located between domain I and domain II in the complex model of Enzyme A and TPP, where the pyrimidine ring of TPP was bound to domain II and the phosphate group was bound to domain I.

[0073] TPP consists of an aminopyrimidine ring, a thiazoline ring and pyrophosphate (Figure 6). The aminopyrimidine ring binds to closely fit inside the binding pocket of Enzyme A. The side chains of Phe396 and Ile369 are bound via hydrophobic interactions to sandwich the aminopyrimidine ring. Especially, a strong bond via π stacking was deduced

between the side chain of Phe396, the cyclic phenyl group, and the aminopyrimidine ring. The main-chain oxygen of Gly120 and the side-chain oxygen of Glu371 formed hydrogen bonds with the nitrogen atom at position 1 in the aminopyrimidine ring. The hydrogen bond between the nitrogen atom at position 1 in the pyrimidine ring of TPP and Glu residue is important for the catalytic reaction via TPP, and is known to be conserved in an enzyme that uses TPP as a coenzyme. The side chain of Arg399 is predicted to form the binding pocket not only via the hydrophobic bond but also via hydrogen bonds with the side chains of the peripheral amino acid residues Glu371 and Ser122. As to the interactions with the thiazoline ring, hydrophobic bonds with Ile184 and Ile369 were observed. As to the interactions with pyrophosphate, hydrogen bonds with the main chain of Gly152, the side chain of Lys285 and the side chain of His79 were deduced.

[0074] The bond with the TPP coenzyme requires Mg. Mg is located between the two phosphate groups of TPP, and Mg was deduced to bind with the side chains of Asp151 and Asn180 and the main chain of Met182. As to DXS, the sequence of GDGX25-30N is known to be conserved as a TPP binding motif [3]. The amino acid sequence Gly150-Asp151-Gly152-Asn180 including Asp151 and Asn180 that were deduced to form bonds with Mg in Enzyme A matches this motif.

[0075] Thus, the carbons in the aminopyrimidine ring and the thiazoline ring of TPP were deduced to have hydrophobic interactions with hydrophobic residues Ile184, Ile369, Phe396 and Arg399 of Enzyme A. Moreover, two nitrogen atoms in the aminopyrimidine ring were deduced to form hydrogen bonds with Gly120 and Glu371. Besides the hydrogen bonds with His79, Gly152 and Lys285, the pyrophosphate was deduced to form hydrogen bonds with Asp151, Asn180 and Met182 via Mg. Figure 7 shows the amino acid residues that were deduced to be responsible for the bonds with TPP.

[0076] (2) Interaction with pyruvic acid

Pyruvic acid as the substrate reacts with TPP and forms a hydroxyethyl-TPP intermediate. From the complex model of Enzyme A and the hydroxyethyl-TPP intermediate, a hydrophobic interaction with Val77 and a hydrogen bond with His432 were deduced as interactions with the pyruvic acid-derived hydroxyethyl group. These amino acid residues were considered to be involved in the bond with pyruvic acid. Figure 8 shows amino acid residues that were predicted to have interactions with the hydroxyethyl groups.

[0077] (3) Interaction with glyceraldehyde 3-phosphate

As the amino acid residues that interact with the substrate glyceraldehyde 3-phosphate (GAP), hydrogen bonds with His48, Tyr393, Arg421, Asp428 and Arg479 were deduced (Figure 9).

His48 and Asp428 form hydrogen bonds with the aldehyde groups of GAP. Tyr393, Arg421 and Arg479 were deduced to form hydrogen bonds with the phosphate groups of GAP.

[0078] The above-described deduced active sites are shown in Tables 2-4.

Table 2: Amino acid residues predicted to have interaction with hydroxyethyl-TTP intermediate detected in Enzyme A

hydroxyethyl-TTP	Enzyme_A		interaction
Amino-Pyrimidine	Gly120	main-chain	hydrogen bond
	Ile369	side-chain	hydrophobic interaction
	Glu371	side-chain	hydrogen bond
	Phe396	side-chain	hydrophobic interaction
	Arg399	side-chain	hydrophobic interaction
Thiazolium	Ile184	side-chain	hydrophobic interaction
	Ile369	side-chain	hydrophobic interaction
PO4	His79	side-chain	hydrogen bond
	Gly152	main-chain	hydrogen bond
	Lys285	side-chain	hydrogen bond
Mg	Asp151	side-chain	hydrogen bond
	Asn180	side-chain	hydrogen bond
	Met182	main-chain	hydrogen bond
Hydroxyethyl	Val77	side-chain	hydrophobic interaction
	His432	side-chain	hydrogen bond

Table 3: Amino acid residues predicted to have interaction with glyceraldehyde 3-phosphate detected in Enzyme A

GAP	Enzyme_A		interaction
Glyceraldehyde	His48	side-chain	hydrogen bond
	Asp428	side-chain	hydrogen bond
PO4	Tyr393	side-chain	hydrogen bond
	Arg421	side-chain	hydrogen bond
	Arg479	side-chain	hydrogen bond

Table 4: Amino acid residues at deduced binding sites in Enzyme A

binding site	residue
TTP	His79
	Gly120
	Gly152
	Ile184
	Lys285
	Ile369
	Glu371
	Phe396
	Arg399
Mg	Asp151
	Asn180
	Met182
Pyrvate	Val77
	His432
GAP	His48
	Asp428
	Tyr393
	Arg421
	Arg479

[0079] Furthermore, the binding modes between Enzyme A and TPP coenzyme, the binding modes between Enzyme A and pyruvic acid, the binding modes between Enzyme A and glyceraldehyde 3-phosphate are shown in Figures 6, 10 and 11, respectively.

Since Enzyme A retained active sites that bind TPP coenzyme and substrates pyruvic acid and GAP like template *D. radiodurans*-derived DXS, it was predicted to have the enzymatic activity of DXS. Other than *D. radiodurans*-derived DXS, these amino acid residues are also known to be highly conserved in DXS from *E. coli* and the like and *S.*

cerevisiae-derived TK [3].

[0080] In *E. coli* DXS, substitution of the amino acid residues corresponding to Glu370, Arg399 and Arg479 of Enzyme A to Ala is found to result deactivation [3]. According to Document [8], enzymatic activity was generally deactivated in all of the experiments of mutating amino acid residues of *E. coli* DXS corresponding to His48, Glu371 and Asp428 of Enzyme A. These amino acid residues are predicted to be important for DXS activity in Enzyme A as well.

[0081] 2.4. Effect of mutation G225D in Enzyme A

Mutation G225D in Enzyme A was present in the disordered region (residues 196-238) whose conformation was unspecified. In order to deduce the effect of this mutation on the conformation of Enzyme A, the previous findings, the conformational location of the disordered region and the relationship with the active site were examined.

[0082] (1) Regarding mutation in known disordered region

So far, two mutations caused in the disordered region of each of Muscat and *E. coli* DXS, i.e., a total of four mutations, were all reported to increase the enzymatic activity.

In Muscat (*Vitis vinifera*)-derived DXS, mutation K284N was reported to result an increase in the activity that was about twice as high as that of the wild-type in Vmax and Kcat/Km, and overexpression was reported to greatly increase the amount of monoterpene produced [9].

[0083] Moreover, inventions related to mutations K284N and R306C in Muscat and mutations K213N and K234C in *E. coli* are known (Japanese Patent Application Publication No. 2014-500710, US20130276166). This invention relates to a method for increasing the amount of terpene produced by enhancement of DXS activity, where the amount of terpene produced increased by the single-residue mutation in all of the four cases.

[0084] The positions of mutations in Enzyme A and *E. coli* and Muscat DXS that resulted increase in the enzymatic activity are shown in Figure 12.

K284N and R306C in Muscat, and K213N and K234C in *E. coli* all existed in the disordered region (blue). Mutation G225D in Enzyme A also existed in the disordered region. Based on the amino acid sequence alignment, the active site (green) of DXS was conserved and Enzyme A was predicted to have a reaction mode similar to the reaction modes of these DXS. The active site existed on the N-terminal side (magenta) of the

disordered region.

[0085] Thus, multiple mutations in the disordered region of DXS increased the enzymatic activity of DXS, and mutagenesis in this region of interest was predicted to give some effect to the DXS activity.

[0086] (2) Conformational location of mutation G225D (disordered region) in Enzyme A

Figure 13 shows the location of the disordered region (residues 196-238) that has mutation G225D in Enzyme A in a blue dotted line.

The region of interest is located in the vicinity of the binding site of TPP coenzyme essential for DXS activity. Active sites Asn180, Met182 and Ile184 exist in the loop (magenta) on the N-terminal side of the region of interest. The side chain of Asn180 and the main chain of Met182 bind to Mg. The side chain of Ile184 form a hydrophobic bond with TPP. It is considered important that this loop has a suitable structure such that TPP essential for DXS activity can bind with Mg. In document [9], the physiological role of the region of interest was unclear but the region of interest was considered to exist near the active site, and the mutation in the region of interest rationally seemed to have an effect on the activity of the enzyme. Since the region of interest exists in the vicinity of the TPP binding site both in terms of the amino acid sequence and the conformation as shown in Figures 12 and 13, it is highly likely that the mutation in the region of interest has an effect on these active sites.

[0087] (3) Preparations of model of disordered region of Enzyme A and mutant G225D enzyme

Next, in order to examine the effect of mutation G225D in Enzyme A on the conformation of the disordered region, a model structure was prepared for the disordered region across residues 196-238 (43 residues) of Enzyme A. In document [9] that reported mutation K284N in Muscat, a model of the disordered region of Muscat DXS was prepared to observe the change in the electrostatic potential by the mutation. For reference, a similar analysis was conducted for Enzyme A as well. A conformational model was prepared by homology modeling based on a fragment structure of an amino acid sequence that was highly homologous with the disordered region of Enzyme A. As a template structure, a fragment 1AL7 having the highest amino acid matching degree (34%) among the conformations registered in PDB was used. Even though the disordered region was predicted to have a fluctuated structure, it was considered to serve as a reference of a folding

that is likely to form the region of interest. The prepared model is shown in Figure 14 (left).

[0088] Furthermore, a conformational model of the mutant G225D enzyme was prepared by substituting the mutation site Gly225 with Asp. Since Gly225 is located on the surface, substitution with Asp exposes the side chain of Asp on the surface. The results from mapping electrostatic potential on the surface profile of the prepared conformational model are shown in Figure 14 (right). Blue represents a positive (positively charged) region while red represents a negative (negatively charged) region.

[0089] While there is a strong positively charged region in the wild type, a weakened positive charge and a strengthened negative charge can be confirmed in the mutant G225D enzyme. In the wild type, the side chains of positively charged Arg227, Arg228, Lys230 and K234 aggregate, and form a strong positively charged region. Since the uncharged Gly225 existing in this region was replaced with the negatively charged Asp, the positive charge near Asp225 seemed to have weakened. This result shows similar tendency to document [9], that is, the change in the electrostatic potential due to mutation K284N in Muscat, i.e., the change in the electrostatic potential from a positive charge to a negative charge on the surface of the disordered region. The structural change in the disordered region caused by G225D in Enzyme A was predicted to have an effect similar to the mutation in Muscat on the active site including the TPP binding site, suggesting that the mutant G225D of Enzyme A had an increased enzymatic activity similar to the mutant K284N of Muscat due to this effect.

[0090] 2.5. Effect of mutation G225D in Enzyme A on astaxanthin synthesis pathway

Accordingly, mutation G225D in Enzyme A was confirmed to occur in the disordered region of Enzyme A, and this region was confirmed to exist in the vicinity of the TPP binding site that was essential for the activity. So far, multiple mutations in this region have been found to enhance the enzymatic activity of DXS, suggesting that G225D mutation causes a structural change similar to that caused by the mutations that are known to enhance the DXS activity.

[0091] From the location of the disordered region confirmed with the conformational model, mutation in the disordered region seems to have some effect on the TPP binding region to enhance the enzymatic activity at least as described in the document. In the case of Muscat, Kcat/Km of the mutant was shown to be about twice as high as that of the wild type

by an enzymonological experiment *in vitro*, and thus the mutation in the disordered region was predicted to result a structural change that was more suitable for the binding of TPP.

[0092] In order to greatly increase a monoterpene in a cell, isopentenyl diphosphate (IPP) as a raw material of the monoterpene needs to be increased. Feedback inhibition of DXS by IPP as a product of the deoxyxylulose pathway and competitive inhibition of IPP with TPP have been reported [10]. Moreover, it is known that once IPP reaches a certain amount, DXS, i.e., the first enzyme of the deoxyxylulose pathway, is inhibited so that IPP can no longer increase.

[0093] Since the amount of the IPP supplied is regulated to stay at a certain level by DXS due to this feedback inhibition, an increase in the enzymatic activity Kcat/Km of DXS would not simply increase the IPP supply to significantly increase terpene. In a known example, the mutation in the disordered region increased the amount of the synthesized terpene and this mutation was predicted to increase the IPP supply, suggesting that feedback inhibition of DXS by IPP was no longer effective.

[0094] IPP and TPP competitively bind to DXS *in vitro*, and are found to inhibit DXS [10]. The mutation in the disordered region that caused a structural change in the TPP binding region suggests not only that it gives a structure suitable for TPP binding but also that feedback inhibition by IPP no longer works because of the effect on IPP binding.

[0095] Mutation G225D in Enzyme A was also predicted to result a structural change of the TPP binding region and increase the amount of IPP produced because feedback inhibition by IPP no longer works. As a result, the supply of IPP as a raw material of astaxanthin synthesis increased, by which the amount of synthesized astaxanthin was increased (Figure 15).

[0096] 3. Construction of conformational model of Enzyme C and analysis of mutant

From the homology of the amino acid sequences and a comparative conformational analysis, Enzyme C was found to be one kind of polyprenyl diphosphate synthases, namely, decaprenyl diphosphate synthase that synthesizes decaprenyl diphosphate from farnesyl diphosphate (FPP) and seven isopentenyl diphosphates (IPP).

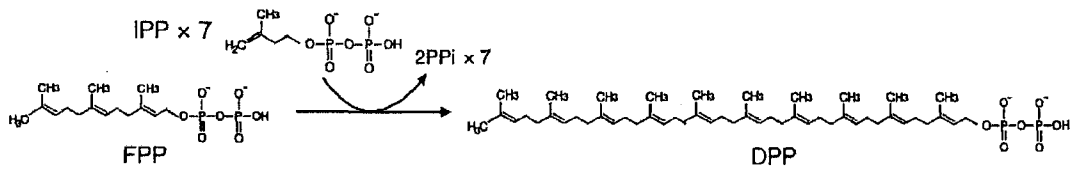
[0097] Mutation A305V identified by a genome analysis based on the constructed conformational model of Enzyme C caused steric hindrance due to repulsion of the atoms with the peripheral amino acid residue, and was deduced to destabilize the conformation of

Enzyme C. A decrease in Enzyme C activity due to the destabilized conformation decreases the amounts of FPP and IPP digested by Enzyme C. As a result, the increase in the amounts of FPP and IPP that can be used for astaxanthin synthesis seemed to increase the amount of astaxanthin produced.

[0098] 3.1. Enzymatic reaction of decaprenyl diphosphate synthase

Decaprenyl diphosphate synthase is an enzyme that has an activity of catalyzing condensation of FPP with IPP and repeats condensation with IPP to synthesize decaprenyl diphosphate (DPP) from FPP and seven IPPs. The enzymatic reaction of decaprenyl diphosphate synthase is shown below.

Chemical formula 2



DPP synthase

[0099] 3.2. Construction of conformational model of Enzyme C

(1) Construction of conformational model of Enzyme C by homology modeling

In order to examine the effects of the active site and the mutations in Enzyme C, a conformational model (Figure 16) was constructed by homology modeling using, as a template structure, the conformation of *Rhodobacter capsulatus* (*R. capsulatus*)-derived decaprenyl diphosphate synthase (PDB ID: 3MZV) that had the highest amino acid matching degree (amino acid matching degree of 76.2%) among the conformations registered in PDB, and whose conformation had been determined by X-ray crystallography [4].

The homology modeling was conducted based on the conformation alignment between Enzyme C and the template structure (Figure 17).

[0100] (2) Preparation of Enzyme C-substrate complex model

Since the template structure 3MZV is not bonded with a substrate, the conformational data of *Escherichia coli*-derived octaprenyl pyrophosphate synthase (octaprenyl diphosphate synthase) (PDB ID: 3WJN, 3WJO) [11] was used to superimpose

3WJN on the conformational model of Enzyme C and embed FPP as the substrate into the conformational model of Enzyme C, thereby preparing a complex model of Enzyme C and FPP. Next, in the same manner, 3WJO was superimposed and IPP was embedded to prepare a complex model of Enzyme C and FPP and IPP.

Figure 18 shows the template structure and the constructed model structure. Similar to the template structure, Enzyme C forms a homodimer.

[0101] 3.3. Regarding Enzyme C

(1) Conformational comparison

The decaprenyl diphosphate synthase as Enzyme C belongs to the polyprenyl diphosphate synthase family (Pfam PF00348 Polyprenyl synthetase). Figure 19 shows the state of the substrate binding with Enzyme C.

Polyprenyl diphosphate synthase condensates FPP and IPP in the head-to-tail direction (following document [4], the phosphate group side is referred to as the head while the isoprenyl group side is referred to as the tail), thereby synthesizing various polyprenyl diphosphates. By continuing condensation reaction of FPP and IPP, decaprenyl diphosphate synthase extends the prenyl chain deep into the substrate binding site (as represented by an arrow in the right view of Figure 19) to synthesize C50-decaprenyl diphosphate.

[0102] Enzyme C and *R. capsulatus*-derived decaprenyl diphosphate synthase as the template have an amino acid matching degree as high as 76.3% with a conserved active site (Figure 17). RMSD of the conformation by Cα superimposition was 0.063Å, where the two enzymes were very similar. When the matching of the amino acid residues was distinguished by colors, a region having different types of amino acid residues was limited to the molecular surface, the active site and the region binding the substrate entirely consisted of matching amino acid residues (Figure 20).

[0103] (2) Comparison by amino acid sequences

Next, the amino acid sequence of Enzyme C was compared with *Paracoccus*-derived decaprenyl diphosphate synthase. *Paracoccus zeaxanthinifaciens* (Q8L1I6)- and *Paracoccus denitrificans* (A1B3M9)-derived amino acid sequences have been submitted to UniProt (http://www.uniprot.org) as already known amino acid sequences of *Paracoccus*-derived decaprenyl diphosphate synthases. When they were compared with the amino acid sequence of Enzyme C, the amino acid matching degree was 75.1% (degree

of similarity 89.2%), showing high homology (Table 5).

The amino acid sequence also suggested that Enzyme C was decaprenyl diphosphate synthase. Figure 21 shows the alignments.

Table 5: Comparison of amino acid sequences between Enzyme C and decaprenyl diphosphate synthases

Protein	Organism	UniProt	Identity (similar)
Decaprenyl diphosphate synthase	*Paracoccus zeaxanthinifaciens*	Q8L1I6	86.2% (95.2%)
	Paracoccus denitrificans	A1B3M9	75.1% (89.2%)

[0104] 3.4. Deduction of active site of Enzyme C

In a complex model of Enzyme C and the substrates, the FPP binding site and the IPP binding site as the substrates and the Mg binding site required for catalysis were deduced. The active sites deduced from these results, and the active center and the substrate binding sites of other polyprenyl diphosphate synthases were highly conserved, and thus Enzyme C was deduced to have a reaction mode similar to that of polyprenyl diphosphate synthase.

[0105] The complex model of Enzyme C and the substrates is shown in Figure 22.

Similar to decaprenyl diphosphate synthase as the template structure, Enzyme C was expected to form a homodimer. Figure 22 shows the binding sites of FPP and IPP in chain A (light red). FPP binds to the tunnel region of chain A while FPP and IPP bind to each other in a head-to-tail form with the phosphate group of FPP directing toward the isopentenyl group of IPP. A catalytic reaction occurs between the phosphate group of FPP and the isopentenyl group of IPP in the presence of Mg.

[0106] In order to deduce the active site of Enzyme C, amino acid residues present in the vicinity of FPP and IPP were detected in the Enzyme C-substrate complex model to deduce the interaction between the substrate and the amino acid residues. For FPP, hydrogen bonds between phosphate groups and Arg102, Lys179 and Lys244, and hydrophobic interactions between polyprenyl groups and Ala88, Thr89, His92 and Phe125 were deduced (Figure 23, top image).

For IPP, hydrogen bonds between phosphate groups and Lys54, Arg57, His86 and Arg103, and a hydrophobic interaction between an isopentenyl group and Phe216 were

deduced (Figure 23, center image).

[0107] This catalytic reaction requires Mg. As Mg binding sites, two DDXXD motifs are known in a known polyprenyl diphosphate synthase [11]. Although the coordinates of Mg are not determined in the template structure, Asp93, Asp94, Asp97 and Asp220, and Asp221 and Asp224 corresponding to the DDXXD motifs exist near phosphate groups in Enzyme C similar to the known Mg binding sites, and thus these amino acid residues were deduced to bind Mg (Figure 23, bottom image).

[0108] The deduced active sites are shown in Tables 6-8. In addition, the binding modes between Enzyme C and FPP and IPP as well as the binding modes between Enzyme C and Mg are shown in Figures 24 and 25, respectively.

Table 6: Amino acid residues predicted to have interactions with FPP and IPP detected in Enzyme C

Substrate		Enzyme_C		interaction
FPP	Polyprenyl	Ala88	side-chain	hydrophobic interaction
		Thr89	side-chain	hydrophobic interaction
		His92	side-chain	hydrophobic interaction
		Phe125	side-chain	hydrophobic interaction
	PO4	Arg102	side-chain	hydrogen bond
		Lys179	side-chain	hydrogen bond
		Lys244	side-chain	hydrogen bond
IPP	Isopentenyl	Phe216	side-chain	hydrophobic interaction
	PO4	Lys54	side-chain	hydrogen bond
		Arg57	side-chain	hydrogen bond
		His86	side-chain	hydrogen bond
		Arg103	side-chain	hydrogen bond

[0109]

Table 7: Amino acid residues predicted to have interaction with Mg detected in Enzyme C

Mg	Enzyme_C		Interaction
Mg 1	Asp93	side-chain	hydrogen bond
	Asp94	side-chain	hydrogen bond
	Asp97	side-chain	hydrogen bond
Mg 2	Asp220	side-chain	hydrogen bond
	Asp221	side-chain	hydrogen bond
	Asp224	side-chain	hydrogen bond

[0110]

Table 8: Amino acid residues of deduced active sites in Enzyme C

binding site	residue
FPP	Ala88
	Thr89
	His92
	Arg102
	Phe125
	Lys179
	Lys244
IPP	Lys54
	Arg57
	His86
	Arg103
	Phe216
Mg	Asp93
	Asp94
	Asp97
	Asp220
	Asp221
	Asp224

[0111] Each of the amino acid residues at the deduced bonds matches that of *R. capsulatus*-derived decaprenyl diphosphate synthase as the template structure (Figure 17). In addition, since the FPP binding site, the IPP binding site and the Mg binding site of Enzyme C retain the binding site of *E. coli*-derived octaprenyl diphosphate synthase used for making the substrate complex model, Enzyme C is considered to take a reaction mode similar to octaprenyl diphosphate synthase.

[0112] In particular, Arg102, Lys179 and Lys244 that bind to the phosphate group of FPP, and Asp93, Asp94, Asp97, Asp220, Asp221 and Asp224 that bind to Mg seem to be important residues that are directly responsible for the activity of catalyzing transfer of the phosphate groups. Experiments of preparing rat and yeast FPP synthase mutants reported

that amino acid residues corresponding to Arg102 and Arg103 as phosphate binding sites and Asp94, Asp97, Asp220, Asp221 and Asp224 as Mg binding sites of Enzyme C were important for enzymatic activity, and that these amino acid residues were highly conserved among polyprenyl diphosphate synthases [14, 15].

[0113] In addition, in FPP synthase, side chains of Phe and Gln were found to be important for substrate binding through preparation of mutants of the amino acid residues corresponding to Phe216 and Gln217 of Enzyme C [16]. Phe216 and Gln217 also existed in the region of the active site in the complex model of Enzyme C and thus were predicted to be important for the activity.

[0114] 3.5. Effect of mutation A305V in Enzyme C

In order to deduce the effect of mutation from Ala305 to Val identified in Enzyme C on the conformation, a conformational model of mutant A305V enzyme of Enzyme C was prepared.

[0115] (1) Preparation of mutant model by A305V single residue substitution

In order to observe the effect of substitution from Ala to Val, a model was first prepared by fixing the conformation of the rest of the amino acid residues (assuming it to be a rigid body) and allowing a single residue substitution from Ala305 to Val, to compare the model with the wild type.

[0116] Figure 26 shows conformational models of the wild-type and mutant A305V enzymes. Ala305 in the wild type was present in the α-helix (pink), where the side chain provided packing with the amino acid residue of the adjacent α-helix (cyan) via a hydrophobic interaction.

[0117] Figure 27 shows the structure of the amino acid residue adjacent to Ala305.

The carbon atom of the methyl group as the side chain of Ala305 makes contact with the peripheral amino acid residues Tyr208, Ala211, His301 and Ala302, where the interatomic distances between the carbons were all less than 4.0Å. The substitution from Ala305 to Val results in an addition of two methyl groups to the side chain. In the conformational model including the substitution from Ala305 to Val, the interatomic distances between the carbon of the methyl group of the side chain of Val and the carbons of Tyr208 and Ala211 were 2.42Å and 2.47Å, respectively.

[0118] The lower limit of the contact distance between carbons via a noncovalent bond is

2.9Å. Since the interatomic distances of the carbons measured with respect to Val305 was lower than this value, these carbon atoms would repel and cause steric hindrance with the peripheral amino acid residues. In a space-filling representation, the interatomic distances were shorter than the van der Waals radii, confirming repulsion between the atoms. Accordingly, A305V mutation was predicted to induce steric hindrance in Enzyme C due to repulsion of the atoms, which leads to destabilization of the conformation.

[0119] (2) Change in intramolecular energy due to mutation A305V

In order to examine the destabilization of the structure caused by A305V, the intramolecular energies of the wild-type and mutant A305V Enzyme C were computed in terms of unit kilojoules/mol (KJ/mol) from a set of the interatomic bond length, the bond angle, the torsion, the bond energy and the like.

[0120] As a result, mutant A305V enzyme gave an intramolecular energy of -16,295 (KJ/mol) while the wild type gave -17,912 (KJ/mol), showing an increase of 9.02% and confirming destabilization of the structure. This increase in the intramolecular energy were particularly observed at the amino acid residues of Tyr208, Ala211 and Val305, and thus repulsion caused by these amino acid residues seemed to result the increase in the intramolecular energy. Comparison of the intramolecular energies between the wild-type and mutant A305V Enzyme C is shown in Figure 28.

[0121] (3) Structural change caused by mutation A305V

Next, an energy minimization calculation was performed on the mutant model so as to examine if repulsion due to A305V can be avoided by moving the conformation of the peripheral amino acid residues. As a result, it was found that the conformation of the amino acid residues of the α-helix adjacent to the α-helix in which Val305 exists needs to be moved in order to avoid repulsion of the side chain of Val305 while accepting the side chain. Specifically, it was predicted that this structural change not only affects the side chain but also the main chain, and thus the original packing between the α-helices does not occur in the mutant A305V enzyme and the structure surrounding the two helices was destabilized. Furthermore, the active sites that bond substrate IPP and Mg (Phe216, Gln217, Asp220) are present in the α-helix adjacent to mutation A305V. The structural change in the main-chain structure of the α-helix as the basis of the active sites dislocates the amino acid residues of the active sites, and this would have an effect on the substrate bonds and activity themselves

(Figure 29, right).

[0122] Figure 29 shows the structures around Ala305 and Val305 in the wild type and the mutant A305V following energy minimization calculation, while Figures 30, 31 and 32 show the structural changes in mutant A305V.

[0123] (4) Decrease in Enzyme C activity due to structural destabilization

Thus, from the conformational model of A305V, occurrence of the steric hindrance with the peripheral amino acid residues, the increase in the intramolecular energy and the structural change in the adjacent two α-helices were deduced, suggesting that mutation A305V destabilizes the conformation of Enzyme C. Conformational destabilization caused by point mutation has also been reported frequently in genetic disorders and the like. For example, a protein that has a destabilization-inducing mutation may be deactivated in shorter time than usual for being unable to maintain the original conformation in a solvent, and the mutant protein that cannot give original packing in the cell is eliminated by the function of the cell itself. Accordingly, the mutant A305V enzyme that does not have the original activity and that has an unstable structure may be eliminated in the bacterium, which is deduced to consequently decrease the Enzyme C activity in the bacterium.

[0124] 3.6. Effect of mutation A305V in Enzyme C on astaxanthin synthesis pathway

Decaprenyl diphosphate synthase is one of the enzymes in the coenzyme C10 (CoQ10) synthesis pathway. *Paracoccus zeaxanthinifaciens*- or *Paracoccus denitrificans*-derived decaprenyl diphosphate synthase that was confirmed to be highly homologous with Enzyme C this time is found to be an enzyme required for CoQ10 production [Japanese Patent Application Publication No. 2005-211020, Japanese Patent Application Publication No. 2006-517794]. FPP and IPP as the substrates of decaprenyl diphosphate synthase also serve as substrates of geranyl-geranyl diphosphate (GGPP) synthase CrtE in the astaxanthin synthesis pathway. Therefore, in a usual *Paracoccus* cell, decaprenyl diphosphate synthase and CrtE are considered to compete for substrates FPP and IPP.

[0125] Mutation A305V identified in Enzyme C was deduced to decrease the Enzyme C activity by destabilizing the molecular conformation. The decreased Enzyme C activity means decreased amounts of the substrates FPP and IPP used, which would increase the

amounts of FPP and IPP that can be used in the astaxanthin synthesis pathway.

[0126] CrtE synthesizes one molecule of GGPP from one molecule of FPP and one molecule of IPP. Meanwhile, Enzyme C requires one molecule of FPP and seven molecules of IPP to synthesize one molecule of decaprenyl diphosphate. In terms of IPP, Enzyme C digests IPP seven times as much as CrtE in a single reaction. Thus, the decrease in the Enzyme C activity seems very effective for increasing the IPP supplied to the astaxanthin synthesis pathway.

[0127] As a result, the amount of the synthesized astaxanthin is deduced to increase significantly (Figure 33).

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CLAIMS

1. A mutant carotenoidogenic bacterium, comprising any of genes (a)-(c) below:

(a) a gene encoding a protein comprising a mutant amino acid sequence in which at least the 225th amino acid residue in the amino acid sequence of 1-deoxy-D-xylulose 5-phosphate synthase of a carotenoidogenic bacterium has been substituted with other amino acid residue;

(b) a gene encoding a protein comprising a mutant amino acid sequence in which at least the 305th amino acid residue in the amino acid sequence of decaprenyl diphosphate synthase of a carotenoidogenic bacterium has been substituted with other amino acid residue; and

(c) both of the genes (a) and (b) above.

2. The bacterium according to Claim 1, wherein the amino acid sequence of 1-deoxy-D-xylulose 5-phosphate synthase is the sequence represented by SEQ ID NO:2.

3. The bacterium according to either one of Claims 1 and 2, wherein the 225th amino acid residue has been substituted from glycine to aspartic acid.

4. The bacterium according to any one of Claims 1-3, wherein the amino acid sequence of decaprenyl diphosphate synthase is the sequence represented by SEQ ID NO:4.

5. The bacterium according to any one of Claims 1-4, wherein the 305th amino acid residue has been substituted from alanine to valine.

6. The bacterium according to any one of Claims 1-5, which has acquired carotenogenic capacity that is higher than the carotenogenic capacity of a carotenoidogenic bacterium without the gene encoding the protein comprising the mutant amino acid sequence.

7. The bacterium according to Claim 6, which has acquired carotenogenic capacity

that is at least 5 times or more the carotenoid production amount of a carotenoidogenic bacterium without the gene encoding the protein comprising the mutant amino acid sequence.

8. The bacterium according to any one of Claims 1-7, wherein the carotenoidogenic bacterium belongs to the genus *Paracoccus*.

9. The bacterium according to Claim 8, wherein the bacterium belonging to the genus *Paracoccus* is strain E-396.

10. The bacterium according to any one of Claims 1-9, wherein the carotenoid is astaxanthin.

11. A method for producing a carotenoid, comprising culturing the bacterium according to any one of Claims 1-10, and collecting the carotenoid from the resulting cultured product.

12. The method according to Claim 11, wherein the carotenoid production amount is at least 5 times or more the carotenoid production amount of a carotenoidogenic bacterium without the gene encoding the protein comprising the mutant amino acid sequence.

13. The method according to either one of Claims 11 and 12, wherein the carotenoid is astaxanthin.

14. A method for screening for a carotenoidogenic bacterium, comprising subjecting a carotenoidogenic bacterium to a mutation treatment, and selecting a bacterium having any of characteristics (a)-(c) below from the bacteria subjected to the mutation treatment:

(a) a characteristic where the activity of 1-deoxy-D-xylulose 5-phosphate synthase is increased compared to said activity in the bacterium before the mutation treatment;

(b) a characteristic where the activity of decaprenyl diphosphate synthase is decreased compared to said activity in the bacterium before the mutation treatment; and

(c) both of the characteristics (a) and (b) above.

15. A method for producing a carotenoid, comprising culturing the bacterium selected by the method according to Claim 14, and collecting a carotenoid from the resulting cultured product.

16. A gene encoding a protein comprising a mutant amino acid sequence in which at least the 225th amino acid residue in the amino acid sequence of 1-deoxy-D-xylulose 5-phosphate synthase has been substituted with other amino acid residue.

17. A gene comprising either of DNA (a) or (b) below:

(a) DNA comprising the nucleotide sequence represented by SEQ ID NO:5; or

(b) DNA that hybridizes with DNA having a nucleotide sequence complementary to said DNA (a) under stringent conditions, and that encodes a protein having 1-deoxy-D-xylulose 5-phosphate synthase activity.

18. A gene encoding a protein comprising a mutant amino acid sequence in which at least the 305th amino acid residue in the amino acid sequence of decaprenyl diphosphate synthase has been substituted with other amino acid residue.

19. A gene comprising either of DNA (a) or (b) below:

(a) DNA comprising the nucleotide sequence represented by SEQ ID NO:7; or

(b) DNA that hybridizes with DNA having a nucleotide sequence complementary to said DNA (a) under stringent conditions, and that encodes a protein with decreased decaprenyl diphosphate synthase activity.

20. A recombinant vector comprising any of genes (a)-(c) below:

(a) the gene according to Claim 16 or 17;

(b) the gene according to Claim 18 or 19; and

(c) the genes (a) and (b) above.

21. A transformant comprising the recombinant vector according to Claim 20.

22. A method for producing a carotenoid, comprising culturing the transformant according to Claim 21, and collecting a carotenoid from the resulting cultured product.

Fig. 1

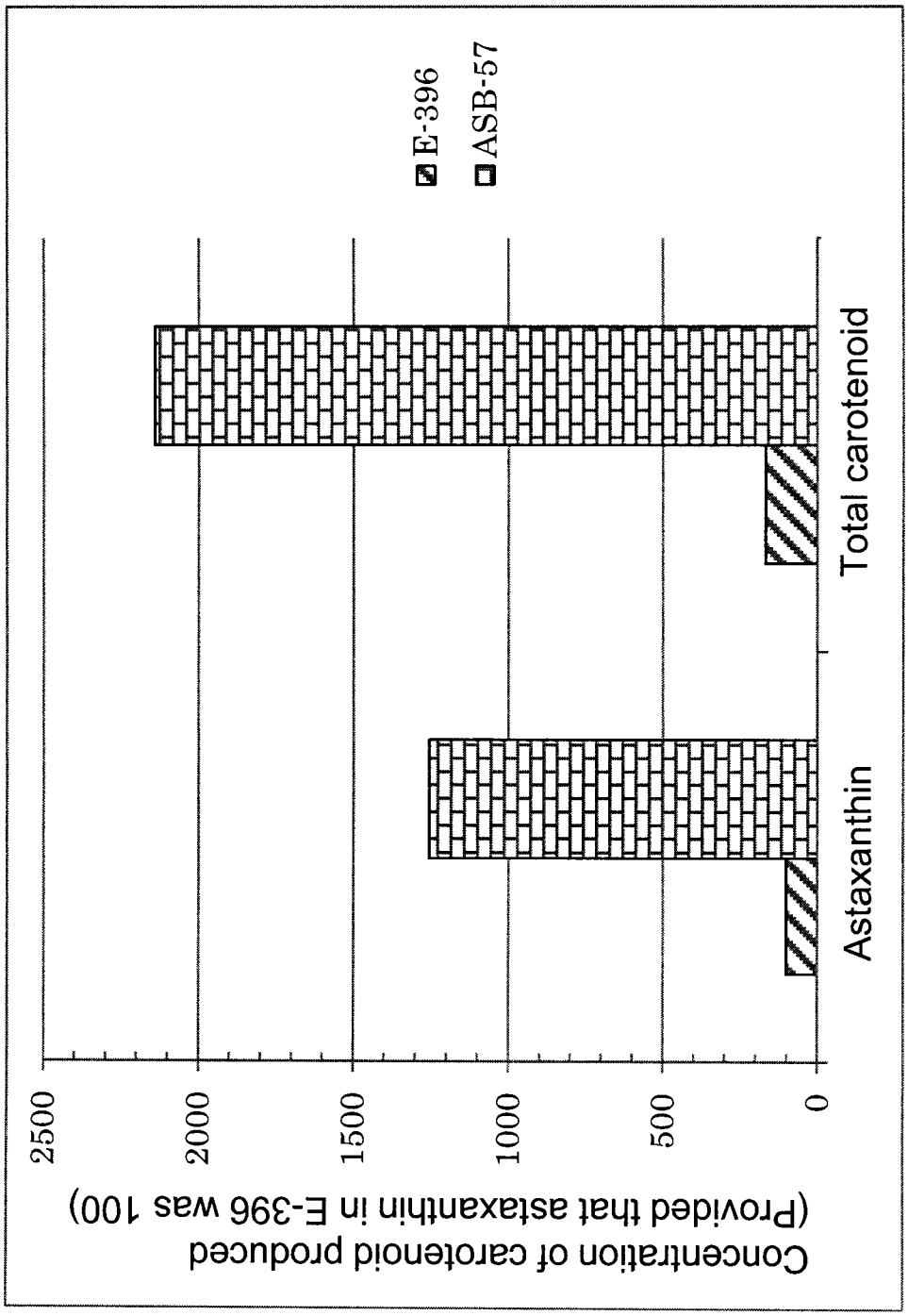
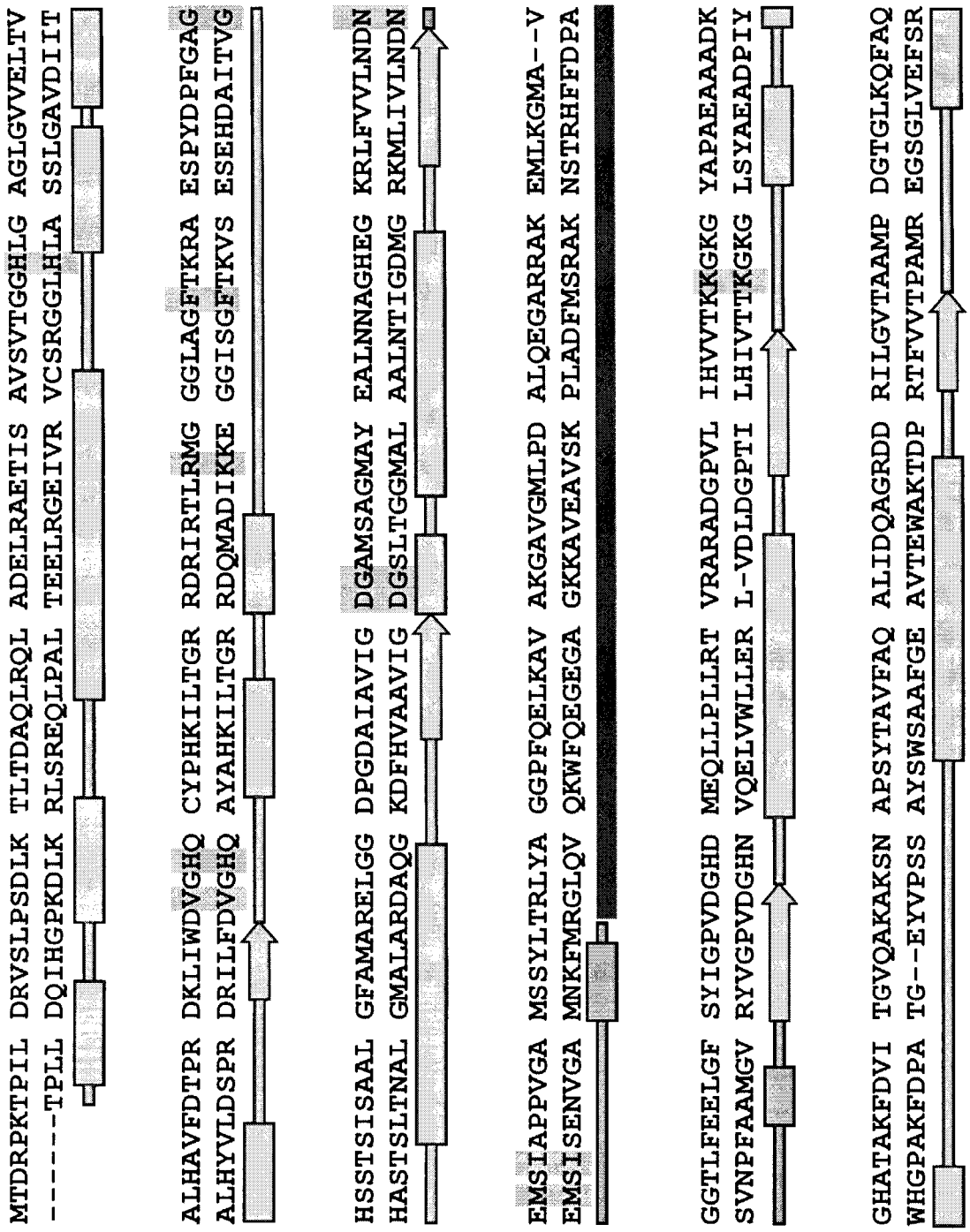

Concentration of carotenoid produced
(Provided that astaxanthin in E-396 was 100)
2500
2000
1500
1000
500
0
E-396
ASB-57
Astaxanthin
Total carotenoid

Fig. 2

Protein	1-deoxy-D-xylulose-5-phosphate synthase
UniProt	Q9RUB5 (DXS_DEIRA) EC:2.2.1.7
Organism	*Deinococcus radiodurans*
Pfam	PF13292 DXP_synthase_N
PDB ID	2O1X Resolution: 2.90[Å] dimer
Identity	44.1% 7-630

2O1X

Fig. 3A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2o1x ------TPLL DQIHGPKDLK RLSREQLPAL TEELRGEIVR VCSRGGLHLA SSLGAVDIIT
EnzymeA ALHAVFDTPR DKLIWDVGHQ CYPHKILTGR RDRIRTLRMG GGLAGFTKRA ESPYDPFGAG 120
2o1x ALHYVLDSPR DRILFDVGHQ AYAHKILTGR RDQMADIKKE GGISGFTKVS ESEHDAITVG
EnzymeA HSSTSISAAL GFAMARELGG DPGDAIAVIG DGAMSAGMAY EALNNAGHEG KRLFVVLNDN 180
2o1x HASTSLTNAL GMALARDAQG KDFHVAAVIG DGSLTGGMAL AALNTIGDMG RKMLIVLNDN
EnzymeA EMSIAPPVGA MSSYLTRLYA GGPFQELKAV AKGAVGMLPD ALQEGARRAK EMLKGMA--V 238
2o1x EMSISENVGA MNKFMRGLQV QKWFQEGEGA GKKAVEAVSK PLADFMSRAK NSTRHFFDPA
EnzymeA GGTLFEELGF SYIGPVDGHD MEQLLPLLRT VRARADGPVL IHVVTKKGKG YAPAEAAADK 298
2o1x SVNPFAAMGV RYVGPVDGHN VQELVWLLER L-VDLDGPTI LHIVTTKGKG LSYAEADPIY
EnzymeA GHATAKFDVI TGVQAKAKSN APSYTAVFAQ ALIDQAGRDD RILGVTAAMP DGTGLKQFAQ 358
2o1x WHGPAKFDPA TG--EYVPSS AYSWSAAFGE AVTEWAKTDP RTFVVTPAMR EGSGLVEFSR

Fig. 3B

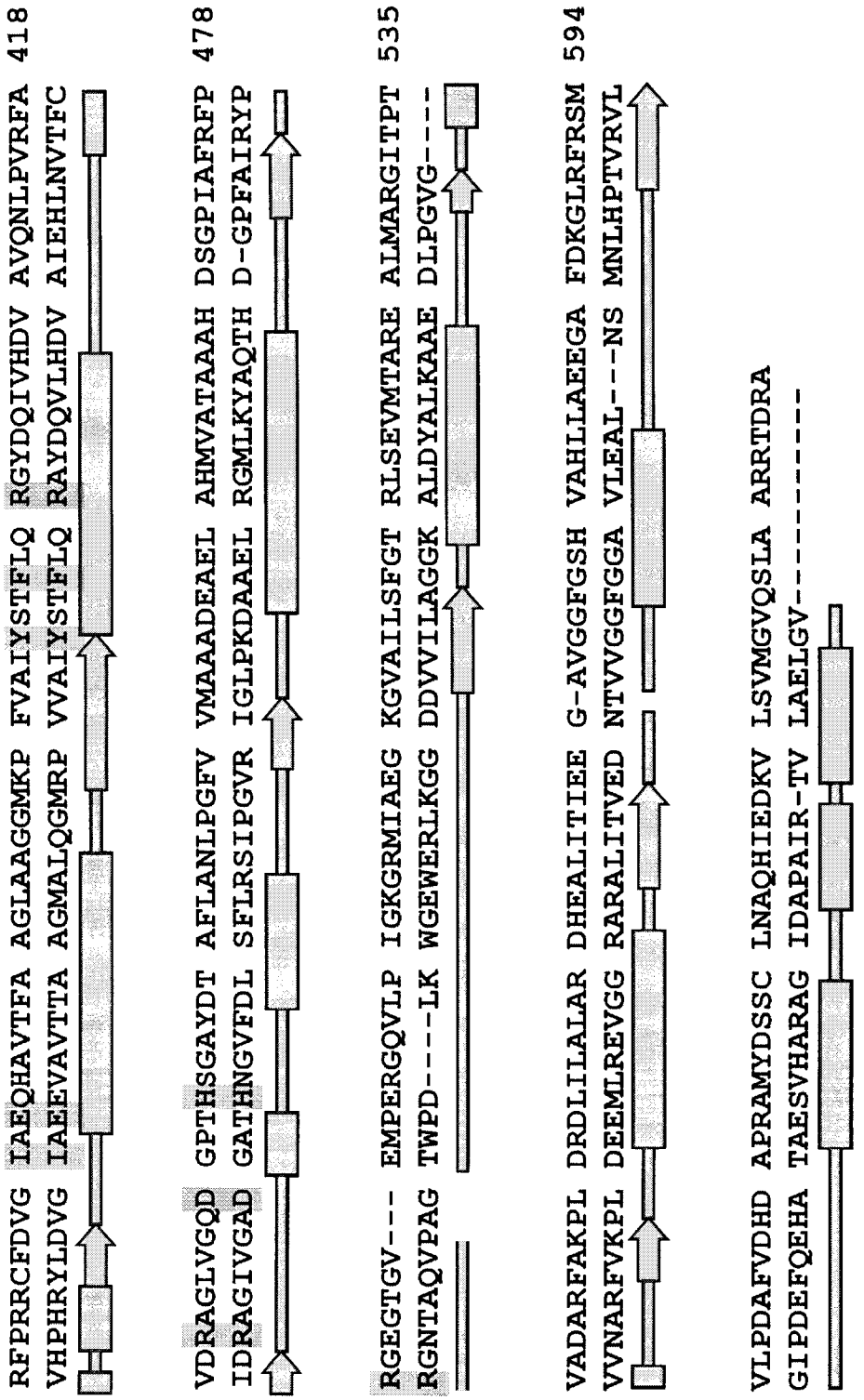

EnzymeA RFPRRCFDVG IAEQHAVTFA AGLAAGGMKP FVAIYSTFLQ RGYDQIVHDV AVQNLPVRFA 418
2o1x VHPHRYLDVG IAEEVAVTTA AGMALQGMRP VVAIYSTFLQ RAYDQVLHDV AIEHLNVTFC
EnzymeA VDRAGLVGQD GPTHSGAYDT AFLANLPGFV VMAAADEAEL AHMVATAAAH DSGPIAFRFP 478
2o1x IDRAGIVGAD GATHNGVFDL SFLRSIPGVR IGLPKDAAEL RGMLKYAQTH D-GPFAIRYP
EnzymeA RGEGTGV--- EMPERGQVLP IGKGRMIAEG KGVAILSFGT RLSEVMTARE ALMARGITPT 535
2o1x RGNTAQVPAG TWPD----LK WGEWERLKGG DDVVILAGGK ALDYALKAAE DLPGVG----
EnzymeA VADARFAKPL DRDLILALAR DHEALITIEE G-AVGGFGSH VAHLLAEEGA FDKGLRFRSM 594
2o1x VVNARFVKPL DEEMLREVGG RARALITVED NTVVGGFGGA VLEAL---NS MNLHPTVRVL
EnzymeA VLPDAFVDHD APRAMYDSSC LNAQHIEDKV LSVMGVQSLA ARRTDRA
2o1x GIPDEFQEHA TAESVHARAG IDAPAIR-TV LAELGV----------

Fig. 4

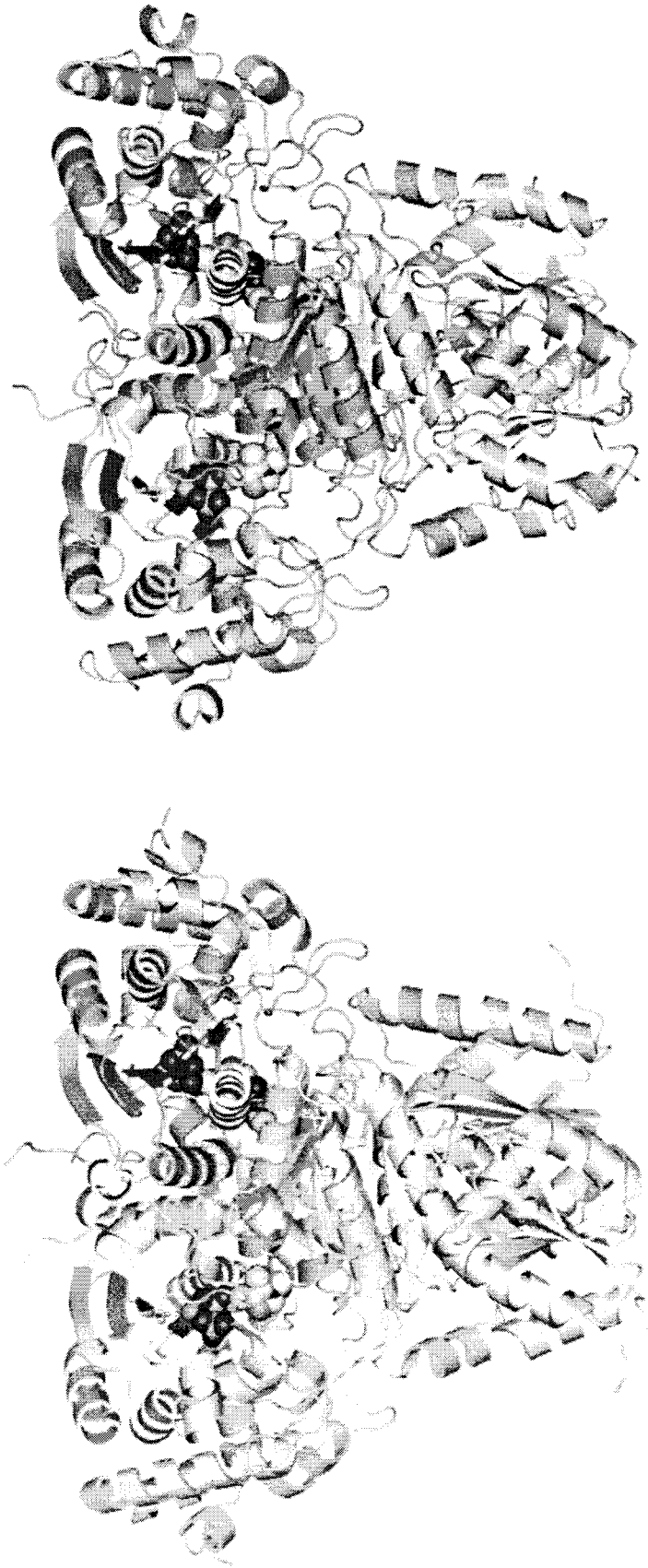
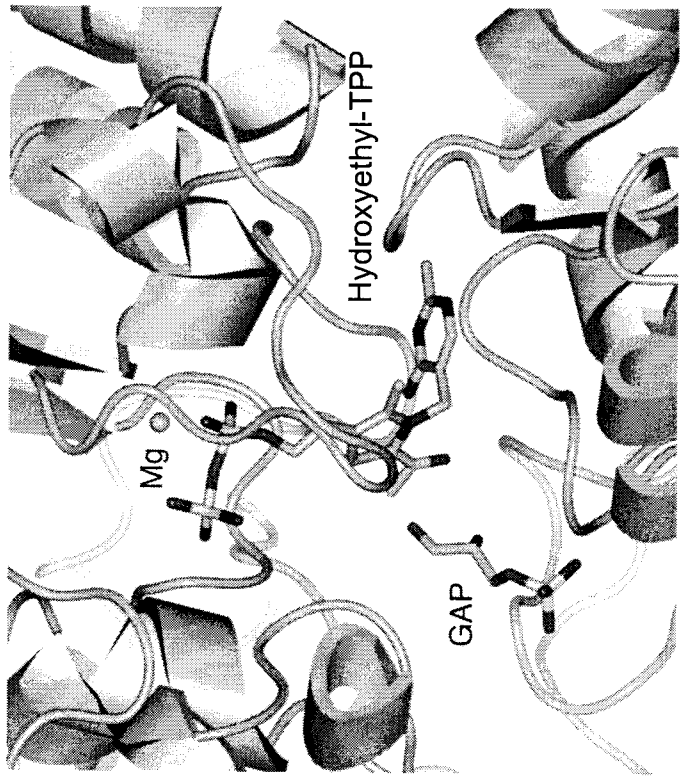


Fig. 5


Hydroxyethyl-TPP
Mg
GAP

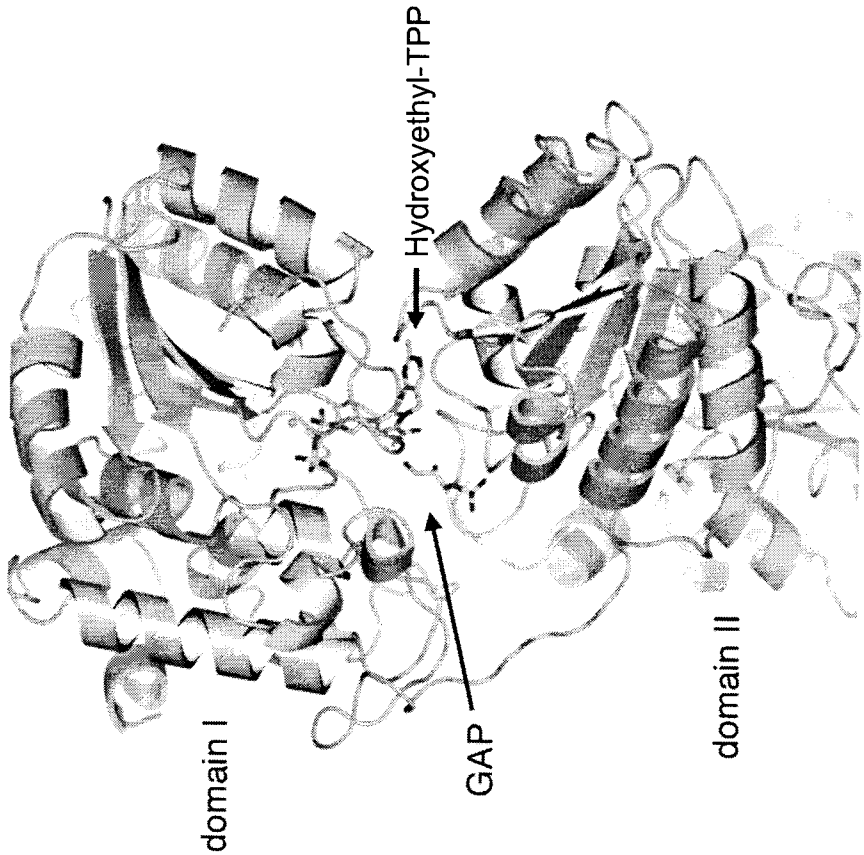

Hydroxyethyl-TPP
domain I
GAP
domain II

Fig. 6

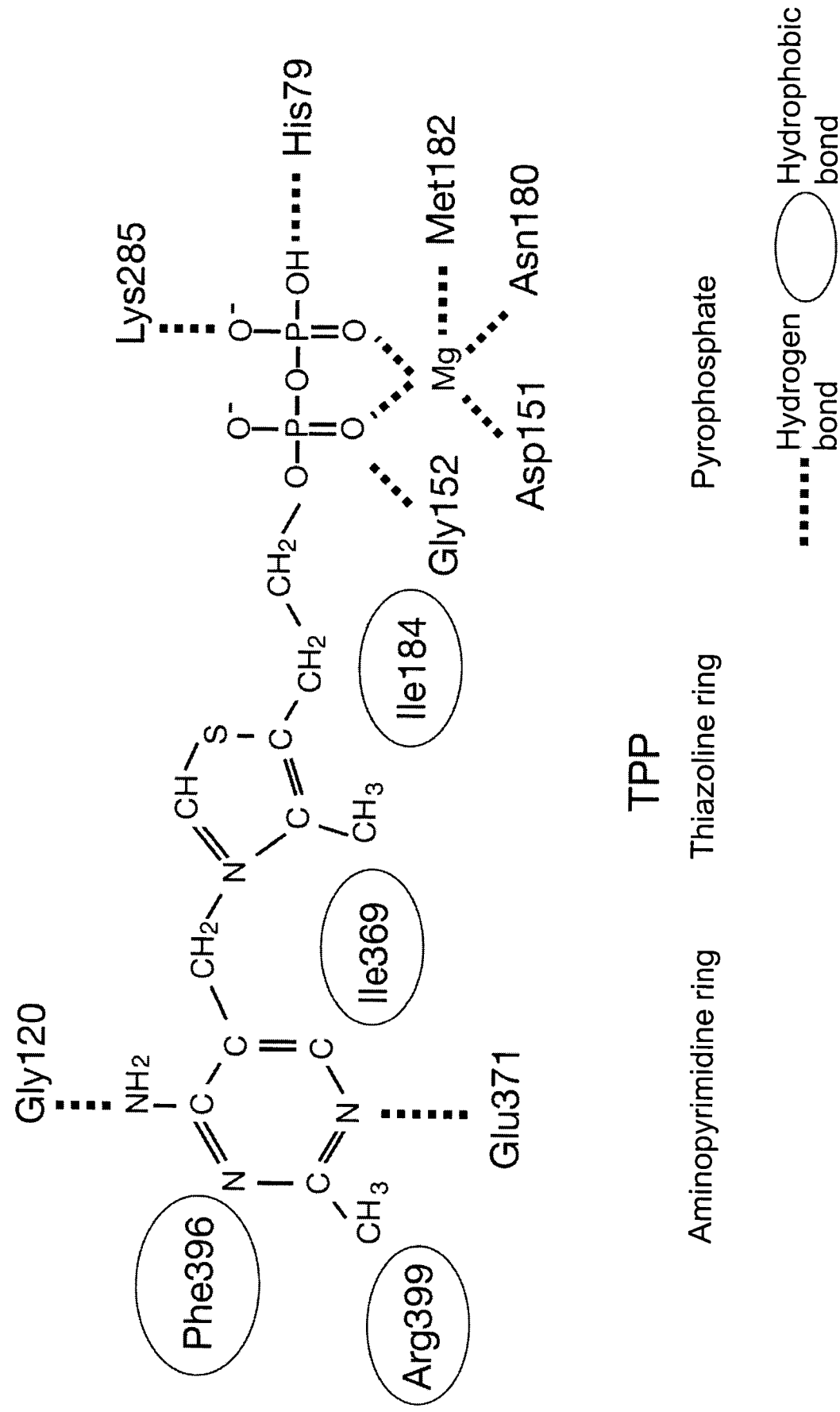
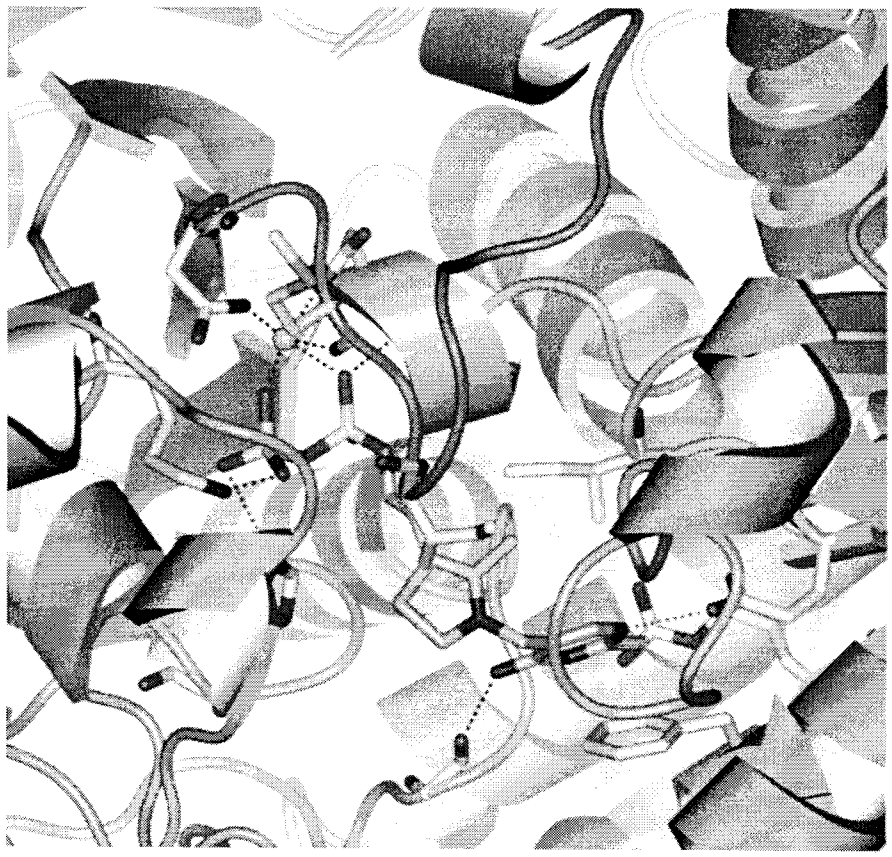

Lys285
His79
Met182
Asn180
Gly152
Asp151
Gly120
Glu371
Ile184
Ile369
Phe396
Arg399
TPP
Aminopyrimidine ring
Thiazoline ring
Pyrophosphate
Hydrogen bond
Hydrophobic bond

Fig. 7



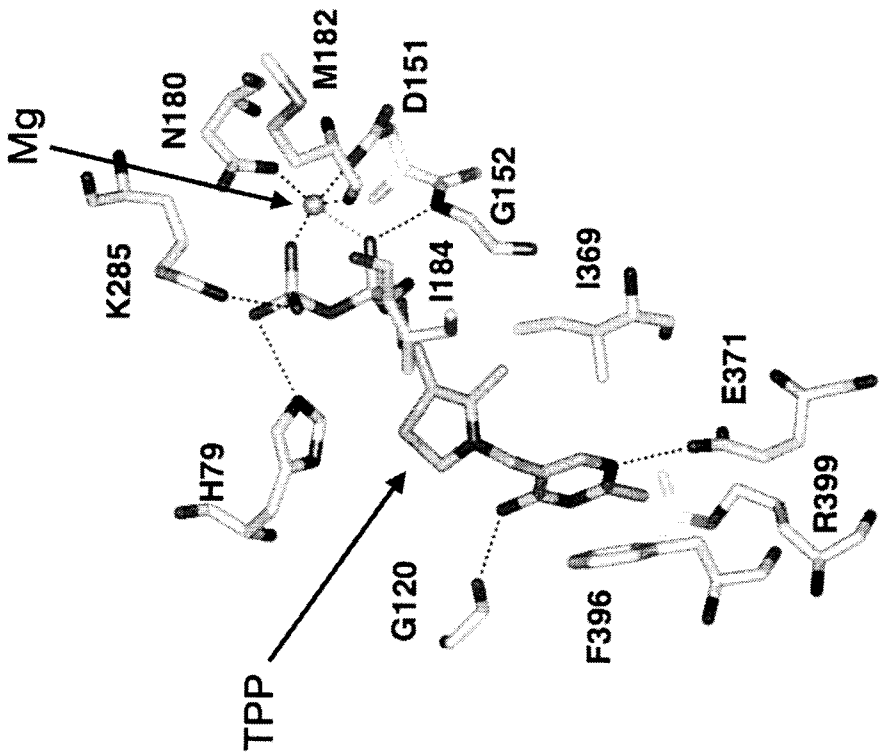
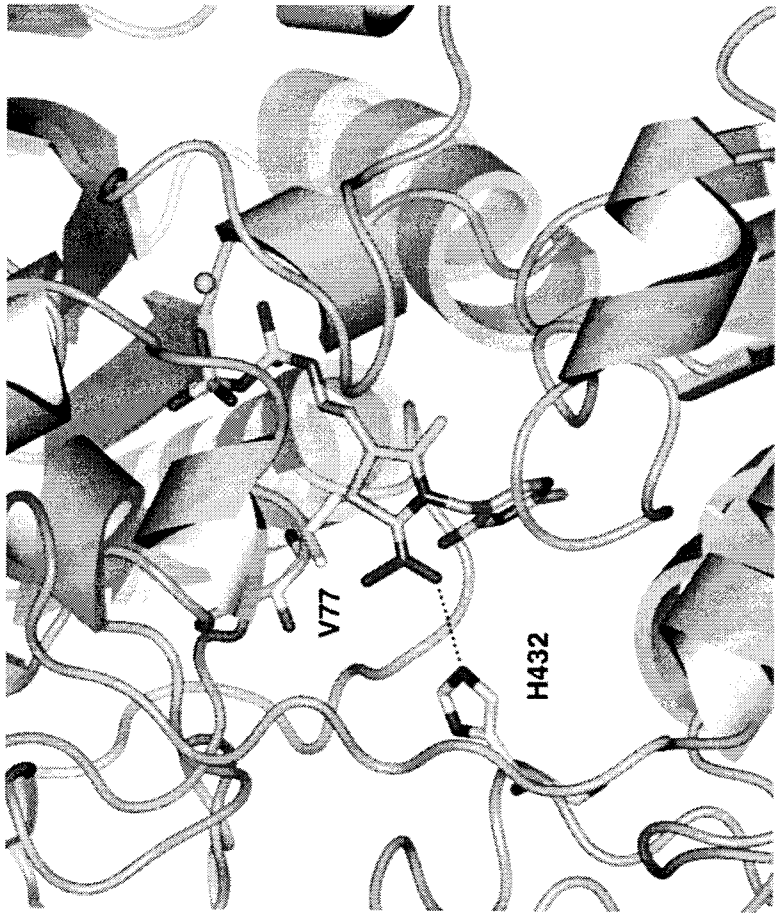

Mg
N180
M182
D151
G152
K285
I184
I369
E371
H79
R399
G120
F396
TPP

Fig. 8



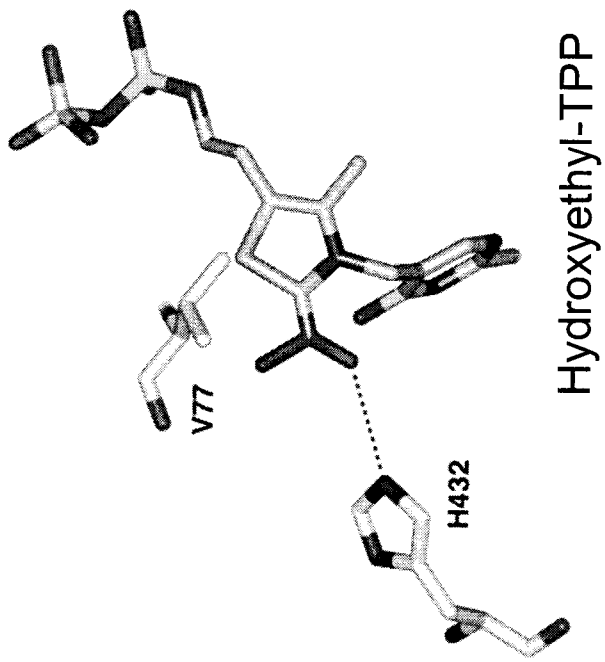
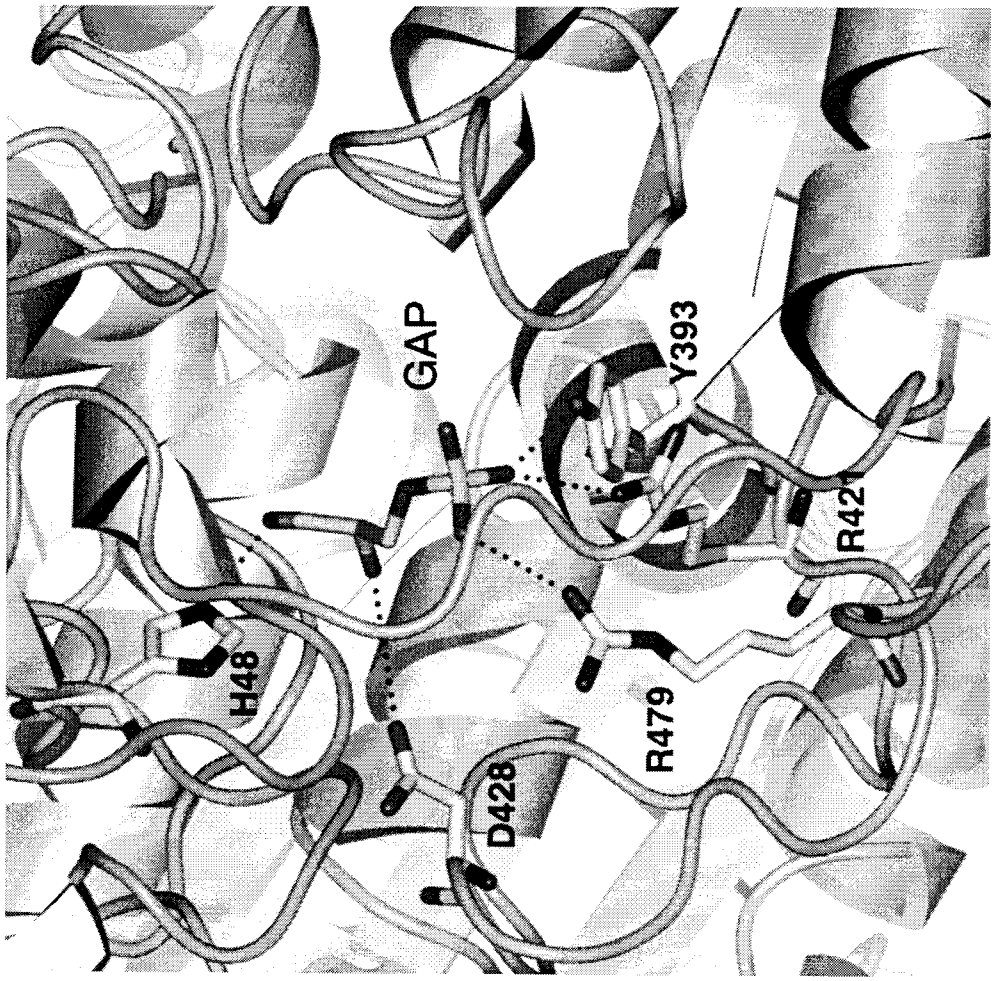


Fig. 9



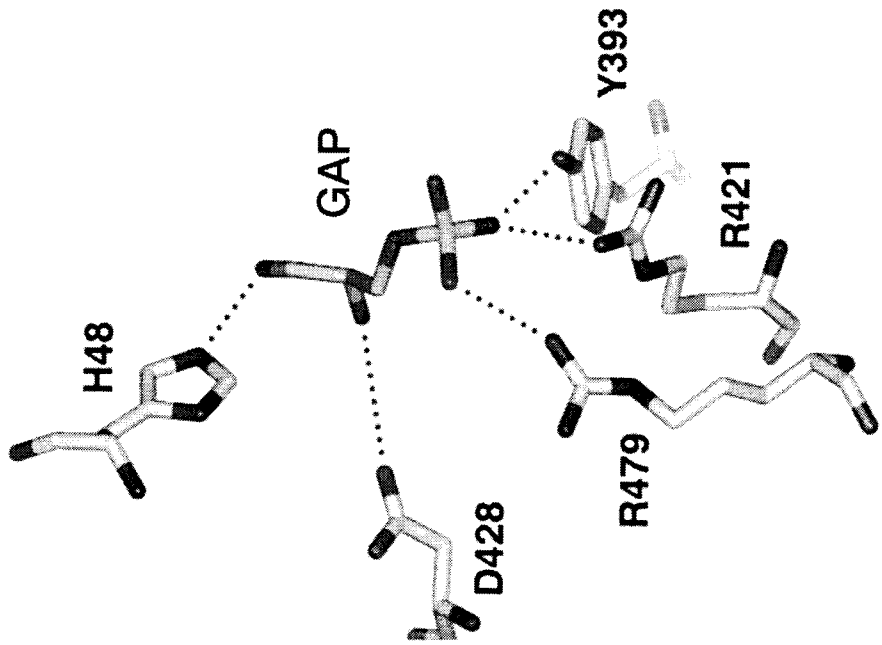


Fig. 10

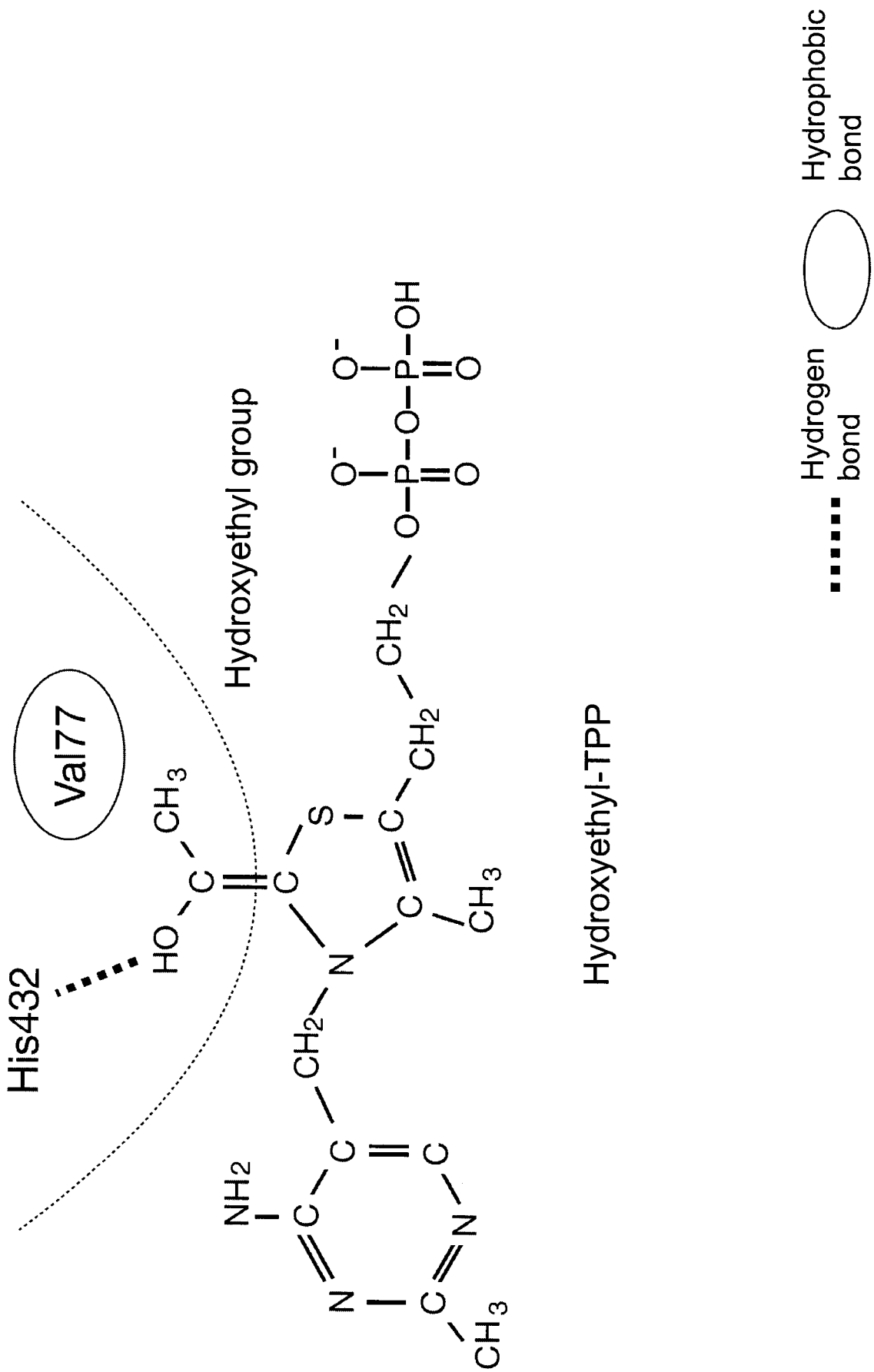

Hydrophobic bond
Hydrogen bond
Val77
His432
Hydroxyethyl group
Hydroxyethyl-TPP

Fig. 11

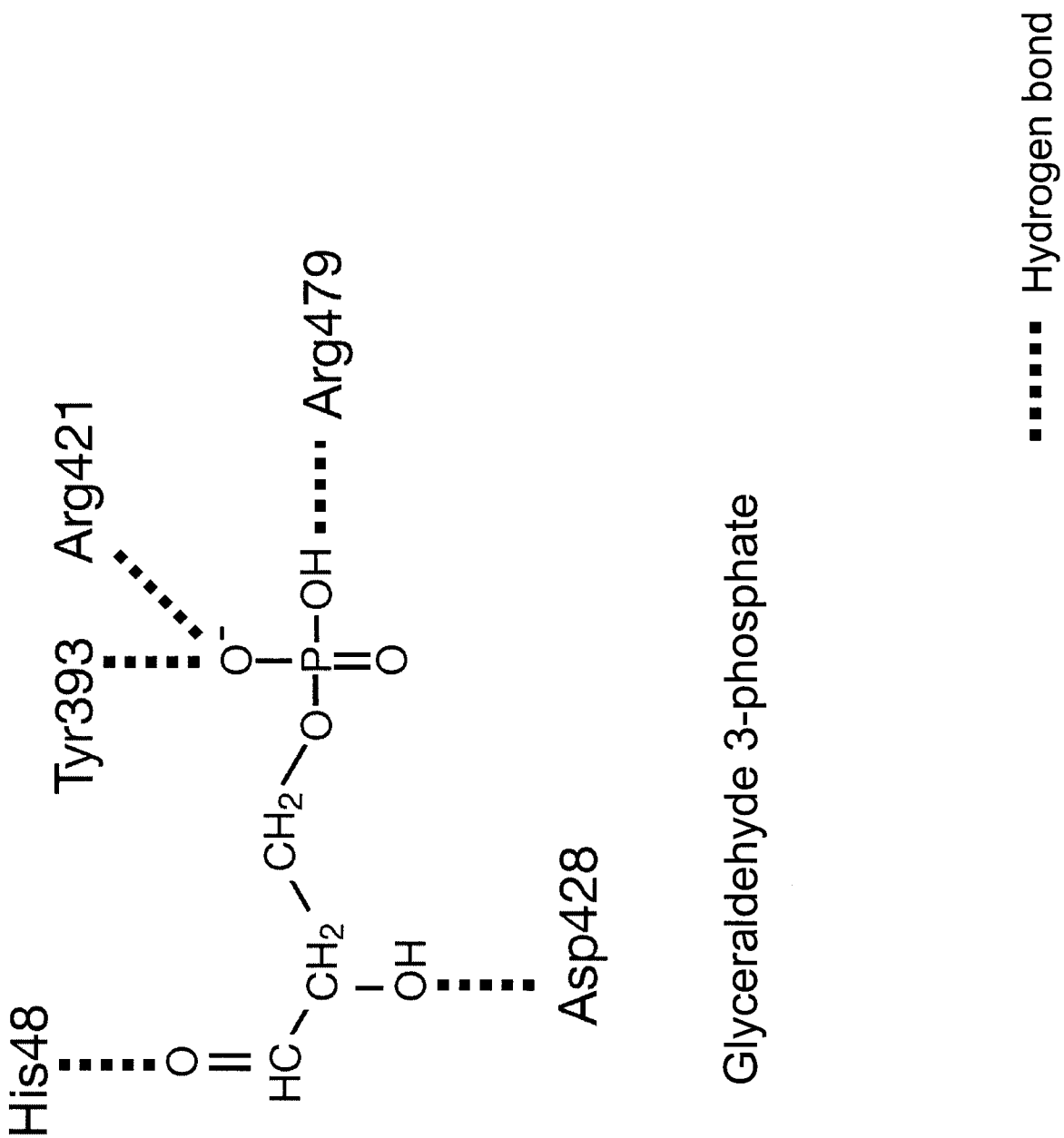

His48
Tyr393
Arg421
Arg479
Asp428
Glyceraldehyde 3-phosphate
Hydrogen bond

Fig. 12

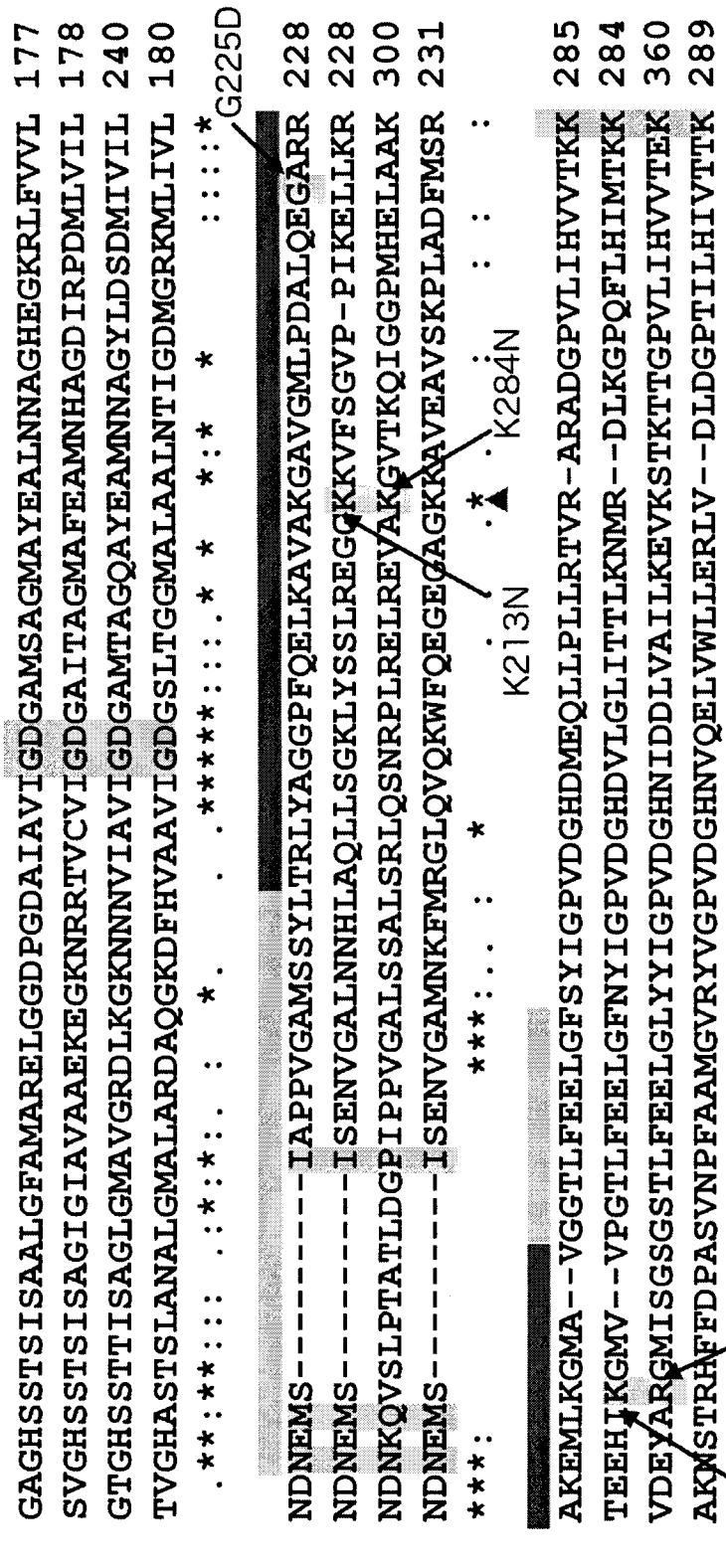
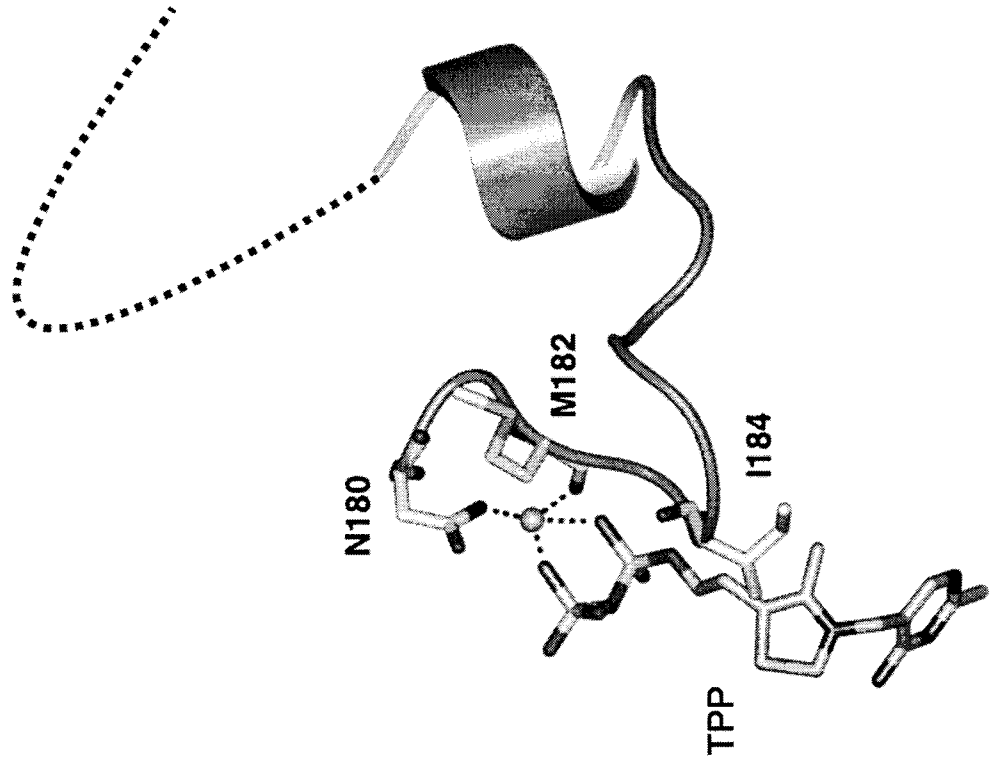

EnzymeA GAGHSSTSISAALGFAMARELGGDPGDAIAVIGDGAMSAGMAYEALNNAGHEGKRLFVVL 177
DXS_ECOLI SVGHSSTSISAGIGIAVAAEKEGKNRRTVCVIGDGAITAGMAFEAMNHAGDIRPDMLVIL 178
DXS_VITVI GTGHSSTTISAGLGMAVGRDLKGKNNNVIAVIGDGAMTAGQAYEAMNNAGYLDSDMIVIL 240
DXS_DEIRA TVGHASTSLANALGMALARDAQGKDFHVAAVIGDGSLTGGMALAALNTIGDMGRKMLIVL 180
EnzymeA NDNEMS----------IAPPVGAMSSYLTRLYAGGPFQELKAVAKGAVGMLPDALQEGARR 228
DXS_ECOLI NDNEMS----------ISENVGALNNHLAQLLSGKLYSSLREGGKKVFSGVP-PIKELLKR 228
DXS_VITVI NDNKQVSLPTATLDGPIPPVGALSSALSRLQSNRPLRELREVAKGVTKQIGGPMHELAAK 300
DXS_DEIRA NDNEMS----------ISENVGAMNKFMRGLQVQKWFQEGEQAGKKAVEAVSKPLADFMSR 231
EnzymeA AKEMLKGMA--VGGTLFEELGFSYIGPVDGHDMEQLLPLLRTVR-ARADGPVLIHVVTKK 285
DXS_ECOLI TEEHIKGMV--VPGTLFEELGFNYIGPVDGHDVLGLITTLKNMR--DLKGPQFLHIMTKK 284
DXS_VITVI VDEVARGMISGSGSTLFEELGLYYIGPVDGHNIDDLVAILKEVKSTKTTGPVLIHVVTEK 360
DXS_DEIRA AKMSTRHFFDPASVNPFAAMGVRYVGPVDGHNVQELVWLLERLV--DLDGPTILHIVTTK 289
G225D
K213N
K284N
K234C
R306C

Fig. 13



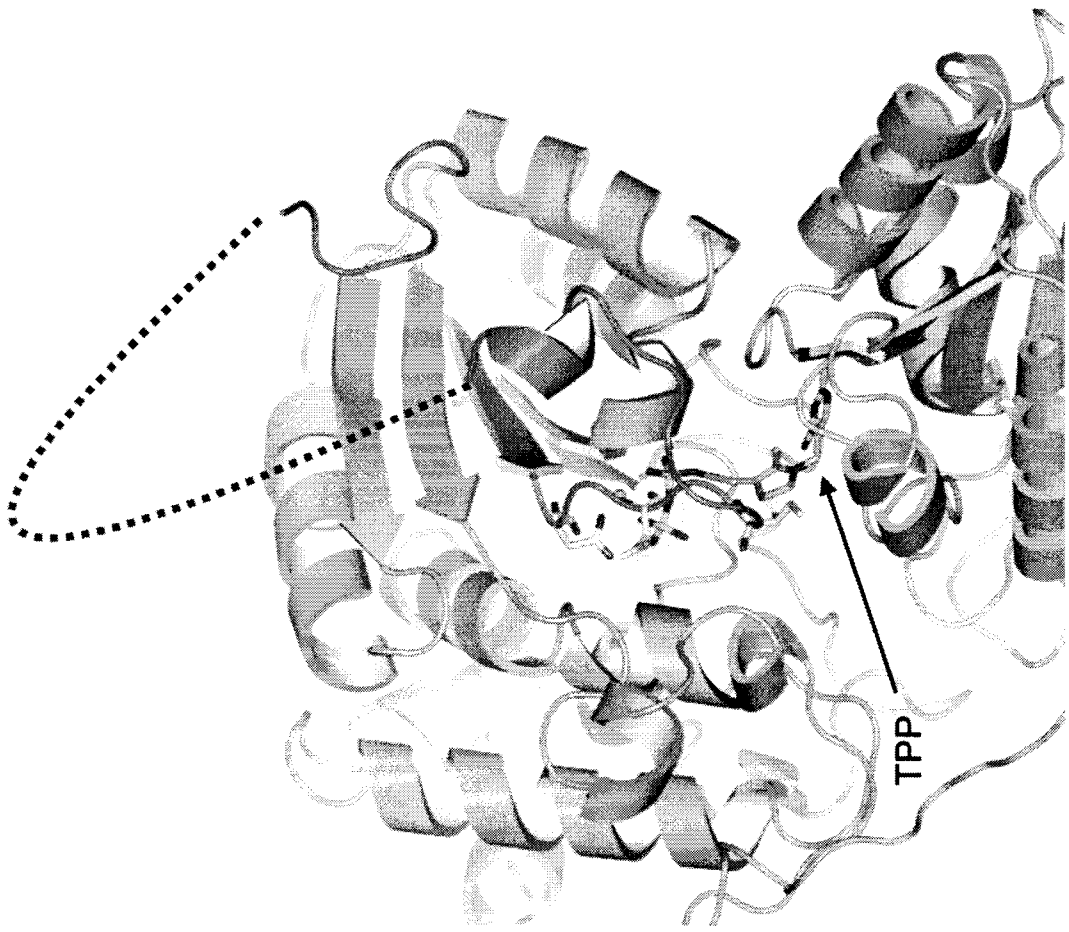


Fig. 14

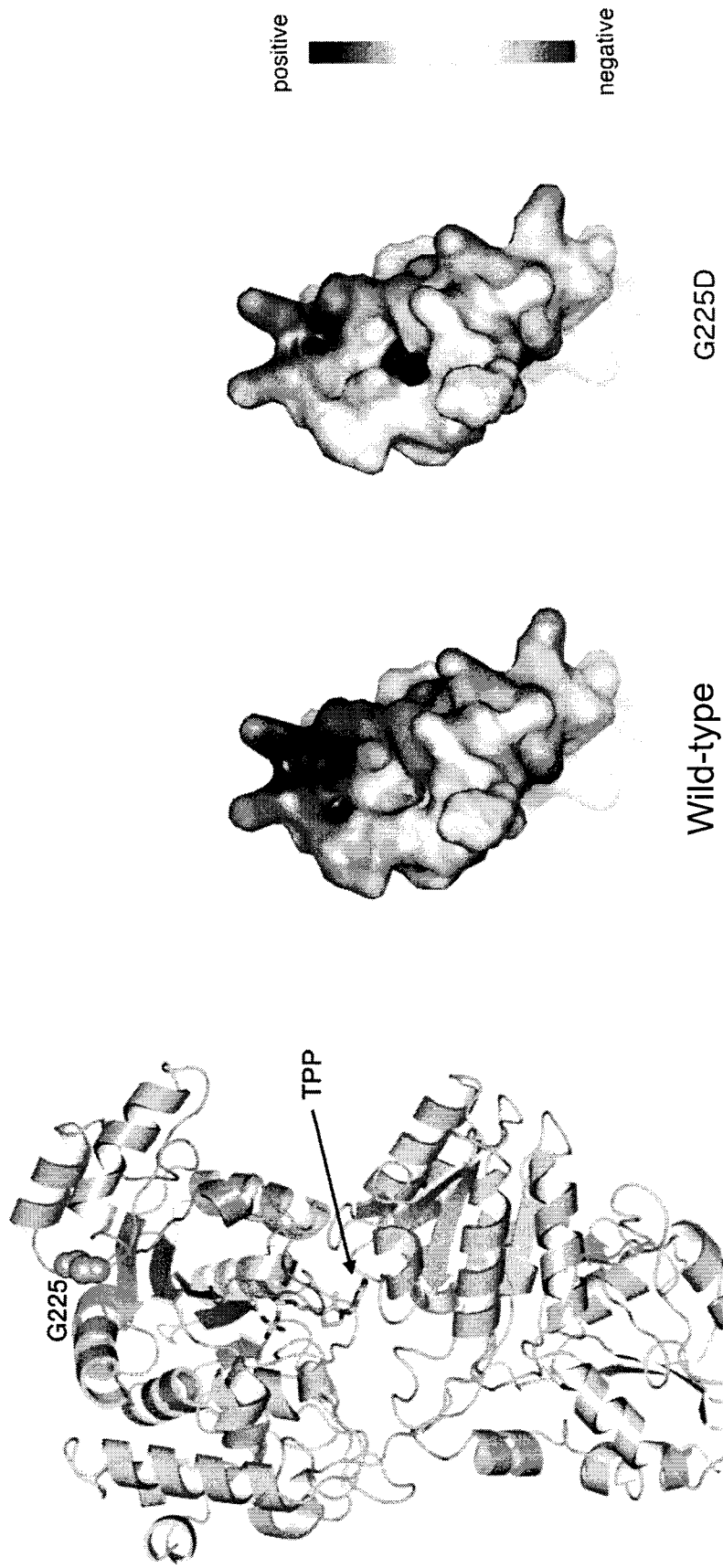


Fig. 15

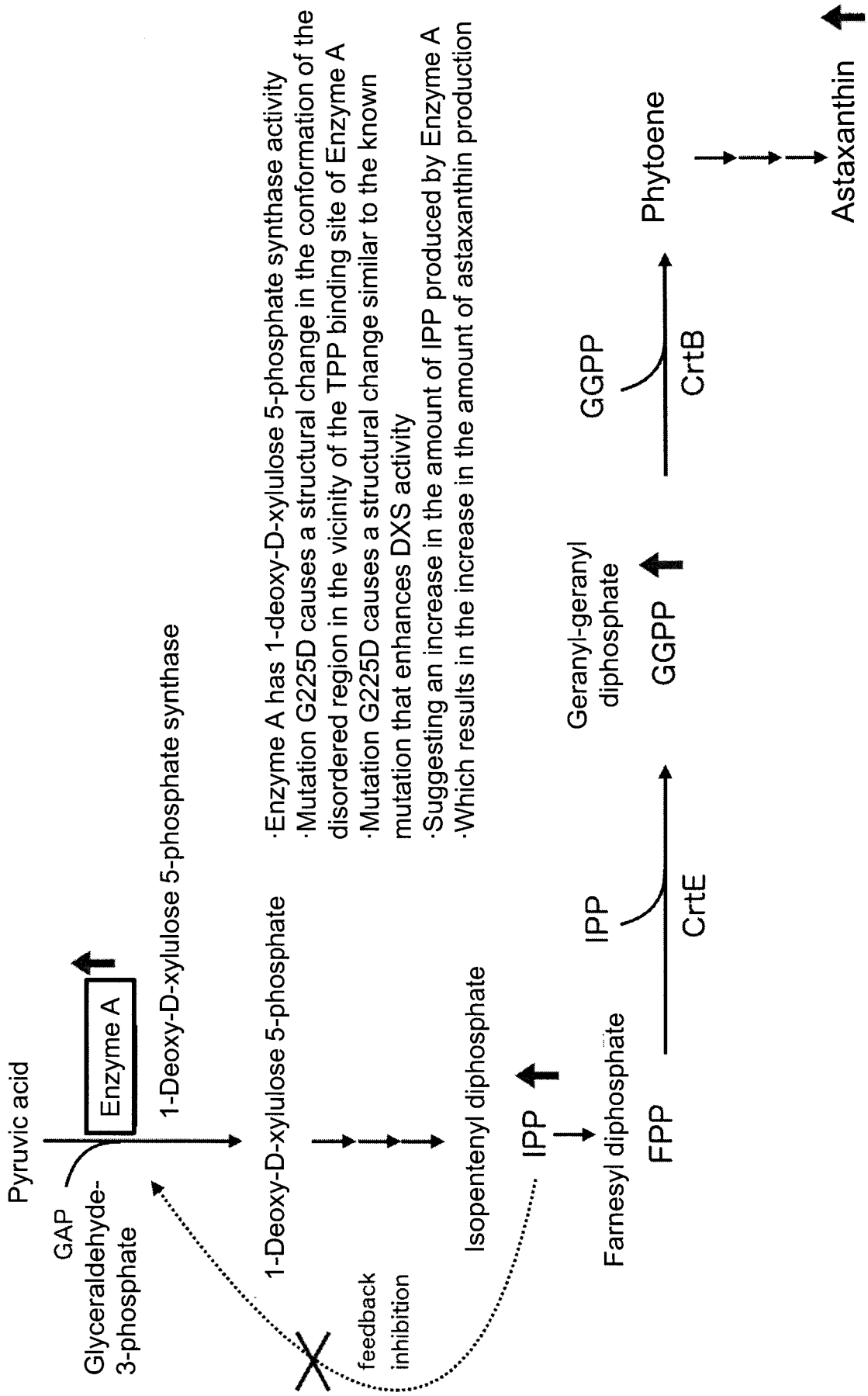

Pyruvic acid
GAP
Glyceraldehyde-3-phosphate
Enzyme A
1-Deoxy-D-xylulose 5-phosphate synthase
1-Deoxy-D-xylulose 5-phosphate
·Enzyme A has 1-deoxy-D-xylulose 5-phosphate synthase activity
·Mutation G225D causes a structural change in the conformation of the disordered region in the vicinity of the TPP binding site of Enzyme A
·Mutation G225D causes a structural change similar to the known mutation that enhances DXS activity
·Suggesting an increase in the amount of IPP produced by Enzyme A
·Which results in the increase in the amount of astaxanthin production
feedback inhibition
Isopentenyl diphosphate
IPP
Farnesyl diphosphate
FPP
IPP
CrtE
Geranyl-geranyl diphosphate
GGPP
GGPP
CrtB
Phytoene
Astaxanthin

Fig. 16

Protein	Decaprenyl diphosphate synthase
UniProt	Q07D00 (Q07D00_RHOCA) EC:2.5.1.91
Organism	*Rhodobacter capsulatus*
Pfam	PF00348 Polyprenyl synthetase
PDB ID	3MZV Resolution: 1.90[Å] dimer
Identity	76.2% 10-332

3MZV

Fig. 17

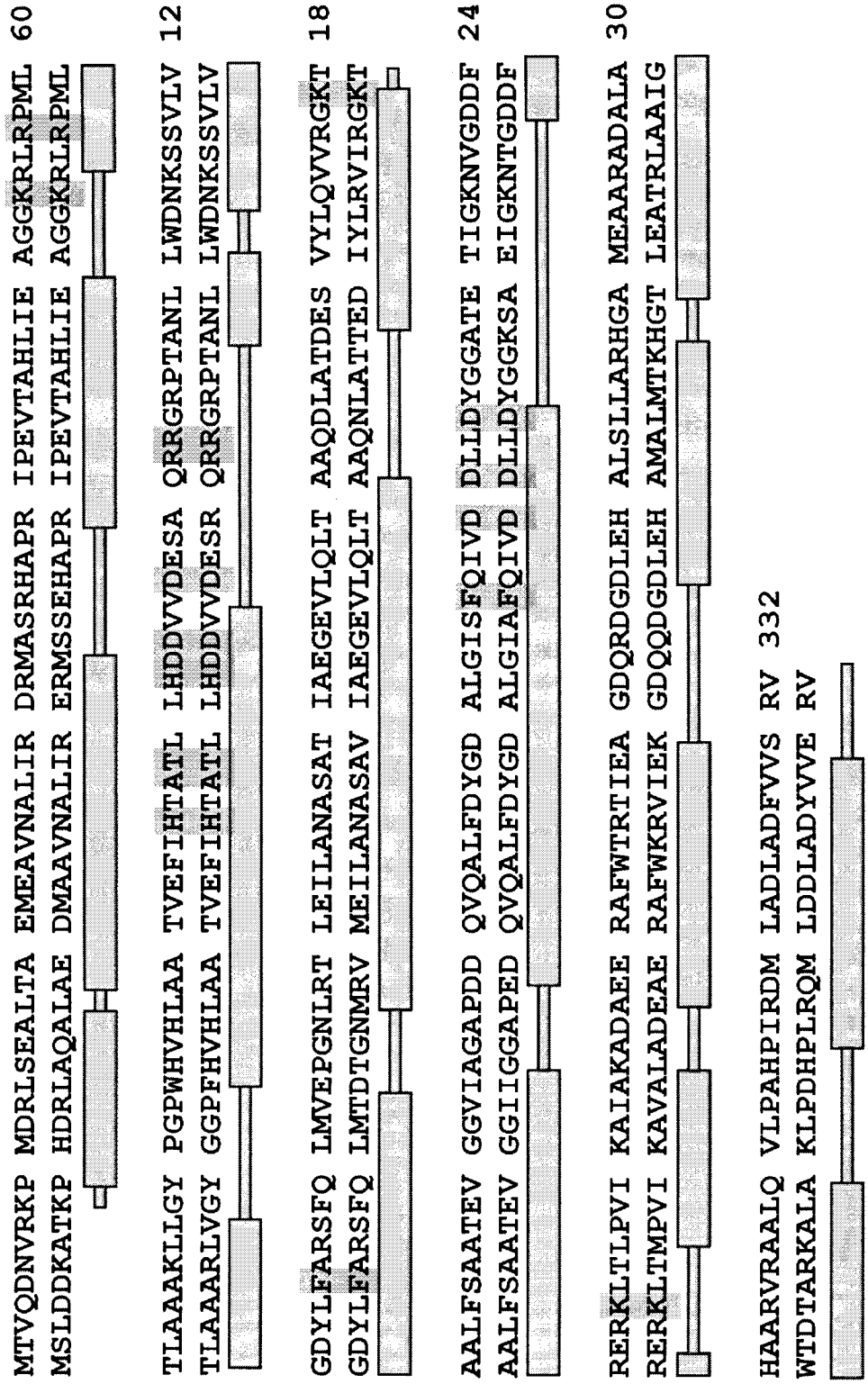
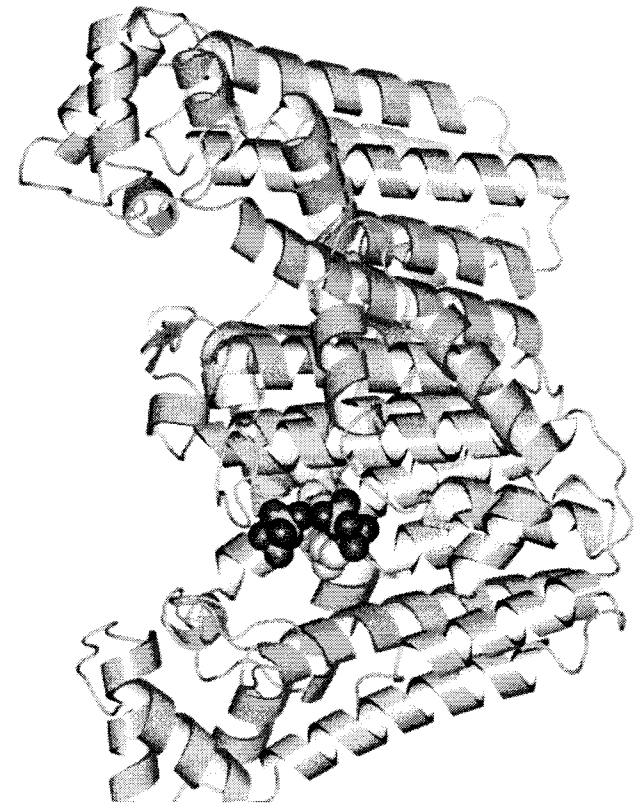

EnzymeC MTVQDNVRKP MDRLSEALTA EMEAVNALIR DRMASRHAPR IPEVTAHLIE AGGKRLRPML 60
3mzv MSLDDKATKP HDRLAQALAE DMAAVNALIR ERMSSEHAPR IPEVTAHLIE AGGKRLRPML
EnzymeC TLAAAKLLGY PGPWHVHLAA TVEFIHTATL LHDDVVDESA QRRGRPTANL LWDNKSSVLV 120
3mzv TLAAARLVGY GGPFHVHLAA TVEFIHTATL LHDDVVDESR QRRGRPTANL LWDNKSSVLV
EnzymeC GDYLFARSFQ LMVEPGNLRT LEILANASAT IAEGEVLQLT AAQDLATDES VYLQVVRGKT 180
3mzv GDYLFARSFQ LMTDTGNMRV MEILANASAV IAEGEVLQLT AAQNLATTED IYLRVIRGKT
EnzymeC AALFSAATEV GGVIAGAPDD QVQALFDYGD ALGISFQIVD DLLDYGGATE TIGKNVGDDF 240
3mzv AALFSAATEV GGIIGGAPED QVQALFDYGD ALGIAFQIVD DLLDYGGKSA EIGKNTGDDF
EnzymeC RERKLTLPVI KAIAKADAEE RAFWTRTIEA GDQRDGDLEH ALSLLARHGA MEAARADALA 300
3mzv RERKLTMPVI KAVALADEAE RAFWKRVIEK GDQQDGDLEH AMALMTKHGT LEATRLAAIG
EnzymeC HAARVRAALQ VLPAHPIRDM LADLADFVVS RV 332
3mzv WTDTARKALA KLPDHPLRQM LDDLADYVVE RV

Fig. 18



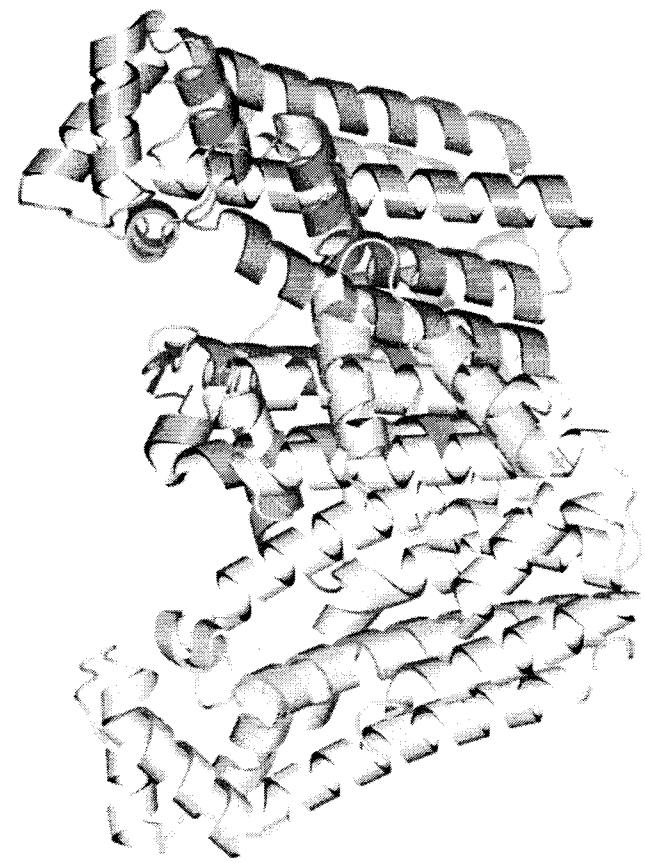
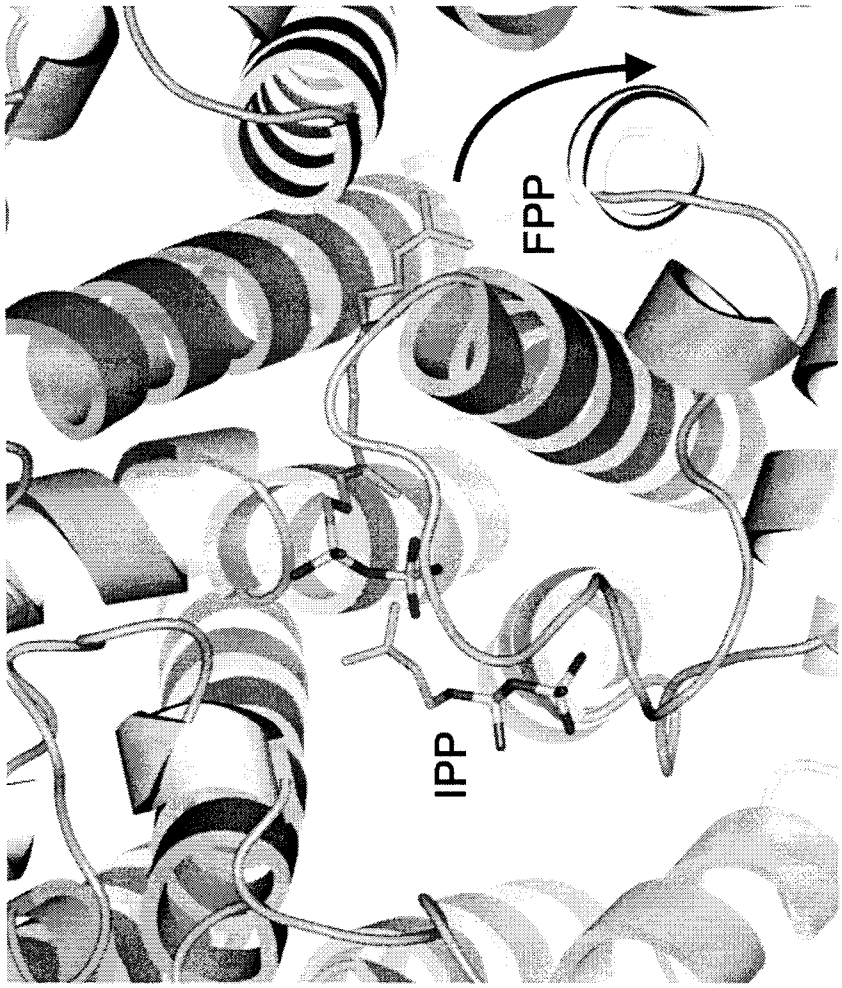


Fig. 19



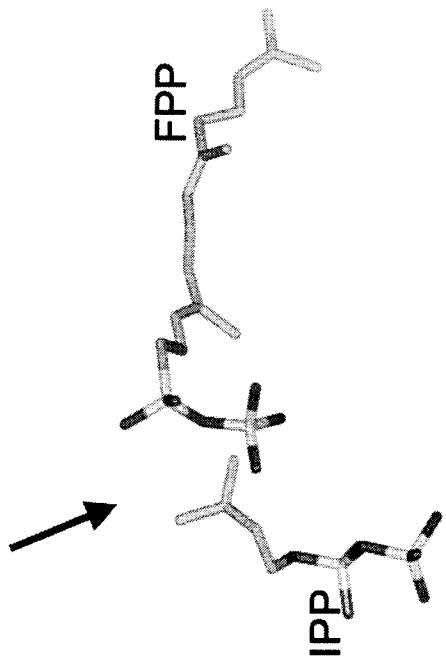
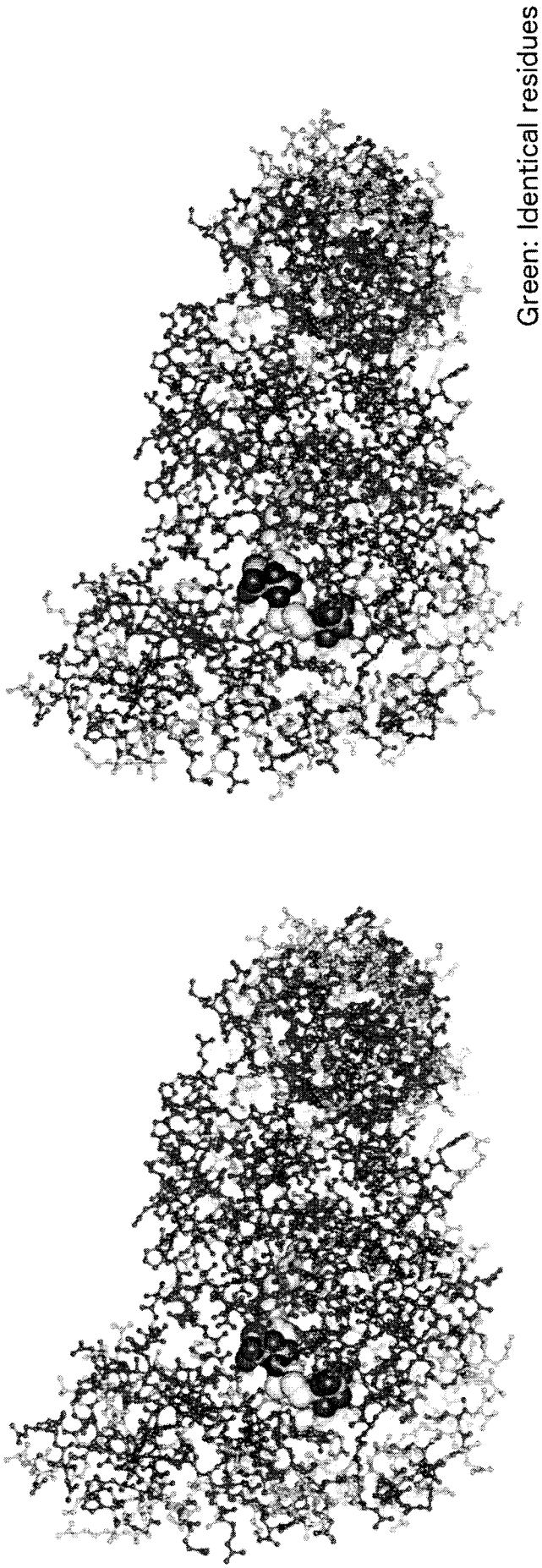


Fig. 20

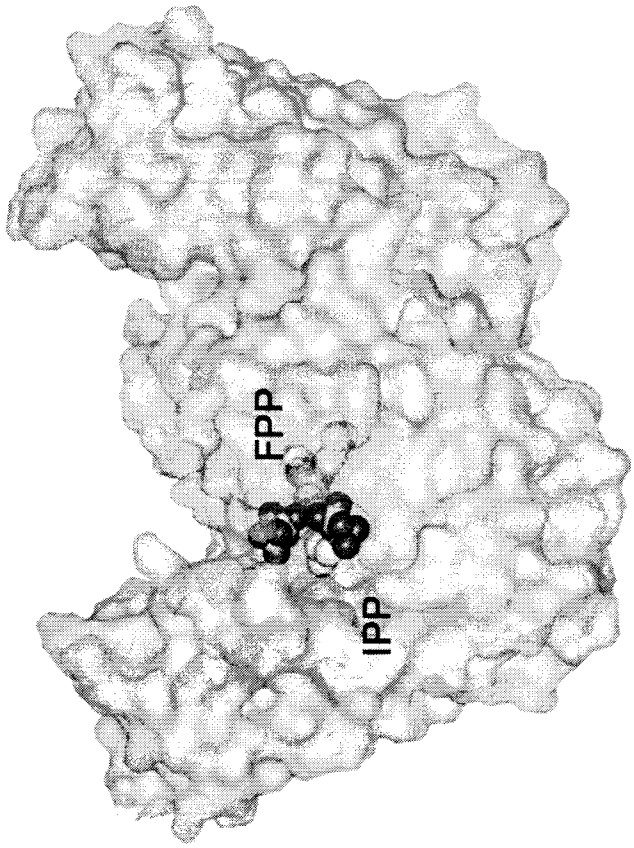


```
>>tr|Q8L1I6|Q8L1I6_PARZE                                  (333 aa)
 initn: 1887 init1: 1859 opt: 1866  Z-score: 2009.0  bits: 380.1 E(5): 2.1e-109
Smith-Waterman score: 1866; 86.2% identity (95.2% similar) in 333 aa overlap (1-333:1-333)

               10        20        30        40        50        60
EnzymC MTVQDNVRKPMDRLSEALTAEMEAVNALIRDRMASRHAPRIPEVTAHLIEAGGKRLRPML
       :.:::..::::.::::::. ::::::::::.:::::::::::::::::::::::::::::
Q8L1I6 MNVQEDVRKPLDRLAEALAPEMEAVNALIRERMASRHAPRIPEVTAHLIEAGGKRLRPML

               70        80        90       100       110       120
EnzymC TLAAAKLLGYPGPWHVHLAATVEFIHTATLLHDDVVDESAQRRGRPTANLLWDNKSSVLV
       :::::::::: ::.:::::::::::::::::::::::: :::::::::::::::::::::
Q8L1I6 TLAAAKLLGYGGPYHVHLAATVEFIHTATLLHDDVVDESRQRRGRPTANLLWDNKSSVLV

              130       140       150       160       170       180
EnzymC GDYLFARSFQLMVEPGNLRTLEILANASATIAEGEVLQLTAAQDLATDESVYLQVVRGKT
       ::::::::::::::::::..:::::.::.:::::::::::::::::::::.:..::::::::::
Q8L1I6 GDYLFARSFQLMVEPGSMRTLEILSNAAATIAEGEVLQLTAAQDLATNEDIYLQVVRGKT

              190       200       210       220       230       240
EnzymC AALFSAATEVGGVIAGAPDDQVQALFDYGDALGISFQIVDDLLDYGGATETIGKNVGDDF
       :::::::::::::::.:: ::.:::::::::::.:::::::::::.:.: ::.:::::
Q8L1I6 AALFSAATEVGGVIAGVPDAQVRALFDYGDALGIAFQIVDDLLDYGGTAEAIDKNTGDDF

              250       260       270       280       290       300
EnzymC RERKLTLPVIKAIAKADAEERAFWTRTIEAGDQRDGDLEHALSLLARHGAMEAARADALA
       :::::::::::::.:.:  ::::::.:::: :::.:::::::: ::::::::  :: :::
Q8L1I6 RERKLTLPVIKAVARATPEERAFWSRTIEKGDQKDGDLEHALELLARHGAMADARRDALD

              310       320       330
EnzymC HAARARAALQVLPAHPIRDMLADLADFVVSRVA
        :::::::.::.:: :::::::.:::::::: :.:
Q8L1I6 WAARARASLQILPEHPIRDMLSDLADFVVERIA
```

EnzymC : Enzyme_C
Q8L1I6 : Decaprenyl diphosphate synthase (Paracoccus zeaxanthinifaciens)

Fig. 21

Fig. 22



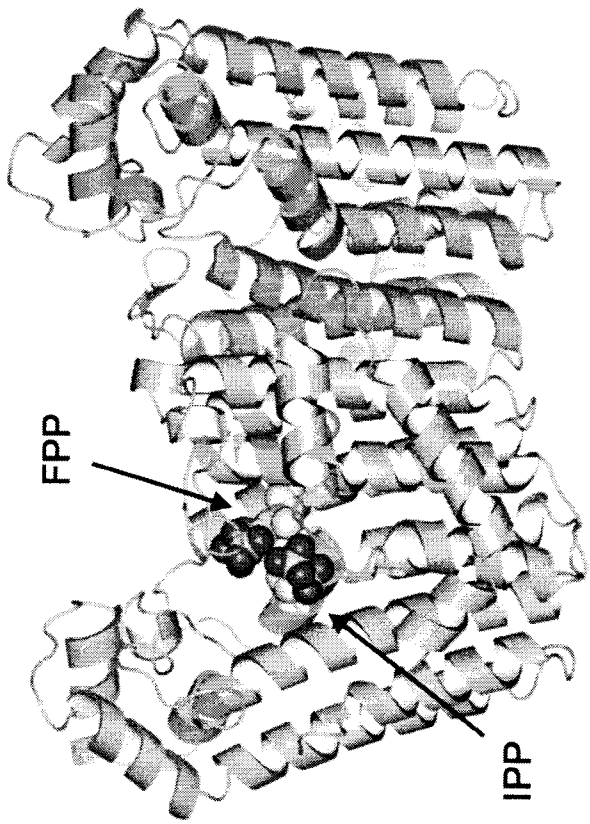


Fig. 23

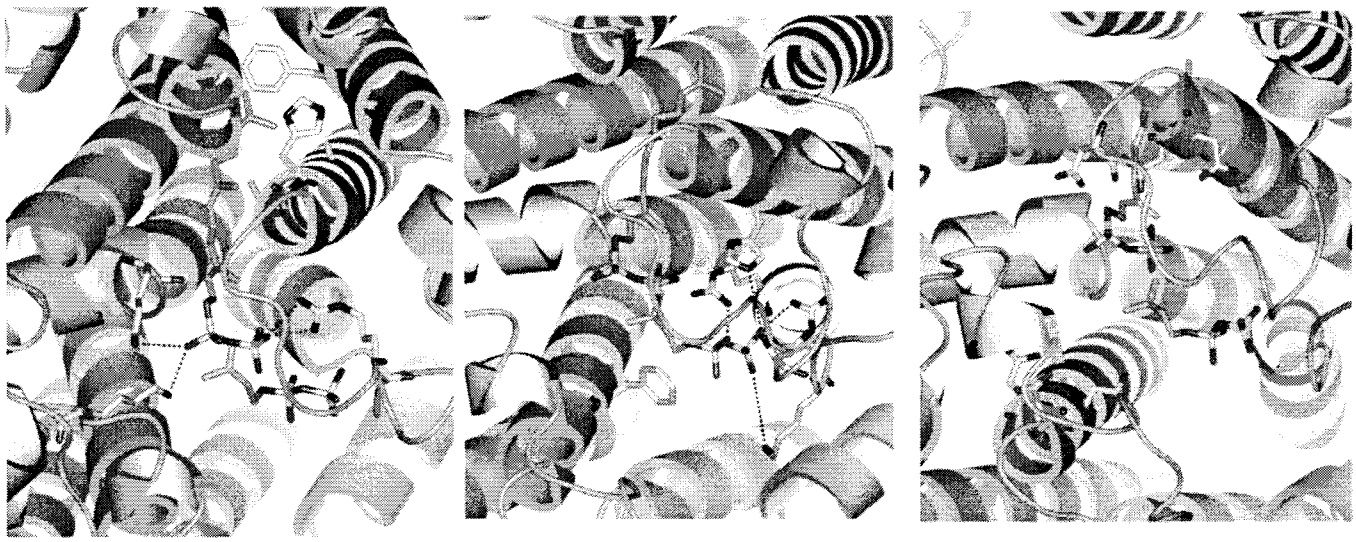

F125
FPP
H92
A88
T89
K179
R102
K244
IPP
FPP
R57
H86
R103
F216
IPP
K54
FPP
D97
D93
D94
D221
D224
D220
IPP

Fig. 24

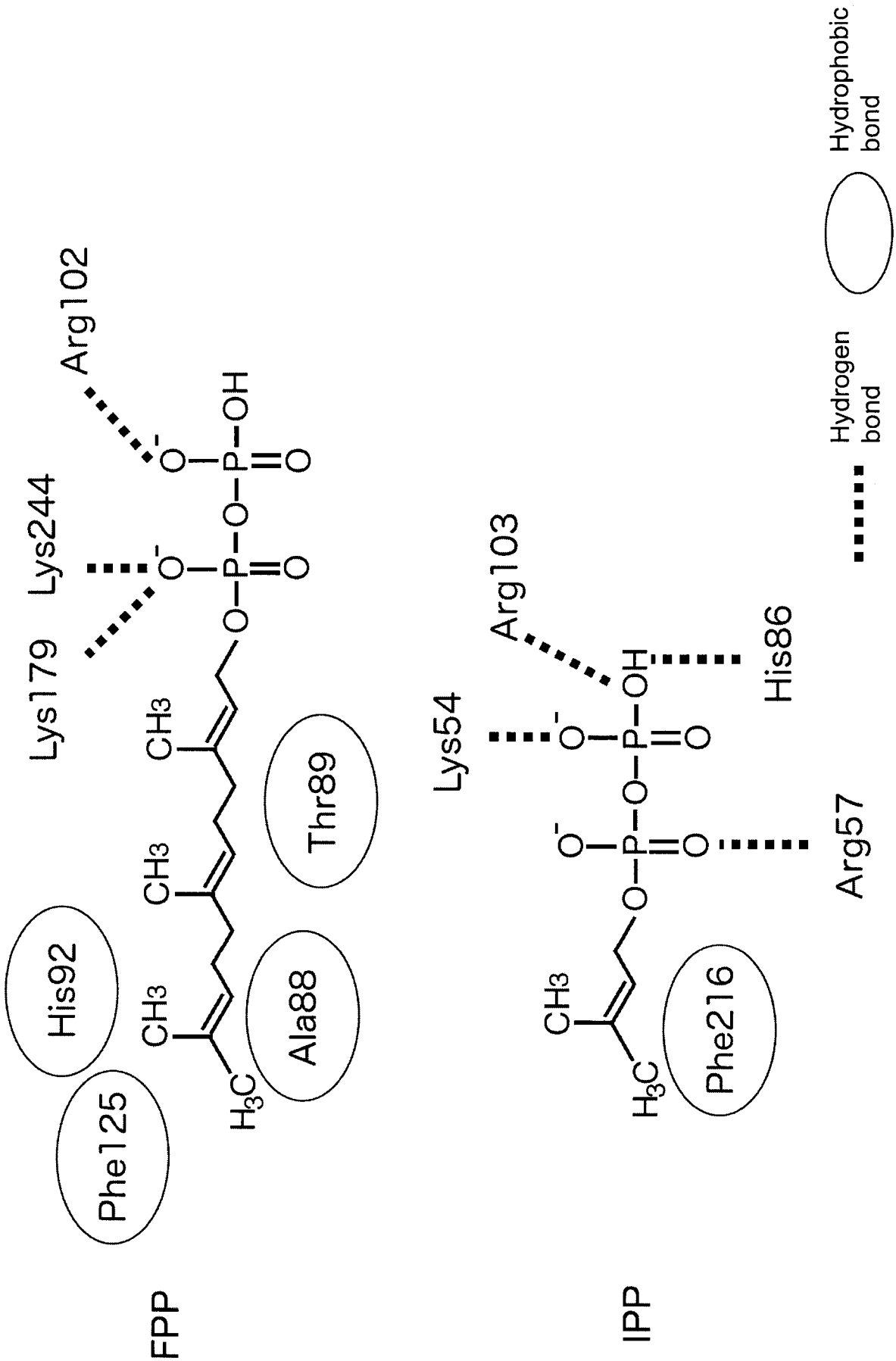

Arg102
Lys244
Lys179
His92
Phe125
Thr89
Ala88
FPP
Arg103
Lys54
His86
Arg57
Phe216
IPP
Hydrophobic bond
Hydrogen bond

Fig. 25

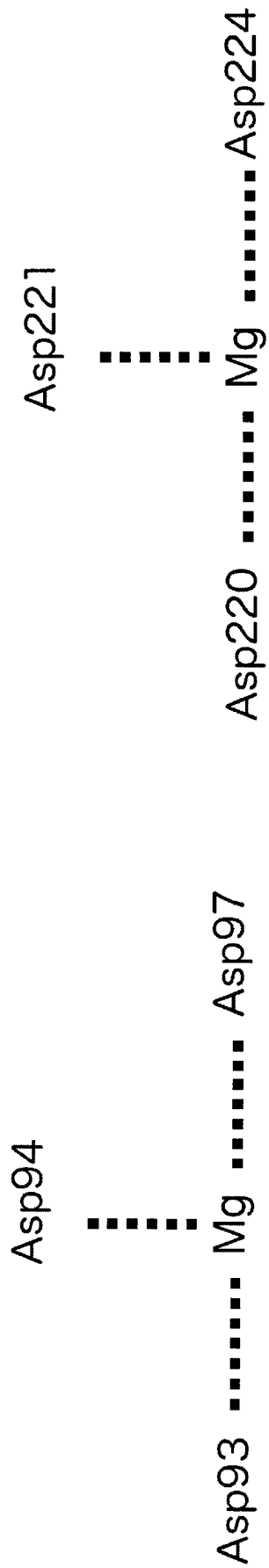
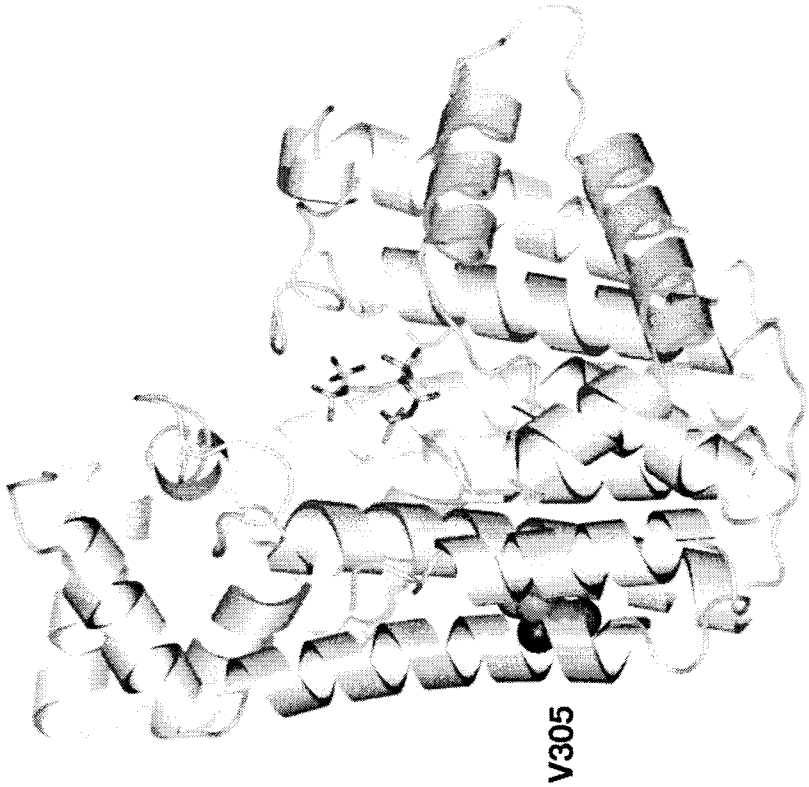


Fig. 26



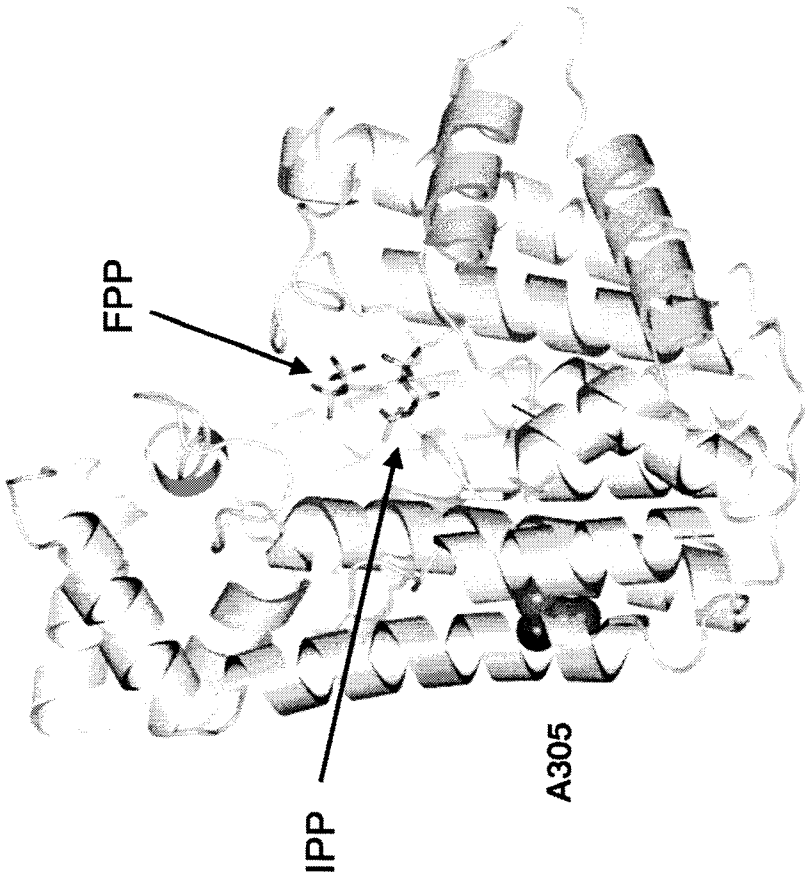


Fig. 27

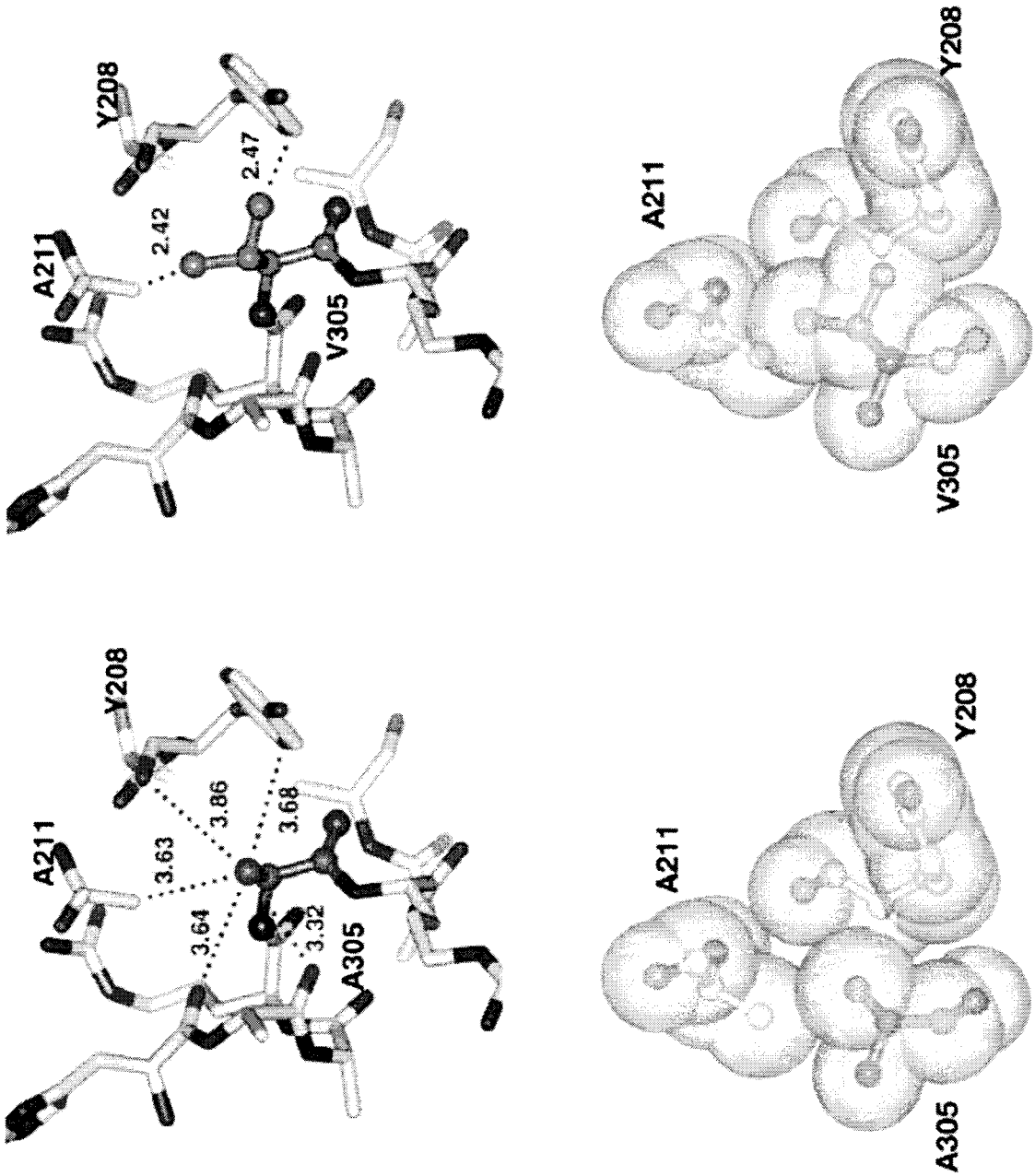

Y208
2.47
2.42
A211
V305
A211
Y208
V305
Y208
3.86
3.68
3.63
A211
3.64
3.32
A305
A211
Y208
A305

Fig. 28

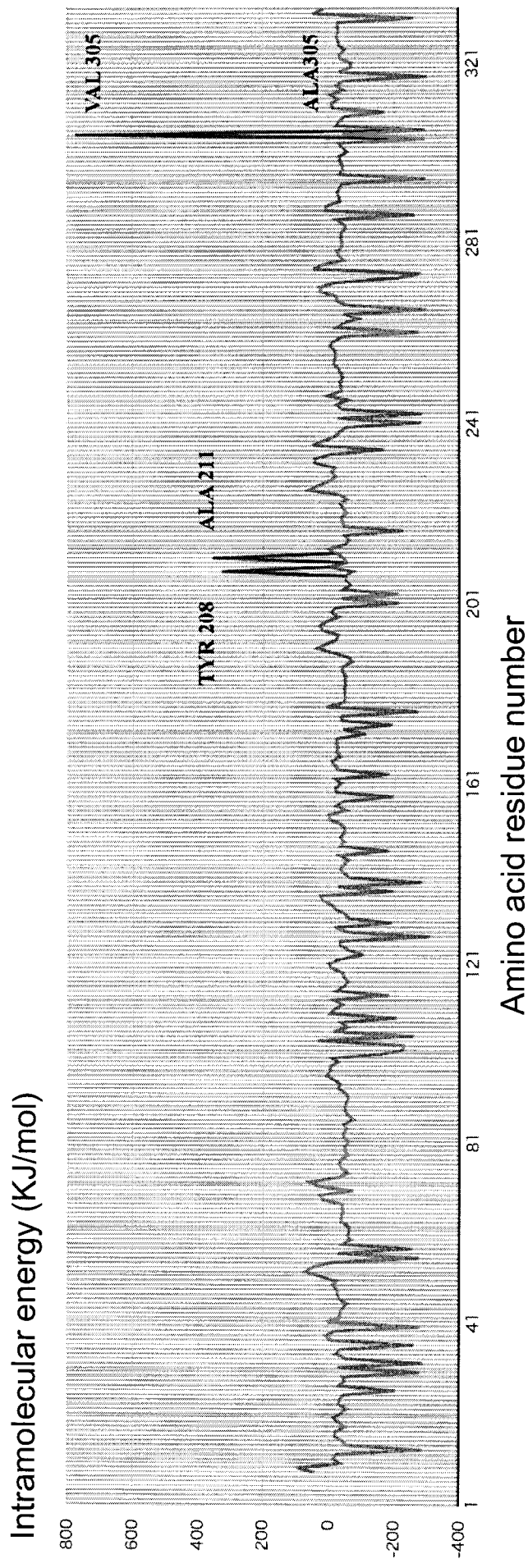
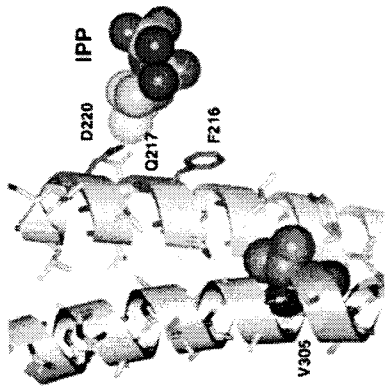
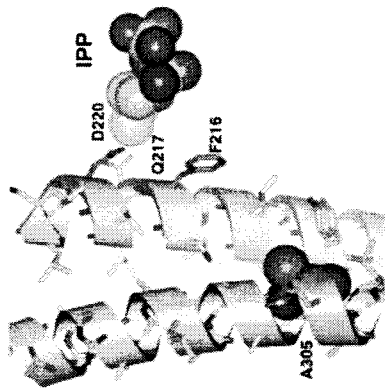

VAL 305
ALA305
ALA 211
TYR 208
Intramolecular energy (KJ/mol)
800
600
400
200
0
-200
-400
1
41
81
121
161
201
241
281
321
Amino acid residue number

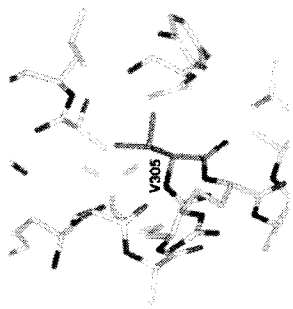
Fig. 29



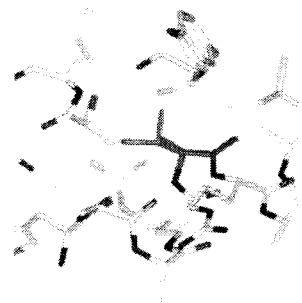
A305V Enzyme C



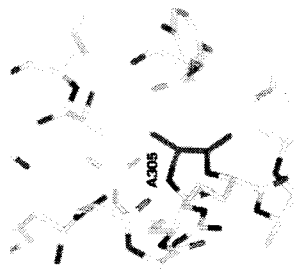
Wild-type Enzyme C



A305V Enzyme C



Superimposition of wild-type and A305V Enzyme C



Wild-type Enzyme C

Fig. 30

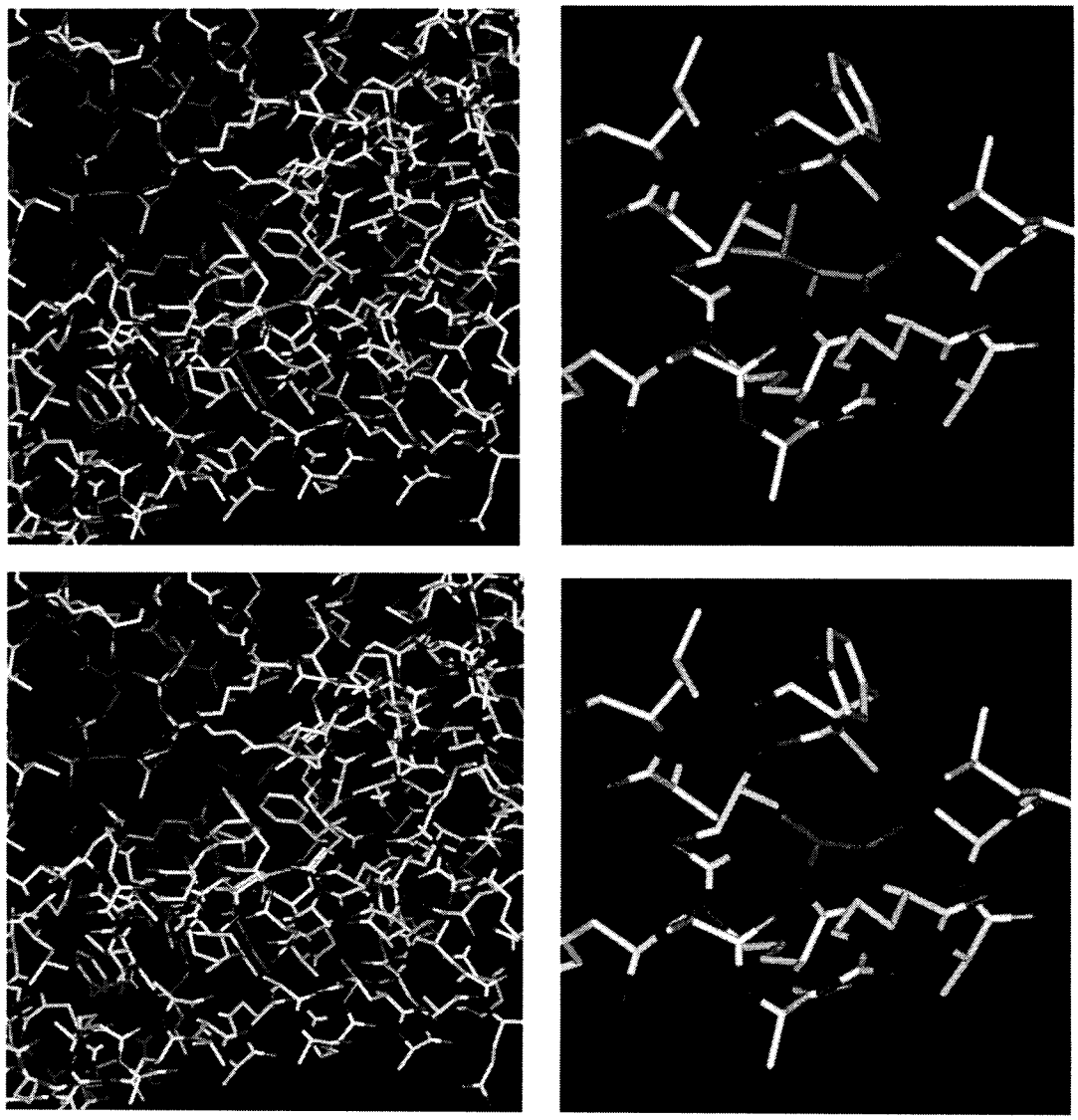
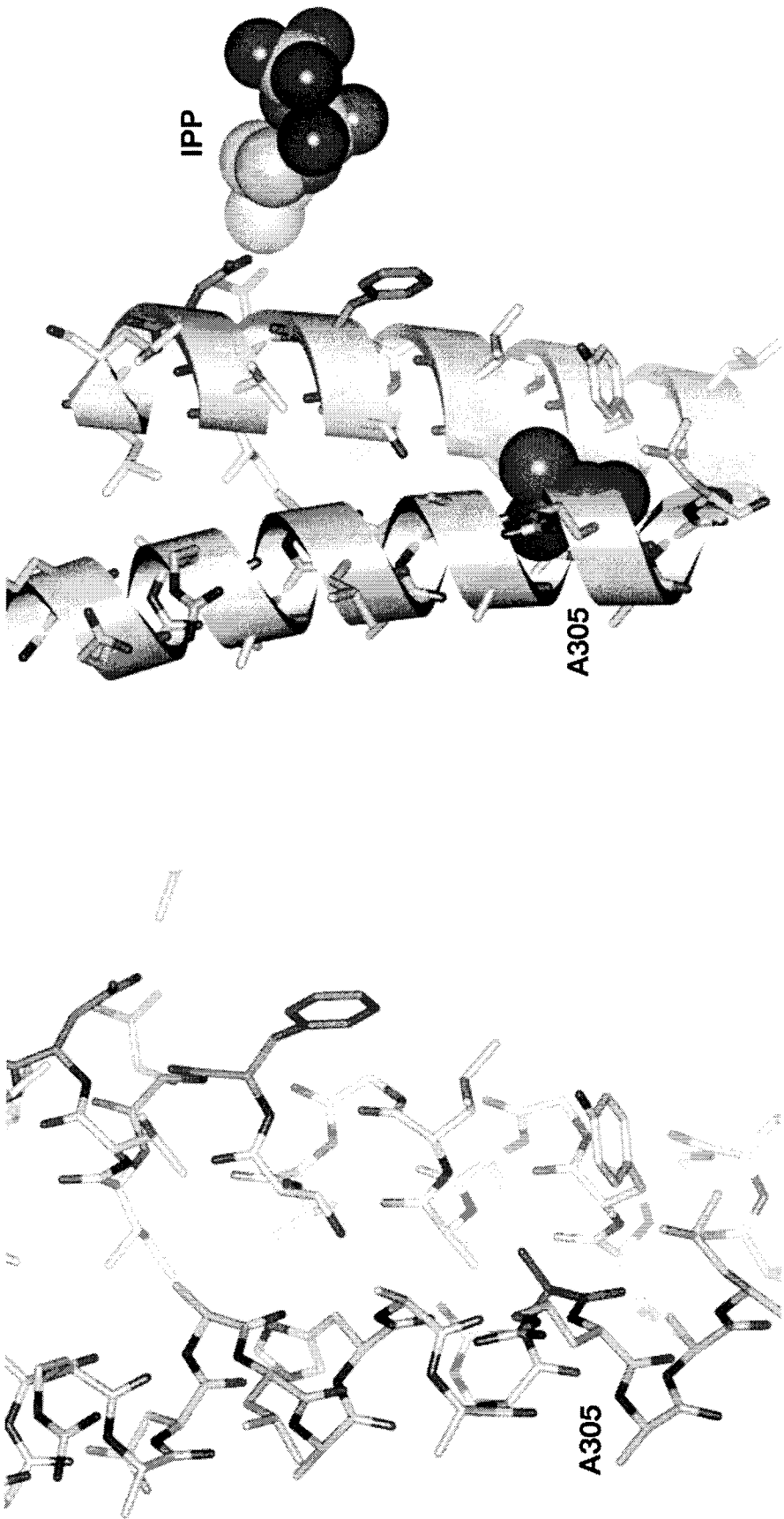


Fig. 31



Wild-type Enzyme C

Fig. 32

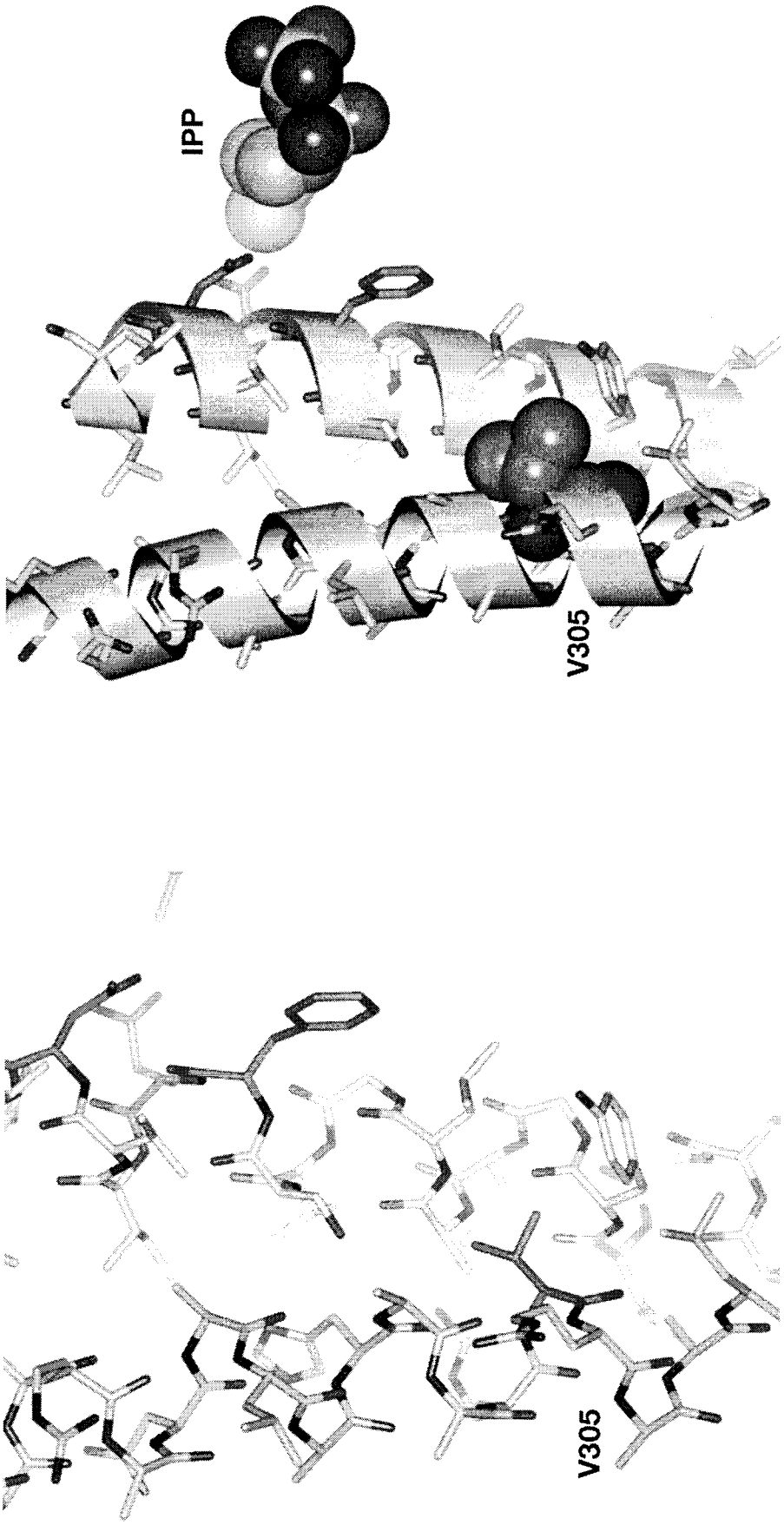
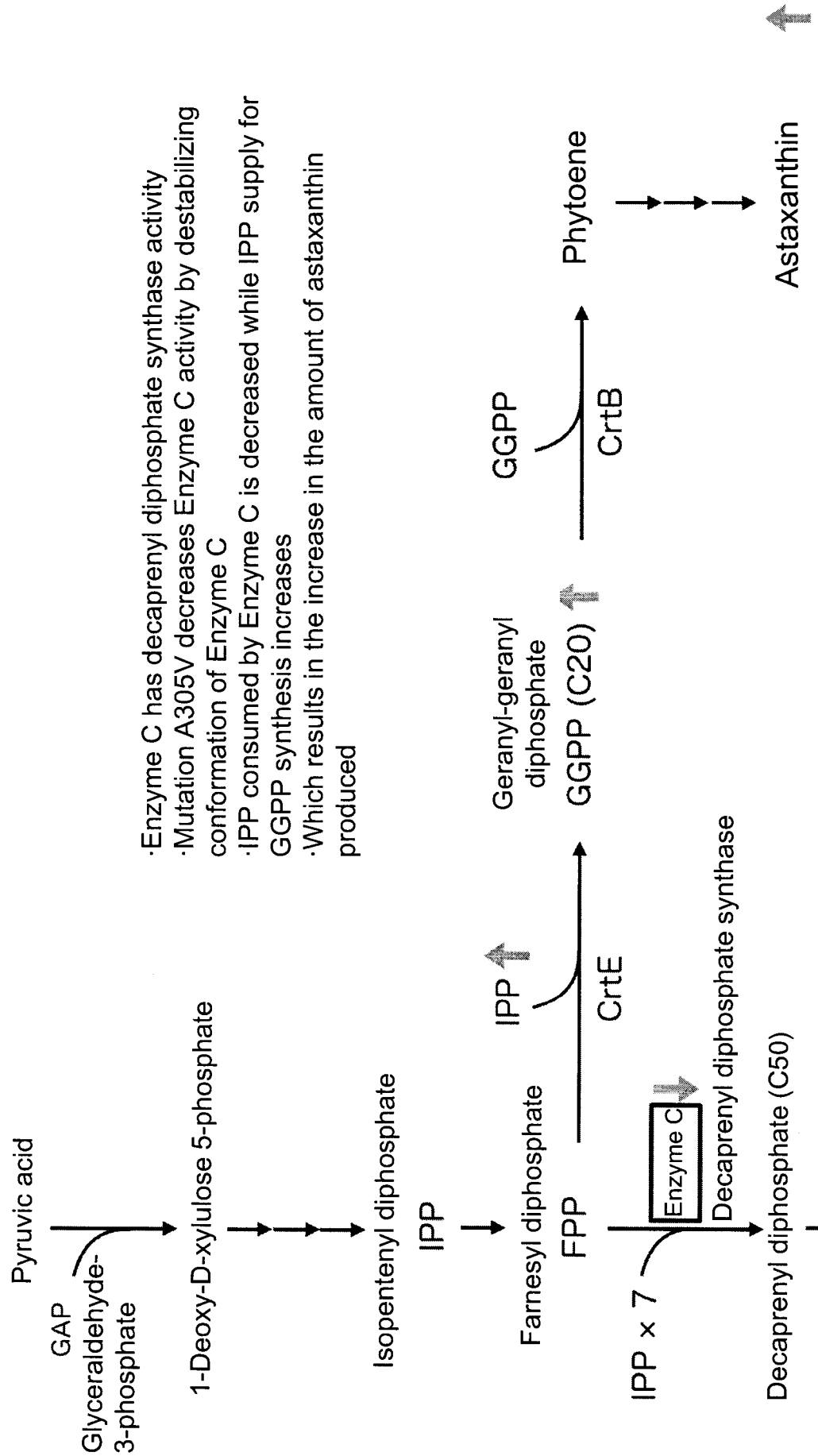


Fig. 33


·Enzyme C has decaprenyl diphosphate synthase activity
·Mutation A305V decreases Enzyme C activity by destabilizing conformation of Enzyme C
·IPP consumed by Enzyme C is decreased while IPP supply for GGPP synthesis increases
·Which results in the increase in the amount of astaxanthin produced
Pyruvic acid
GAP
Glyceraldehyde-3-phosphate
1-Deoxy-D-xylulose 5-phosphate
Isopentenyl diphosphate
IPP
Farnesyl diphosphate
FPP
IPP
CrtE
Geranyl-geranyl diphosphate
GGPP (C20)
GGPP
CrtB
Phytoene
Astaxanthin
IPP x 7
Enzyme C
Decaprenyl diphosphate synthase
Decaprenyl diphosphate (C50)
CoQ10