Alteration in relative activities of phenylalanine dehydrogenase towards different substrates by site-directed mutagenesis

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Abstract Glycine-124 and leucine-307 of phenylalanine dehydrogenase from *Bacillus sphaericus* were altered by site-specific mutagenesis to the corresponding residues in leucine dehydrogenase: alanine and valine, respectively. These two residues have previously been implicated from molecular modelling as important in determining the substrate discrimination of the two enzymes. Single and double mutants displayed lower activities towards L-phenylalanine and enhanced activity towards almost all aliphatic amino acid substrates tested compared to the wild-type, thus confirming the predictions made from molecular modelling.

Key words: Phenylalanine dehydrogenase; Mutagenesis; Substrate specificity; Amino acids

1. Introduction

Phenylalanine dehydrogenase (EC 1.4.1.20) catalyses the reversible NAD^+-linked, oxidative deamination of L-phenylalanine to phenylpyruvate and is found in a limited number of Gram-positive, aerobic bacteria [1]. It belongs to a family of amino acid dehydrogenases, whose members include both glutamate dehydrogenase (EC 1.4.1.2–4) and leucine dehydrogenase (EC 1.4.1.9). Phenylalanine dehydrogenase (PheDH) and leucine dehydrogenase (LeuDH) show considerable amino acid sequence homology. For example in PheDH from *Bacillus sphaericus* and LeuDH from *Bacillus stearothermophilus* identical residues are found at 50% of the positions [2]. However, the substrate specificities of the two enzymes are different. LeuDH only accepts aliphatic and not aromatic amino acids as substrates [3,4]. On the other hand PheDH, besides reacting with aromatic amino acids, shows only slight activity towards aliphatic substrates [5,6,7,8].

In seeking to decipher the rules governing the activity of these enzymes with different substrates, it is now possible to compare their amino acid sequences in the light of the known structure of glutamate dehydrogenase (GDH) from *Clostridium symbiosum* [2,9–11]. Though the sequences of both PheDH and LeuDH show a relatively low level of sequence identity with GDH, the alignment suggests that residues inferred as being responsible for the overall shape of the active site and for catalysis, as well as those involved in the recognition of the nicotinamide cofactor, are conserved in all three enzymes [2,12]. This justifies the attempt to model the structure of the amino acid dehydrogenases using the GDH structure as a framework [2]. Glycine-124 and leucine-307 in PheDH (numbering based on the *B. sphaericus* sequence; the corresponding residues in *Clostridium symbiosum* GDH are 163 and 377, respectively) are replaced by alanine and valine, respectively, both in LeuDH [2] and in another branched-chain amino acid dehydrogenase, valine dehydrogenase from *Streptomyces coelicolor* [13]. It appears that in PheDH the glycine and leucine residues at these two positions allow the access of a large planar aromatic substrate into the binding pocket (Fig. 1).

In this paper we describe the substitution of glycine-124 and leucine-307 in the PheDH sequence of *Bacillus sphaericus* for alanine and valine, respectively, by site-directed mutagenesis. The resultant mutants show decreased activity towards L-phenylalanine and increased catalytic activity towards most aliphatic amino acid substrates tested.

2. Materials and methods

2.1. Materials

Restriction enzymes, T4 DNA ligase and calf intestinal alkaline phosphatase were from Northumbrian Biologicals (UK). Reagents for site-directed mutagenesis were supplied in a kit by Boehringer Corp. (Lewes, UK). DNA sequencing was performed using the 7-deaza-dGTP kit and Sequenase Version 2.0 (United States Biochemicals, USA). Plasmid DNA was prepared by using the Wizard miniprep from Promega Corp. (USA). The Geneclean II Kit was obtained from BIO 101 (La Jolla, CA, USA). Oligonucleotides for mutagenesis were synthesised by Dr. A.J.G. Moir and Mr. P.E. Brown (University of Sheffield) using an Applied Biosystems 381A DNA synthesiser. Oligonucleotides for DNA sequencing were synthesised using the Cyclone Plus DNA synthesizer (Milligen, USA). Sepharose CL-6B and butyl-Sepharose 4 fast flow were from Pharmacia Biotech (UK). Procion red P3BN was from Imperial Chemical Industries (UK). All other chemicals were of analytical grade.

2.2. Bacterial strains and plasmids

Escherichia coli TG1 (*Δlac-proAB, supE, thi*1, *hsdΔ*5/F'*tra*D36, *proA*$^+$*B*$^+$, *lacI*^{q}, *lacZΔ*M15) and CJ236 (*dut*1, *ung*1, *thi*1, *relA*1/pCJ1059-(cm^r)) were obtained from Boehringer Corp. (Lewes, UK). *E. coli* strain containing plasmid pBDH1DBL (*pdh* gene of *B. sphaericus* inserted in the *Bam*H1 site of pUC8) was provided by Sagami Chemical Research Centre (Kanagawa, Japan). M13mp19 was purchased from Pharmacia Biotech (UK). The plasmid ptac85 [15] was used to express the wild-type and mutant *pdh* genes.

2.3. Plasmid construction and mutagenesis

The wild-type and mutant *pdh* genes were subcloned into the *Bam*H1 site of M13mp19 and into ptac 85 using standard procedures [16]. The orientation of the gene in the plasmid was determined by *Eco*RI digests

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since there are two asymmetrically positioned *Eco*RI sites in the *pdh* gene. The mutagenic oligonucleotides 5′-TTTACACAGCTACTGAC-AT-3′ (the underlined codon GCT replacing GGT) and 5′-GCCGG-CGGCGTGATCCAG-3′ (the underlined codon GTG replacing TTG) were used to create the G124A and L307V mutations, respectively. The mutagenic oligonucleotides were hybridised to the antisense strand and mutants were preferentially selected by using the uracilated-DNA template method [17,18]. Mutants were screened directly by sequencing using the dideoxy-chain termination method. [19]. The entire DNA sequences of the selected positive mutants were then determined to confirm that there were no other secondary mutations. The mutant genes were cloned into the expression vector ptac85, the genes of the positive clones were recloned back into M13mp19, and the entire DNA sequences were again determined to confirm that no other mutations had occurred during the cloning procedure.

2.4. Expression and purification of enzymes

The wild-type and mutant *pdh* genes were expressed under control of the *tac* promoter of ptac85. Potassium phosphate buffer, pH 7.9, containing 0.1 mM EDTA and 5 mM 2-mercaptoethanol, was used throughout the purification procedure which was carried out at room temperature unless indicated otherwise.

E. coli TG1 cells containing the expression plasmid were grown in LB medium supplemented with 0.1 mg/ml ampicillin at 37°C. 0.5 mM of isopropyl β-D-thiogalactopyranoside was added to the culture in log phase and the culture was allowed to grow for a further 3 h before harvesting. The harvested cell pellet was resuspended in 0.1 M phosphate buffer and broken by sonication. Cell debris was removed by centrifugation at 20000 × *g* for 30 min. The clarified supernatant was brought to 30% ammonium sulphate saturation to remove unwanted proteins. The resultant soluble proteins were then brought to 60% ammonium sulphate saturation. The precipitate was dissolved in 0.01 M phosphate buffer and dialysed against three changes of 0.01 M phosphate buffer at 4°C before applying to a Procion red P3BN column (12 × 4 cm prepared by immobilising the dye on Sepharose CL-6B according to a standard procedure [20]). Unbound materials were washed off the column with 0.01 M phosphate buffer and the enzyme was eluted with 0.2 M NaCl dissolved in the same buffer. The eluate was brought to 30% saturation with ammonium sulphate and loaded onto a butyl-Sepharose column (5 × 2 cm) pre-equilibrated with 30% ammonium sulphate in 0.01 M buffer. Unbound proteins were washed off with the pre-equilibration solution. The enzyme was eluted with 20% saturation of ammonium sulphate in 0.01 M phosphate buffer and stored as a precipitate in 60% ammonium sulphate. Before use, the enzyme was pelleted by centrifugation, redissolved in 0.01 M phosphate buffer and dialysed against the same buffer to remove all traces of ammonium sulphate. The concentration of the purified enzyme was determined by measuring its absorbance at 280 nm, using an extinction coefficient of 11.7×10^{-2} absorbance units · g^{-1} · ml · cm^{-1} [6]. The purity of the enzyme was routinely monitored by SDS-PAGE according to the discontinuous method of Laemmli [21].

2.5. Enzyme assay

The enzyme activity was determined at 25°C spectrophotometrically (Kontron Uvikon 941 plus) by measuring the reduction of NAD^+ at 340 nm in a 1-cm cuvette. Reaction mixtures (1 ml) contained 50 μmol glycine-KOH buffer, pH 10.4, 100 μmol KCl, 2.5 μmol NAD^+, 10 μmol of the amino acid substrate and the appropriate amount of enzyme. One unit of enzyme activity was the amount that catalysed the formation of 1 μmol NADH/min.

3. Results and discussion

3.1. Construction of mutants

Using a mixture of the two mutagenic oligonucleotides, about 50% of the putative mutants screened contained the double mutation G124A;L307V, while the other 50% contained only the L307V mutation. Surprisingly, the G124A single mutant was not obtained using the mixture of oligonucleotides nor with the single oligonucleotide. This single mutant was finally constructed by ligating an *Ava*II fragment containing the G124A mutation of the double mutant gene to the *Ava*II fragment of the wild-type *pdh* gene containing the wild-type sequence at position 307.

Sequencing of the entire gene in the three mutants confirmed the presence of the desired mutations. There were, however, three positions that were not consistent with the published sequence [22]. The two codons for Asp-240 and Asp-241 were found to be GAT and GAC instead of GAC and GAT. The putative −35 sequence of the *pdh* gene was found to be TTGAT instead of TTGAAT. These differences in sequence were not introduced in the site-directed mutagenesis procedure since the wild-type gene sequence was found to show the same difference. At present it is not clear whether this is due to mutations occurring in the cloned gene or an error in the published se-

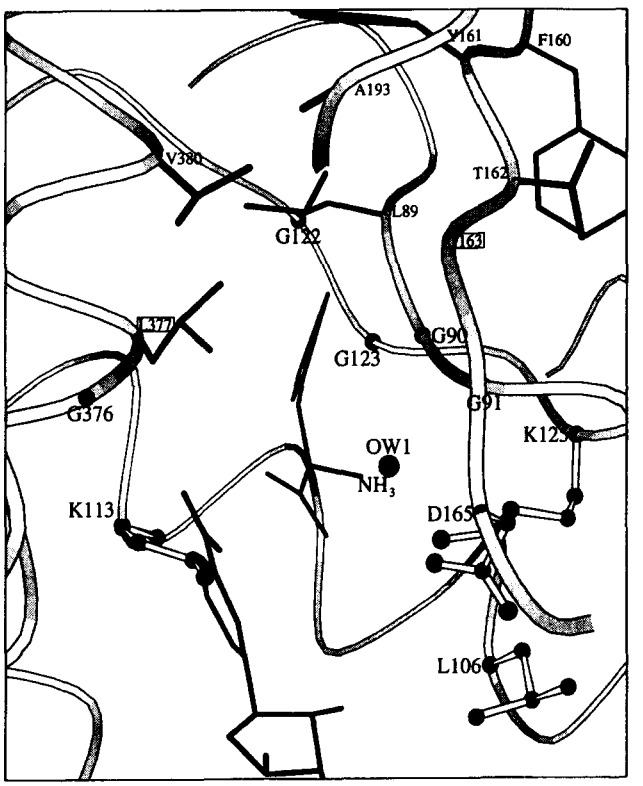

Fig. 1. A MOLSCRIPT [14] diagram of the modelled side chain specificity pocket for phenylalanine in the PheDH from *T. intermedius*. For ease of comparison, the clostridial GDH numbering is retained here. The positions subjected to mutagenesis in this paper (boxed in the figure), glycine-124 and leucine-307 in *B. sphaericus* PheDH are glycine-163 and valine-377 in the clostridial GDH sequence and alanine and valine in LeuDH. PheDH from *Thermoactinomyces intermedius* is used in this modelling exercise. The residues depicted in this diagram are essentially the same as the PheDH from *B. sphaericus* except that A193 in *T. intermedius* is replaced by N in *B. sphaericus*. The ribbon represents the fold of the polypeptide chain. The coordinates of *C. symbiosum* GDH [12] were used to provide the molecular framework of the binding pocket with the side chains modified to take into account of the sequence changes between GDH and PheDH. The strongly conserved side chains in the GDH family which are retained in the PheDH sequence are shown in a ball and stick representation whilst those which are modified in the PheDH sequence are portrayed in a solid chain style and reduced size labelling. The nicotinamide ring of the NAD, in the bottom left of the picture, and the modelled location of the bound phenylalanine substrate, in the centre, are portrayed in solid chain. The water molecule, OW1, thought to be the attacking nucleophile in the catalytic cycle, is shown as a sphere.

quence. These differences in sequence are however not expected to alter the enzyme since the encoded sequence is unaltered.

3.2. Expression and purification of wild-type and mutant enzyme

The mutant and wild-type *pdh* genes were highly expressed in *E. coli* TG1. A litre of culture yielded about 30 mg of PheDH estimated to be at least 99% pure as shown in Fig. 2.

The specific activity of the recombinant wild-type enzyme purified from *E. coli* was determined to be about 87 U/mg.

3.3. Relative activities towards different substrates

The mutant enzymes were tested for activity on a variety of substrates. The specific activities expressed as a percentage of the activity for L-phenylalanine are shown in Table 1.

Both single and double mutants showed a marked decrease in activities towards the aromatic substrates, L-phenylalanine and L-tyrosine, compared to the wild-type enzyme. The specific activities of the single mutant G124A and double mutant G124A;L307V were decreased about 20-fold for L-phenylalanine and 200-fold for L-tyrosine. This could be attributed to the fact that the methyl side chain of alanine is in a position that interferes with the aromatic-ring sterically [2]. The presence of an additional hydroxyl group attached to the aromatic ring might increase this steric hindrance, thus explaining the fact that a greater reduction in activity was observed for L-tyrosine compared to L-phenylalanine in these mutants. The reduction in activities for aromatic substrates in the case of the L307V mutant could be attributed to the fact that the leucine residue at position 307 of PheDH is probably responsible for hydrophobic interaction with the aromatic side chain of the phenylalanine substrate. Substitution of leucine-307 by valine, an amino acid with a shorter side chain, might decrease the strength of this interaction, thus reducing overall activity.

In the two mutants containing the change G124A, the activities towards aliphatic amino acid substrates were enhanced compared to the wild-type. The most striking example of this is for L-isoleucine where the activity is increased 22-fold in the double mutant. Combined with the accompanying opposite change in activity towards L-phenylalanine, this corresponds to a 380-fold decrease in the discrimination between these two substrates. In addition, the activities for L-isoleucine and L-norleucine are higher than for L-phenylalanine in the double mutant. Thus, two rather conservative amino acid substitutions are sufficient to transform a phenylalanine dehydrogenase of narrow substrate specificity into an enzyme with an apparent preference for certain aliphatic substrates.

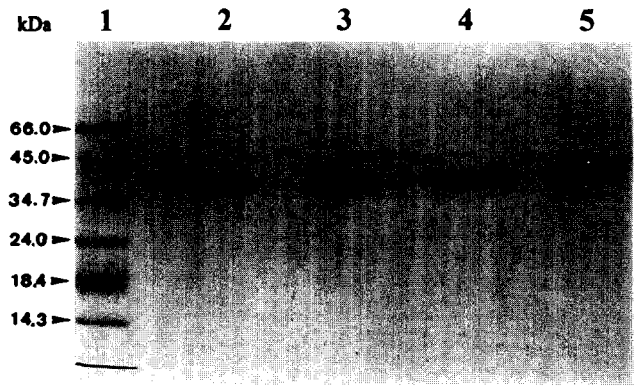

Fig. 2. SDS-PAGE of purified wild-type and mutant PheDH enzymes Lane 1 = molecular weight markers: bovine albumin (66000 Da), egg albumin (45000 Da), porcine stomach mucosa pepsin (34700 Da), bovine pancreas PMSF-treated trypsinogen (24000 Da), bovine milk β-lactoglobulin (2 subunits ~18400 Da), chicken egg white lysozyme (14300 Da); Lane 2 = wild-type PheDH; Lane 3 = G124A; Lane 4 = L307V; Lane 5 = G124A;L307V.

The single mutation L307V produces a substantial decrease in the rates measured with L-phenylalanine and L-tyrosine as substrates, but normally no change in the rates for the straight-chain aliphatic substrate L-norvaline, L-norleucine and L-methionine. On the other hand with branched-chain substrates there was an increase for amino acids with a β-branch (L-valine and L-isoleucine) and a 4-fold decrease for L-leucine, with a γ-branch.

Attempts have previously been made to change the substrate specificity of PheDH from *Thermoactinomyces intermedius*. The conserved amino acid residues ^{124}F-V-H-A-A-^{129}R of this PheDH (corresponding to residues 134–139 in the *B. sphaericus* enzyme) have been replaced by M-D-I-I-Y-Q, the corresponding sequences in LeuDH of *B. stearothermophilus* [23]. Although substrate discrimination of the resultant enzyme was altered, the catalytic efficiency towards all the amino acid substrates was reduced. The altered specificity is the result of the greater reduction in the catalytic efficiency of this enzyme towards L-phenylalanine rather than a positive selection for aliphatic substrates. A chimaeric enzyme made up of the N-terminus of PheDH from *Thermoactinomyces intermedius* and the C-terminus of LeuDH of *Bacillus stearothermophilus* have also been constructed [24]. The resultant enzyme has a glycine corresponding to position 124 and a valine corresponding to position 307. The activity of this enzyme towards L-isoleucine and L-valine was higher than the wild-type *Thermoactinomyces intermedius* PheDH, while L-leucine gave a lower activity. This result was consistent with the L307V mutant obtained here. The valine residue at position 307 probably had an important effect in the chimaeric enzyme. However, other residues might also be important since in the case of L-norvaline and L-norleucine, a much lower activity was obtained in the chimaeric protein, while the L307V mutant obtained here has a similar activity for L-norvaline and L-norleucine compared to the wild-type enzyme.

The sequences of LeuDH from *Thermoactinomyces intermedius* [25] and PheDH of *Rhodococcus sp.* M4 [26] have become available recently. As expected, the LeuDH from *T. intermedius* has an alanine and a valine corresponding to position 124 and 307 in the *B. sphaericus* PheDH enzyme, similar to the *B. stearothermophilus* LeuDH used in the molecular modelling. In the *Rhodococcus* PheDH, however, the residue corresponding to position 124 is glycine but position 307 is alanine rather than leucine as seen in *B. sphaericus* and *T. intermedius*. The effect of this difference is not yet certain because data for the relative activities of the *Rhodococcus* enzyme towards aliphatic substrates are not available. However, on the basis of the result presented here, we anticipate that this will result in a modified specificity profile towards aliphatic substrates. Further analysis of the *Rhodococcus* sequence shows a number of other changes in the substrate binding pocket with respect to *B. sphaericus*, namely leucine-36, phenylalanine-113 and leucine-314, which are replaced by alanine, asparagine and valine, respectively, in the *Rhodococcus* sequence. We predict that these differences

Table 1
Relative activities of wild-type and mutant PheDH with various aromatic and aliphatic substrates

Amino acids	Wild-type	L307V		G124A		G124A;L307V	
		1	2	1	2	1	2
L-Phenylalanine	100	27	100	4.6	100	5.7	100
L-Tyrosine	75	23	85	0.36	7.8	0.42	7.4
L-Leucine	1.1	0.22	0.81	2.6	57	2.5	44
L-Isoleucine	0.38	0.86	3.2	4.1	89	8.6	151
L-Norleucine	3.7	3.7	13	4.0	87	6.8	119
L-Valine	1.4	2.0	7.4	2.4	52	4.2	74
L-Norvaline	1.7	1.6	6.0	3.7	80	3.5	61
L-Methionine	2.2	2.0	7.4	3.1	67	2.8	49

For the wild-type enzyme, activity is expressed as a percentage of activity towards L-phenylalanine. For the mutants, column 1 shows the activity expressed as a percentage of the wild-type activity towards L-phenylalanine, while column 2 shows activity expressed as a percentage of the activity of each individual enzyme towards L-phenylalanine. The concentration of substrate used in each case is 10 mM except for L-tyrosine, where a concentration of 3 mM was used instead.

will have additional bearing on the substrate preferences of this enzyme.

In one respect, the predictions of the modelling are not fully borne out, i.e. LeuDH from various sources does indeed display a broad specificity for amino acids with non-polar side chains, but none of the LeuDH reported shows activity with phenylalanine. In the mutants produced here, L-phenylalanine remains as a substrate. This implies that there are other sequence differences between PheDH and LeuDH which make additional contributions to their substrate specificities.

Further studies will now be required to establish the extent to which the observed changes reflect altered K_m values as opposed to possible changes in maximum catalytic rate.

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