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Structural and mechanistic studies with pterin-4a-carbinolamine dehydratase from *Pseudomonas aeruginosa*. Three-dimensional structure and comparison with the human enzyme.

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Introduction

Mammalian pterin-4a-carbinolamine dehydratase (PCD/DCoH) is a bifunctional protein that catalyzes the dehydration of 4a-OH-tetrahydrobiopterin (4a-carbinolamine) to quinonoid dihydrobiopterin (1). This reaction is a part of the regeneration cycle of tetrahydrobiopterin, the essential cofactor of the aromatic amino acid hydroxylases. PCD/DCoH has a second function as a dimerization cofactor for the transcriptional protein hepatocyte nuclear factor 1-α (HNF1-α) (2). PCD from *Pseudomonas aeruginosa* (PCD/PhhB) also has been proposed to be a bifunctional protein, since it appears to regulate the expression of phenylalanine hydroxylase (PAH) (3). The genes for these proteins are placed next to each other and are controlled by the same operon. Human PCD/DCoH and PCD/PhhB have ~30 % identity and ~60 % similarity.

Recently, based on results obtained with several mutants (4) and on the three-dimensional structure of the complex of PCD/DCoH with a product analogue (5), the location of the active site of human PCD/DCoH has been identified and a reaction mechanism for the dehydration proposed. In the latter three histidines are suggested (4,5) to play a crucial role in the binding of substrate and dehydration catalysis: His61 and His62 form pairs with Asp88 and Glu57, respectively, which, can act as general base catalysts. His79 could play the role as general acid catalyst. In PCD/PhhB all proposed, important active site residues present in human PCD/DCoH are conserved. Here we present the crystal structure of PCD/PhhB and a biochemical comparison with human PCD/DCoH with respect to structure and dehydration

mechanism. Several of the putative active center residues of PCD/PhhB have been mutated and the catalytic properties of the mutants have been studied.

Materials and Methods

PCD/PhhB and the mutant enzymes were expressed in *E.coli* BL21(DE3) and purified to homogeneity by affinity chromatography over a Ni^{2+}-nitriloacetic acid-agarose column as detailed in (4). It should be noted that the enzyme contains no His-tag; it nevertheless binds to the column due to the presence of five closely located histidines. The lysis buffer (no imidazole) and the elution buffer (+ 10 mM imidazole) were sufficient to elute the enzyme. Details of the crystallization of PCD/PhhB and the X-ray crystal structure analysis will be published elsewhere (Ficner et al., in preparation). Enzymatic activity was measured in a so called direct assay using 6-methyl-4a-carbinolamine (6-MeCA) as substrate. The latter was prepared according to Bailey et al. (6) with minor modifications (7). A second, enzyme coupled assay (8) was also used. It contains the natural cofactor tetrahydrobiopterin as substrate, human recombinant PAH and all components required for cofactor regeneration. Binding constants of the product analogue quinonoid 6,6-dimethyl-7,8-dihydropterin (6,6-Me_2-PH_2) were determined as described earlier (7).

Results

Three-dimensional structure of PCD/PhhB

The structure was refined at a resolution of 1.75 Å. The enzyme crystallizes as a homodimer, which appears to be the functional form also in solution. One monomer consists of three α-helices which are packed against a four-stranded anti-parallel β-sheet. The dimer is built by interaction of the α-helices a2 and the β-sheets b3 of two monomers. In Fig. 1 the dimer of PCD/PhhB (right hand side) and a dimer of human PCD/DCoH (left), which normally build a tetramer, are shown side by side for comparison. The PCD/PhhB molecule is slightly larger since it contains additional 11 N-terminal and 3 C-terminal residues. Strikingly, the bend of the saddle like shaped dimer of PCD/PhhB is larger compared to that of PCD/DCoH. The distance of the Cα atoms at the tip of the loops is 18 Å in the PCD/PhhB dimer compared to 28 Å for that of PCD/DCoH. In both enzymes there is one active site per monomer and in PCD/PhhB all residues which have been proposed to be catalytically important are conserved. His73 and His74 form classical pairs with Asp100 and Glu69, respectively, while the side chain of His91 is completely solvent exposed. The two salt bridges of PCD/DCoH that cover the active site cleft (Arg30-Glu64 and Glu80-Arg87) (4), however, are not conserved in PCD/PhhB. Arg30

and Glu64 are exchanged to Ile42 and Gly75, respectively, Glu80 and Arg87 are replaced by some of the additional N-terminal residues.

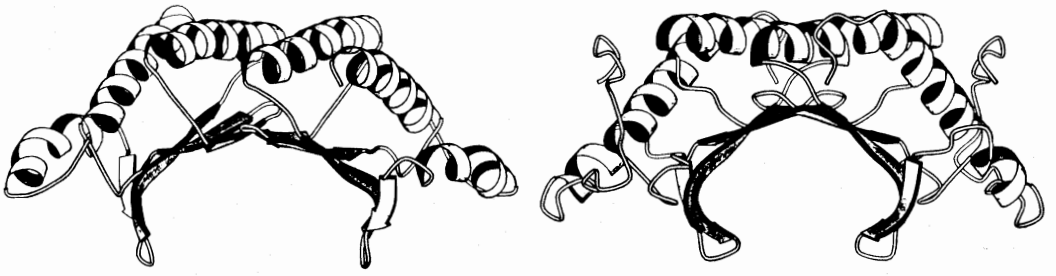

Figure 1: Ribbon rendering of the dimers of PCD/PhhB (left) and PCD/DCoH (right).

Catalytic properties of PCD/PhhB and of some mutants

The enzymatic activity of PCD/PhhB observed in the direct assay using the synthetic substrate 6-MeCA is ~40 % of that found with human PCD/DCoH (Table 1), whereas in the enzyme coupled assay it has approximately the same value (90 %) (4). Also the K_m for 6-MeCA (20 µM) and binding of the product 6,6-Me_2-PH_2 (K_d = 2 µM) are comparable to the values obtained with human PCD/DCoH (25 µM, 0.9 µM). From the pH dependence of the activities using 6-MeCA as substrate a pK ~8.4 was estimated for PCD/PhhB which does not vary substantially with the mutants (Table 1). H74A and W81A are the only inactive mutants found. The H73A mutant shows ~75 % activity compared to wt-PCD/PhhB and a 2-fold higher K_m. In human PCD/DCoH the corresponding histidine appears to take part in substrate binding since the K_m is about 10-fold higher compared to wt-enzyme. His73 and His74 also both have been mutated to alanine, but the correct nucleotide sequence of the corresponding plasmid still has to be confirmed. In PCD/PhhB His73 and His74 are in hydrogen bond contact with Asp100 and Glu69, respectively. An exchange of these amino acids, which leads to an inactive (E57A) and to an insoluble mutant (D88N) with human PCD/DCoH, has somewhat different effects in PCD/PhhB. The E69A mutant exhibits ~75 % activity and the D100N mutant has little activity in the direct assay compared to wt-PCD/PhhB. Mutation of His91, however, has the same effects as in human PCD. A residue (Asp60) which appears to interact with the side chain of the substrate in human PCD/DCoH (5) is a glycine in PCD/PhhB and its mutation to Asp (G72D) lowers the activity (Table 1). On the other hand, in the direct assay using 6-propyl-CA as substrate the activity of this mutant was restored at 100 % (not shown).

Table 1: Catalytic Properties and Binding Constants of quinonoid 6,6-Me_2-PH_2 for PCD/PhhB, hwt-PCD/DCoH and Mutants

The direct assay was performed in 10 mM Tris pH 8.5 at 10 °C. V_{max} is expressed as nmol substrate dehydrated per nmol enzyme subunit per s. The V_{max} value of hwt-PCD/DCoH is taken as 100 %. For PCD/PhhB mutants activities are compared to both wt-enzymes (comparison with wt-PCD/PhhB in parenthesis). pK-Values were estimated in the presence of a constant electrolyte concentration of 0.1 M KCl.

		Activity, direct assay			relative activity, coupled assay	K_d for binding of product
Enzyme	K_m (μM)	V_{max} (nmol/sec)	(%)	pK	(%)	(μM)
wt-PCD/PhhB	20	3.5	40 (100)	8.4	90	2
E69A	25	1.3	15 (40)	8.4	30	6
D100N			< 10	8.3	0	50
H73A	45	2.6	30 (75)	8.5	65	1
H74A		0	0		0	> 170
H91A	60	1.4	15 (40)	7.9	65	10
G72D	20	2.7	30 (75)	8.3	65	1
W81A		0	0		0	> 110
hwt-PCD/DCoH	25	8.6	100	8.2	100	0.9
E57A		0	0		0	> 165
H61A	220	1	12	8.0	0-2	> 55
H62A	75	0.9	10	7.15	16	> 65
H61A,H62A		0	0		0-2	> 140
H79A	10	2.2	25	7.8	40	5
Y69F	8	3.5	40	8.3	80	4.4
D60A	18	2.7	30	8.1	90	1.5

Discussion

The homodimeric enzyme PCD/PhhB shows a high similarity to the dimer of PCD/DCoH, although the amino acid sequence is only ~30 % identical. Strikingly, all active center residues considered to be important are conserved in PCD/PhhB. These structural features support the assumption of a very similar binding mode of substrate and of the same dehydration mechanism for the two proteins. This is fully consistent with the properties of the mutants of PCD/PhhB. On the other hand, different roles of specific amino acids or groups in PCD/PhhB are also apparent. Mutation of Glu69, which forms a pair with His74, to Ala reduced the activity to ~40 %. It is conceivable that in the mutant His74 is sufficiently basic to act as a general base. The important role of this group is evidenced by the lack of activity of the H74A mutant. H73A exhibits considerable activity, and in this case it is possible that a water molecule replaces His73 in the pair with Asp100. His91 is completely exposed to solvent in PCD/PhhB and should have unrestricted mobility. It therefore could act as an acid catalyst, a role which was proposed for the corresponding His79 in PCD/DCoH (4). After exchange of these His to Ala similar effects on the activities were obtained with both enzymes. H91A is the only PCD/PhhB mutant for which a higher pK compared to wt-enzyme was found. This is in agreement with the role of His91 as a residue taking part in catalysis. In contrast to Tyr69 which lies in front of the active site of PCD/DCoH the corresponding Trp81 in PCD/PhhB appears to interact either with the side chain of the substrate or affects binding in a different mode, since the W81A mutant is completely inactive and binding of quinonoid 6,6-Me_2-PH_2 is weak. Further data which support the same binding mode of substrate to PCD/PhhB and PCD/DCoH are the similar K_m's of the wt-enzymes determined in the direct assay and essentially the same binding constants for binding of the quinonoid product. The not conserved Asp60 of PCD/DCoH, which interacts with the side chain of the substrate (5), leads to a more active mutant when assayed with 6-propyl-CA (direct assay). This is consistent with the similar direction of the side chain of bound substrate in PCD/PhhB and PCD/DCoH. In conclusion, the same reaction mechanism and substrate binding mode can be assumed for bacterial PCD/PhhB as was proposed for mammalian PCD/DCoH and a related function of both proteins can be inferred. His74 in the Glu69-pair appears to be the most important residue in dehydration catalysis. It might act as general base, while the role of the general acid, which has been postulated for His79 in PCD/DCoH in (4) can be exerted by His91 in PCD/PhhB. It is thus likely that in the enzymatic catalysis of both PCD´s two -COOH-His pairs operate concertedly. Residues involved in substrate binding/recognition, on the other hand, appear to vary between the two proteins.

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