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Rational design of glycerol dehydratase: Swapping the genes encoding the subunits of glycerol dehydratase to improve enzymatic properties

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Abstract 1,3-propanediol (1,3-PD) is an important material for chemical industry, and there has been always much interest in the production of 1,3-PD using all possible routes. The genes encoding glycerol dehydratase (GDHt) from *Citrobacter freundii*, *Klebsiella pneumoniae* and metagenome were cloned and expressed in *E. coli*. All glycerol dehydratases but the one from metagenome could be detected to show enzyme activities. In order to improve the enzymatic properties of GDHts, the genes encoding α and β-γ subunits were cloned, and the enzyme characteristics were evolved by rational design based on their 3D structures which were constructed by homology modeling. Six heteroenzymes were obtained by swapping the α subunit genes of these three different-source-derived GDHts. The pH, thermal stability and V_{max} of some heteroenzymes were dramatically improved by 2—5 times compared with the wild one (GDHtKP). The GDHt cloned from metagenome, originally proved to be with no enzyme activity, was converted into active enzyme by swapping its subunits with other different GDHts. In addition, the effect of subtle 3D structural changes on the properties of the enzyme was also observed.

Keywords: glycerol dehydratase, metagenome, gene swapping, rational design, heteroenzyme.

1,3-propanediol (1,3-PD) is a special chemical with great potential in chemical industry. It is mainly used as solvent, antifreeze agent and protection reagent, and more importantly as the monomer of polyesters. Currently there are two methods for synthesis of 1,3-PD: chemical and biological ones. The latter consumes less energy, generates less pollution, and shows higher efficiency than the former. In recent years, most of studies have been focused on the 1,3-PD production by biological method[1−3]. In the biological process of producing 1,3-PD, glycerol dehydratase (GDHt, EC 4.2.1.30) is a key and rate-limited enzyme in the pathway of the coenzyme B_{12}-dependent conversion of glycerol to 3-hydroxypropionaldehyde (3-HPA), which is further reduced to 1,3-PD by the NADH-linked 1,3-propanediol dehydrogenase (PDOR, EC 1.1.1.202). In general, GDHt, which is mainly found in *Citrobacter freundii, Klebsiella pneumoniae, Clostridium pasteurianum*, etc.[4−7], consists of three types of subunits, i.e. α, β, γ, and exists in form of $\alpha_2\beta_2\gamma_2$ heterohexamer.

During the lengthy fermentation process of producing 1,3-PD, the activity of GDHt was retarded by 3-hydroxypropionaldehyde and other byproducts such as organic acids through pH fluctuation[8]. It is therefore necessary to evolve the GDHt by optimizing its enzymatic properties to harness the biological method in production of 1,3-PD in commercially viable quantities. However, due to the complexity of GDHt *per se* (such as strict dependence on coenzyme CoB_{12}, complicated structure with six subunits, substrate inactivation, delicate reactivation components, etc.), few researches have been conducted on the modification of its structure since GDHt gene were first cloned in the middle of 1990s[9,10]. Gene swapping is a frequently used method for optimizing the properties of enzymes in recent years. Compared with directed evolution method (or called irrational design, such as error-prone PCR and DNA shuffling), gene swapping is time-saving, and efficient and the targeting is easy. Domains or subunits of the enzyme derived from different genus (some of them even do not show any enzyme activity in their original forms) could be recombined by gene swapping to produce a new one with improved enzymatic property such as enhanced processivity. This could be done using the information of protein sequence and structure plus computational rational design. Therefore, gene swapping could reduce the time consumption compared with the family shuffling technology[11]. Gene swapping can also be used to deduce the relation between function and structure of enzymes[12]. In this paper, we report the cloning and *in vitro* evolu-

tion of the genes encoding GDHts from *C. freundii*, *K. pneumonia*, and metagenome by expressing these genes in the *E. coli*. The purification of the cloned enzymes was considerably facilitated by using the PCR primers with His-tag. The rational design was specially manifested by swapping the subunits of three GDHts *in silico* and then experimentally validated. The whole processing is briefly described as follows. (1) Genes encoding GDHt from different bacterial sources were cloned and expressed, and enzymes characterized. The genes were then sequenced to confirm their primary. (2) Subunits were swapped *in silico* among the three different sources. Each swapped intact enzyme that consists of three subunits was modeled using computer software to get its 3D structure. (3) Each model produced was calculated *in silico* to get the energy minimization, and then the models with the lowest energy profiles were tested experimentally. The results turned out to be very promising: our experiments have turned the original enzyme forms that have no activities into enzymatic active ones, and some recombined enzymes appeared 3—5 times more stable than the wild type ones under some pH and temperature conditions. As far as we know, this was the first time the property of GDHt was ameliorated successfully by rational design at molecular level.

1 Materials and methods

1.1 Bacterial strains, plasmids, media, growth conditions and DNA extraction

C. freundii AS1.1732 and *K. pneumoniae* 10018 were obtained from CGMCC (Beijing, China) and CCICC (Beijing, China), respectively. pSE380 (Invitrogen, USA) vector was used for the over-expression of the genes encoding GDHts from *C. freundii* and *K. pneumoniae*. *E. coli* JM109 (Promega, USA) was used as host. *C. freundii* and *K. pneumoniae* were grown under aerobic condition in a pH 7.0 medium containing peptone (10 g/L), meat extract (10 g/L), glucose (10 g/L) and NaCl (5 g/L). The enrichment of glycerol fermenting microorganisms was performed according to Knietsch *et al.*'s studies[13]. The DNA extraction of *C. freundii*, *K. pneumoniae* and uncultured microorganisms (i.e. metagenome) were also carried out according to previous studies[13,14].

1.2 Gene cloning of GDHt

Primers were designed using software Vector NTI according to the sequences of *dhaBCE* and *gldABC* from *C. freundii* and *K. pneumoniae*, respectively (Table 1).

The PCR primers of *dhaBCE*, *dhaB* and *dhaCE* genes were used to amplify corresponding genes (named *udhaBCE*, *udhaB* and *udhaCE*, respectively) from metagenome. PCR primers were designed in a way to contain some restriction recognition sites (underlined, *Pag* I and *Nco* I are isocaudarner) and to contain a short sequence encoding a 6× His-tag (bold type). *dhaBCE*, *gldABC* and *udhaBCE* were cloned by PCR using corresponding genome or metagenome DNA as templates. The genes encoding α subunit and β-γ subunit of these three different-source-derived GDHts were further cloned by PCR using the corresponding recombinant plasmids as templates. *Dpn* I digestion and gel extraction kit were used to remove the remaining recombinant plasmid to reduce the background effect in the next step screening.

Table 1 Primers and their sequences for gene cloning and swapping

Primers	Sequences
dhaBCE sense primer	5′-TGCTCATGATT**CACCACCATCACCATCAT**ATGGGAAGATCAAAACGATT-3′
dhaBCE anti-sense primer	5′-AAGAAGCTTTCACTGGCTGCCTTTACG-3′
dhaB sense primer	*dhaBCE* sense primer
dhaB anti-sense primer	5′-ATAGAGCTCCCGCCTTATTCAATCGAG-3′
dhaCE sense primer	5′-AAAGAGCTCGTGGAATGCACAACTGAA-3′
dhaCE anti-sense primer	*dhaBCE* anti-sense primer
gldABC sense primer	5′-TGCTCATGATT**CACCACCATCACCATCAT**ATGAAAAGATCAAAACGATT-3′
gldABC anti-sense primer	5′-CGCAAGCTTTTAGCTTCCTTTACGCA-3′
gldA sense primer	*gldABC* sense primer
gldA anti-sense primer	5′-AAAGAGCTCCCGCCTTATTCAATGGT-3′
gldBC sense primer	5′-ATAGAGCTCGTGCAACAGACAACCCAA-3′
gldBC anti-sense primer	*gldABC* anti-sense primer

1.3 Sequence analysis and homology modeling

Those genes inserted into pMD18-T were sequenced (TaKaRa) to confirm the fidelity. The sequences were analyzed by BLAST, ClustalX and Vector NTI and submitted to Genbank using the software of Sequin. SWISS-MODEL server (http://swissmodel.expasy.org//SWISS-MODEL.html), and Swiss-Pdb Viewer software were used to construct the 3D structures of wild and hybrid GDHts based on the homologous template of GDHt from *K. pneumoniae* (PDB code 1iwp)[15]. The 3D Structures of enzymes were compared using the DALI server (http://www.ebi.ac.uk/dali/Interactive.html). Rational design by swapping α subunits for improvement of enzymes properties was done through energy computations (energy minimization) with GROMOS96 implementation of Swiss-Pdb Viewer. The effect of 3D structural changes on enzyme property was analyzed based on the modeled 3D structural information.

1.4 Gene swapping of GDHt and construction of expression vector

According to the results of rational design, which was based on homology modeling and energy minimization computations, hybrid enzymes were constructed by swapping α subunit genes and then amalgamating with β-γ subunit genes through recognition site of *Sac* I (Fig. 1). The connected fragments were inserted into pSE380 to test their enzyme activities.

1.5 Gene expression and cell extract preparation

Expression of genes, extraction of recombinant enzymes, activity assay and electrophoresis by SDS-PAGE were according to ref. [16]. The resuspended cells were disrupted by ultra-sonication on ice.

1.6 Enzyme purification and characterization

GDHt was purified by Ni-nitrilotriacetate affinity chromatography followed by Sephacral S-300 gel filtration. The extract containing His-tagged GDHt was loaded onto Ni-nitrilotriacetate agarose gel column which had previously been equilibrated with Buffer A (100 mmol/L potassium phosphate and 150 mmol/L KCl) containing 5 mmol/L imidazole. After non-specific proteins were washed out by Buffer A containing 50 mmol/L imidazole, the required enzymes were collected by eluting column with Buffer A containing 200 mmol/L imidazole. Sephacral S-300 gel filtration with 100 mmol/L potassium phosphate as elution buffer was used to further remove unbound, excess subunits and salt in the raw enzyme elution obtained with Ni-nitrilotriacetate affinity chromatography. All operations were carried out at 4℃. Protein concentrations were determined by the method of Bradford with bovine serum albumin as the standard. The optimal temperature and pH for GDHt were determined at different temperatures and different pH values of 100 mmol/L potassium phosphate buffer, respectively. In order to study the stability of recombinant enzymes, enzyme samples were incubated at 45℃ and 50℃ with potassium phosphate buffer (pH 8.0) or at 37℃ with potassium phosphate buffer (pH 6.0 and 10.0). Treated enzyme samples were put on ice at definite time intervals (every 30 min) to continuously monitor residual enzyme activity as described previously[17]. K_m and V_{max} values of enzymes, at optimal temperature and pH, were measured according to standard Lineweaver-Burk plots. One-minute assay was employed to measure GDHt's K_m and V_{max} for glycerol and 1,2-propanediol (1,2-PD), since glycerol will trigger suicide inactivation of the GDHt enzyme, and this situation is rarely en-

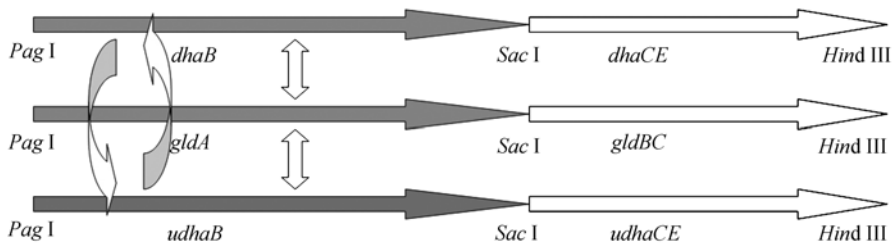

Fig. 1. Schematic illustration of gene swapping between the genes encoding α subunit of glycerol dehydratase. Recombinant GDHt from *C. freundii* was renamed GDHtCF; Recombinant GDHt from *K. pneumoniae* was renamed GDHtKP; hybrid enzyme encoded by *dhaB* and *gldBC* was renamed GDHtCK; hybrid enzyme encoded by *gldA* and *dhaBC* was renamed GDHtKC; hybrid enzyme encoded by *dhaB* and *udhaBC* was renamed GDHtCU; hybrid enzyme encoded by *gldA* and *udhaBC* was renamed GDHtKU; hybrid enzyme encoded by *udhaB* and *dhaBC* was renamed GDHtUC; hybrid enzyme encoded by *udhaB* and *gldBC* was renamed GDHtUK.

countered when using 1,2-PD as substrate.

2 Results

2.1 Gene cloning, swapping and construction of expression plasmid

The whole gene fragments amplified by PCR from the genomes of *C. freundii*, *K. pneumoniae* and metagenome, were nearly of the same sizes, about 2.7 kb. The sequence data of *dhaBCE*, *gldABC* and *udhaBCE* determined by this investigation were deposited in the GenBank database under accession numbers DQ152921, DQ191044 and DQ191045, respectively. After sequencing confirmation of the amplified sequences, the fragments were ligated to pSE380 and transformed into *E. coli* JM109. The confirmed recombinant plasmids were used as the PCR templates to obtain the genes encoding α subunits and β-γ subunits for use in gene swapping. Results of energy minimization calculations showed that the hybrid enzymes GDHtKC and GDHtKU had lower energy profile than wild types (Table 2), suggesting that these two new enzymes was more stable than the wild types. In order to prove this conclusion, we swapped the genes encoding α subunits from one bacterium and reconnected them with genes encoding β-γ subunits from another bacterium. The rationale for choosing the way of recombination of the subunits was solely based on predicted results of energy minimization (Table 2). From Table 2, it is clear that all hybrid-enzymes have lower energy profile than the wild types GDHtKP, and hybrid enzymes GDHtKC, GDHtKU and GDHtUC show lower energy profile than the wild type GDHtCF.

2.2 Expression and purification

In order to confirm the expression of the GDHt genes, it is necessary to detect the enzyme activity from the recombinant *E.coli* cells. Homogenates of the recombinant *E. coli* cells were analyzed by SDS-PAGE. Thick and clear protein bands corresponding to correct molecular weights of 61, 22, and 16 kD of α, β and γ subunits of GDHt are shown in Fig. 2. Only SDS-PAGE of recombinant GDHt from metagenome is presented in Fig. 2.

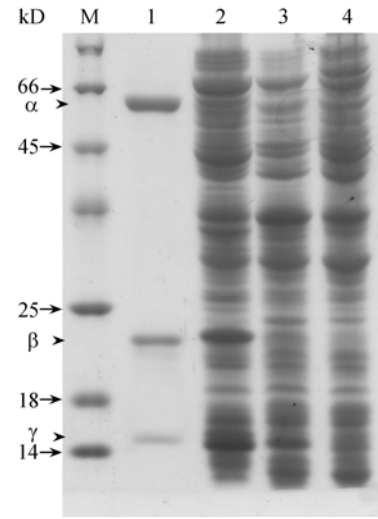

Fig. 2. SDS-PAGE analysis of the crude extract and purified GDHt from metagenome. Lane M, Protein Weight Marker; lane 1, purified GDHt by Ni-nitrilotriacetate and Sephacral S-300; lane 2, crude extract of recombinant induced by IPTG; lane 3, crude extract of recombinant without IPTG; lane 4, the control (crude extract of JM109-pSE380).

2.3 Sequence analysis

The GDHt sequences were compared with other sequences of several homologous enzymes available in GenBank database. The results are shown in Table 3. The similarity of them was all over 70% at nucleotide acid level, and over 90% at amino acid level. The highest similarity between GDHt from metagenome and *C. freundii* (97% in amino acid level) indicated that the sequence from metagenome was GDHt gene. The active sites (all in the TIM barrel of the α subunit) of these three GDHts were highly conservative.

2.4 Property of wild type and hybrid enzymes

Wild type and hybrid GDHts were made homogeneous through purification when measuring their specific activity and kinetic property (Table 3 and Fig. 3). The K_m of wild type and hybrid GDHts were roughly similar. The optimal pH and temperature of wild type GDHts were 8.5 and 45℃, respectively, which were the same as those of hybrid enzymes. The V_{max} of GDHtKU whose sequence partly came from metage nome was less than half that of wild type. Half-life of GDHtKU was 5 h at 45℃, about 5 times longer than that of wild type (GDHtKP), which indicated

Table 2 Information of energy predication about wild and hybrid enzymes[a)]

Type	GDHtCF	GDHtKP	GDHtCK	GDHtKC	GDHtKU	GDHtCU	GDHtUC	GDHtUK
Energy	–98074.0	–81056.0	–97571.8	–101130.5	–100874.8	–97810.0	–98550.5	–98048.2

a) Energy minimizations were computed with the GROMOS96 implementation of Swiss-Pdb Viewer.

Table 3 Similarity analysis of glycerol dehydratases in GenBank[a)]

	2*K. pneumoniae* (DQ191044)	3Ub (DQ191045)	4*C. freundii* (U09771)	5*K. pneumoniae* (U60992)	6Ub (AY205332)
1 *C. freundii*	77%	87%	85%	77%	84%
(DQ152921)	91%	96%	96%	91%	97%
2*K. pneumoniae*		79%	78%	99%	78%
(DQ191044)		91%	91%	99%	91%
3Ub			88%	79%	86%
(DQ191045)			97%	92%	96%
4*C. freundii*				78%	86%
(U09771)				91%	97%
5*K. pneumoniae*					79%
(U60992)					91%

a) For each pairwise alignment, the number of identical nucleic acids (upper) or amino acids (lower) (in % of shorter sequence) is given. Maximum values are underlined.

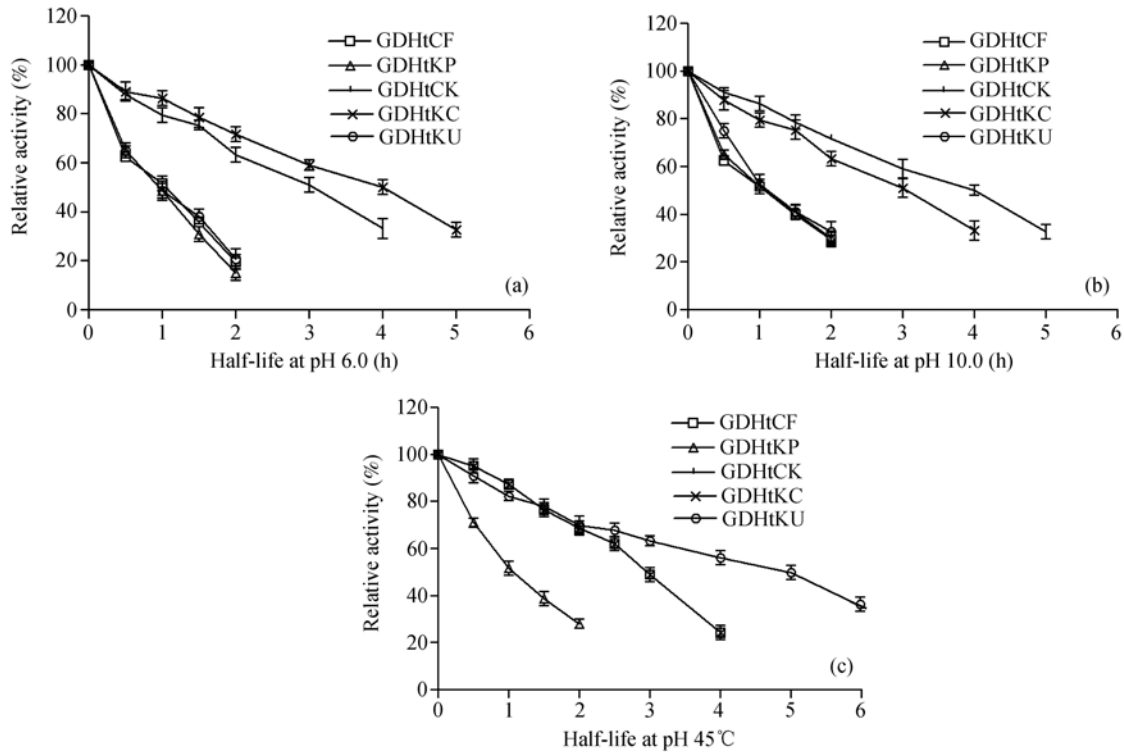

Fig. 3. Tests on the stability of wild type and hybrid glycerol dehydratases. (a) Acid stability at 37℃ in pH 6.0 potassium phosphate buffer; (b) alkali stability at 37℃ in pH 10.0 potassium phosphate buffer; (c) thermal stability at 45℃ in pH 8.0 potassium phosphate buffer.

that GDHtKU was more thermal tolerant than all the others. In addition, the residual activity of GDHtKU was 50% at 50℃ in pH 8.0 potassium phosphate buffers after 20 min treatment, and was 40% at the same condition after 40 min. However the residual activity of wild type GDHts reached zero under the same condition after only 20 min treatment. The specific activity and K_m of hybrid enzyme GDHtCK were similar to those of wild types; however, the V_{max} values, especially that for substrate glycerol, were about two times higher than that of wild type (Table 4). GDHtCK was much less pH-sensitive than the wild types. The specific activity of hybrid enzyme GDHtKC was slightly over half that of wild type, whereas the pH-sensitivity of GDHtKC was similar to that of GDHtCK, i.e. both hybrid enzymes had over 3 h of half life in conditions of pH 6.0 and 10.0, compared with only 1 h for the others including the wild types. The thermal tolerant of GDHtCK and GDHtKC were found to be the same as that of GDHtCF, but 3 times higher than that of another

Table 4 Analysis about properties of wild and hybrid glycerol dehydratases[a)]

Type	Sp. (U/mg)	K_m (mmol/L)		V_{max} (U/mg)		Half-life (h)		
		1,2-PD	glycerol	1,2-PD	glycerol	pH 6.0	pH 10.0	45℃
GDHtCF	91.9	0.25	0.59	73.5	142.9	1.0	1.0	3.0
GDHtKP	85.8	0.24	0.60	61.5	130.1	1.0	1.0	1.0
GDHtCK	90.3	0.22	0.59	89.9	260.8	3.0	4.0	3.0
GDHtKC	56.9	0.23	0.57	71.0	102.6	4.0	3.0	3.0
GDHtKU	37.2	0.35	0.42	30.6	39.7	1.0	1.0	5.0

a) The data were obtained from the average of three independent experiments.

wild type, GHDtKP.

2.5 Analysis of 3D structures

Homology modeling of wild type and hybrid GDHts was performed on the template of *K.pneumoniae* GDHt, PDF code 1iwp. Combining this result with SDS-PAGE analysis, we found that the fully biologically active form of GDHts was an $\alpha_2\beta_2\gamma_2$ heterohexamer, which was the same as 1iwp. There were 25 α helices and 14 β strands in α subunit, 5 α helices and 7 β strands in β subunit, and 7 α helices and 2 β strands in γ subunit. It was observed that these α subunits also contained a TIM (triosephosphate isomerase) barrel structure. The active site was just located inside this barrel formed by eight parallel β-strands. Substrates such as 1,2-PD, and an essential cofactor K^+ were all bound inside this barrel. Our model also showed the active site cavity was covered by a corrin ring of cobalamin that was bound on the interface of α and β subunits. The root-mean-square deviations (RMSD) of the main-chain C_α atoms of the entire hexamer between our enzymes and 1iwp were no more than 0.1 Å for 1754 aligned main chain atoms according to the analytical results from Swiss-Pdb Viewer and DALI. The schematic view of the overall structure of GDHtKP is shown in Fig. 4(a). The differences in amino acid residues resulted in the differences in their 3D structures and it is very likely that this would cause the difference in hybrid enzyme properties. As pointed out above, calculation results showed that the energy profile of hybrid enzymes, especially that of functional GDHtCK, GDHtKC and GDHtKU, were markedly lower than that of GHDtKP (Table 2).

The active sites of functional GDHtCF, GDHtKP, GDHtCK, GDHtKC and GDHtKU were composed of 12 highly conservative amino acids (α-Gln142, α-His144, α-Glu171, α-Glu222, α-Thr223, α-Gln297, α-Val301, α-Thr302, α-Asp336, α-Gln337, α-Ser363 and α-Phe375). Six of them (α-His144, α-Glu171, α-Glu222, α-Gln297, α-Asp336 and α-Ser363) associated with the substrate (Fig. 4(b) showed the schematic illustration for GDHtKP). From the models obtained, we found that the hydrogen bond (H-bond) numbers and distances between substrate and active sites of functional hybrid enzymes were all different from that of wild types (Fig. 5), which might partly explain the

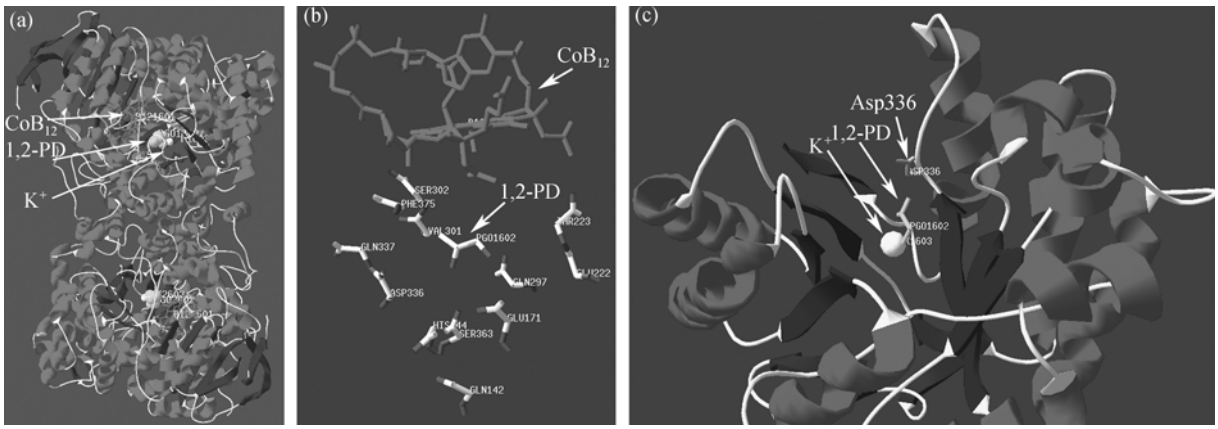

Fig. 4. The structure of glycerol dehydratase from *K. pneumoniae* by homology modeling. (a) Over all structure, CoB_{12}, 1,2-PD and K^+; (b) schematic illustration of the predication active site, substrate and CoB_{12}; (c) schematic illustration of 3 D structure around α-336.

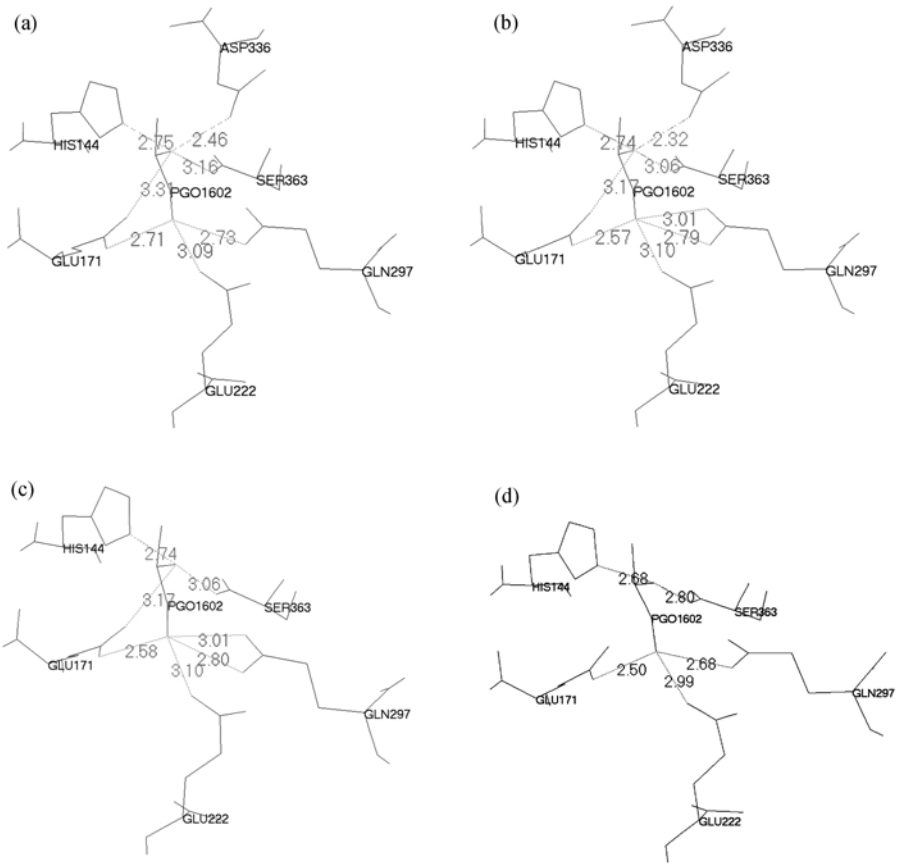

Fig. 5. Schematic illustration of H-bond of GDHtKP (a), GDHtKU (b), GDHtUB (c) and GDHtUK (d) between substrate and active site.

differences in these enzymatic properties. For example, the H-bond distances between the substrate and the six amino acids of the active site for all hybrid enzymes that showed activities differed from the wild type GDHtKP by about 0.00—0.26 Å (usually less than the wild types). Besides, it was observed that hybrid enzymes usually formed two H-bond between substrate and active site position α-Gln297 (Fig. 5(b)), but both wild types GDHtKP and GDHtCF only formed one H-bond (Fig. 5(a)). It was interesting to see that the length of H-bonds formed between substrate and active site of GDHtKU was even 0.14 Å shorter than that of other hybrid enzymes (0.26 Å). More compact structure at the active site was probably one of reasons why GHDtKU is more stable than other enzymes, including the wild types and the hybrid ones. However, this tightened structure probably may not be able to facilitate the binding of substrate to the active site and would result in a decrease in GDHtKU's activity and V_{max} (Table 4).

α-Asp336, one of the six amino acids directly contacting with substrate, was changed into α-Gly336 in GDHtUB (no activity). This kind of amino acid substitution at the active site may account for the activity losses of GDHtUB and other α-Asp336- substitued hybrid enzymes. This amino acid was located in a coil structure nearby the active site consisting of 8 β-strands (Fig. 4(c)). This kind of site-mutation caused a loss in H-bonds between substrate and α-Gly336 of the active site (Fig. 5(c) and (d)), which further led to the shift of associated second structure and hence the distortion of active site. The H-bond numbers and distances between substrate and active site of hybrid enzymes GDHtUB and GDHtUK (both show no activity) were different from that of wild ones (Fig. 5), such as the loss of H-bond between substrate and α-Gly336 mentioned above and between substrate and α-Glu171. The maximal difference in distance of H-bond between hybrid and wild type enzymes was 0.36 Å (Fig. 5(d)), which led to tremendous distortion of the structure of active site. According to the evidence mentioned above, we might presume that the mutation of α-Asp336 in

active site might be the primary reason for the absence of activity of this kind of hybrid enzymes. But we cannot rule out the fact that there may also be other structure transformations affecting the activity of hybrid enzymes along with the mutation of α-Asp336.

3 Discussions

In nature, a majority of microorganisms is not culturable and only a tiny percentage of them (less than 1%) can be grown in artificial environments. This vast amount of non-culturable microorganisms will provide numerous new genes and might be the potential species for study and exploitation[18]. It was reported that several GDHt genes had already been cloned from culturable bacteria. However, very few GDHt genes from metagenome of the non-culturable microorganisms were reported. In this paper, we presented an effective and simple way to isolate and clone the GDHt gene from metagenome using PCR technique.

The similarity between our *in vitro* modeling-constructed enzymes and template (1iwp) were over 90% at amino acid level. This will make the homology modeling a reliable and feasible method for construction of protein 3D structure[15,19]. The root-mean-square deviations (RMSD) between our enzymes and the template 1iwp were no more than 0.1 Å for 1754 aligned main chain atoms, which indicated that the models performed well as we expected. In this paper, recombining the heterogenous subunits through gene swapping was carried out to obtain new enzymes with enhanced property or possessing capacity, and most importantly the part of the hybrid enzymes containing the subunit from metagenome showed the enzyme activity absent in the original genes cloned from metagenome. Homology model showed that the amino acid α-Asp336 in active site of GDHtKU (no activity) may be a critical one since when it was mutated to α-Gly336, and the H-bond number was reduced from two (in α-Asp336) to only one. However, this conclusion needs more evidence. For instance, saturation mutation at α-Asp336 or other related amino acid positions may be able to bring about more definite answers to this issue.

H-bond, especially internal H-bond, plays an important role in maintaining the stability of proteins[20]. The length of internal H-bond between substrate and active sites of hybrid enzymes in our studies had also been markedly changed (distance differences ranged from 0 to 0.36Å) compared with wild type one. The active site of GDHt was shown to be located inside the α subunit, whereas, β-γ subunits is probably associated with reactivation component which makes the reactivating process possible[21,22]. As is well known, the stability of hybrid enzymes was in fact improved by the co-ordinated actions of α and β-γ subunit rather than the α subunit alone, even though the active sites were located in the α subunit. This is because hybrid enzyme GDHtKU containing β-γ subunit of GDHtUB (no activity) and the α subunit of GDHtKP is functional and highly stable. These structural changes could be also used to explain the increase of the pH-stability of the hybrid enzymes (GDHtCK and GDHtKC), as well as the high V_{max} value of GDHtCK. In addition, the low level of energy profile is also a very useful indicator for estimation of the stability of enzyme[23]. It could be seen that the energy profiles from minimization calculations of the hybrid enzyme (GDHtCK, GDHtKC and GDHtKU) based on their 3D structures were much lower than the wild type GDHtKP. This index could be useful for designing the gene swapping, since the overall energy profile could be regarded as one of essential criteria for the protein stability. To get a better understanding of the protein structure-function relation, more powerful tools such as X-ray and NMR should be used to study our hybrid enzymes. Our experimental results would still be very useful since our study supplied for the first time the good evidence for illustration of the structure-function relationships of GDHt. Researchers in this field may be able to use our results to help them to further improve the GDHt and create a novel hybrid enzyme in the manufacture of 1,3-propanediol by the biological pathway.

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