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The PHBH fold: Not only flavoenzymes

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Abstract

p-Hydroxybenzoate hydroxylase, D-amino acid oxidase, cholesterol oxidase and glucose oxidase form a family of structurally related flavoenzymes. Comparison of their three-dimensional structures reveal how the same FAD-binding scaffold has been employed to implement diverse active-site architectures, suited for different types of catalytic reactions. The substrate binding mode differs in each of these enzymes, with the catalytically relevant residues not located on homologous positions. A common feature is provided by the ability of these enzyme to bury their substrates beneath the protein surface. In D-amino acid oxidase and cholesterol oxidase, a loop forms a 'lid' controlling the active site accessibility, whereas in *p*-hydroxybenzoate hydroxylase is the flavin itself, which swings out to allow substrate binding. The crystallographic analysis has revealed that the GTP-dissociation inhibitor of RAB GTPases has a folding topology remarkably similar to *p*-hydroxybenzoate hydroxylase. This finding highlights the versatile nature of this folding topology, which in addition to flavin-dependent catalysis, is suited for diverse functions, such as the regulation of GTPases. © 1998 Elsevier Science B.V.

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In 1979, Wierenga et al. [1] reported the three-dimensional structure of the flavoenzyme *p*-hydroxybenzoate hydroxylase (PHBH) from the soil bacterium *Pseudomonas fluorescens*. PHBH employs a molecule of non-covalently bound flavin-adenine dinucleotide (FAD) to catalyse the conversion of *p*-hydroxybenzoate to 3,4-dihydroxybenzoate. The crystallographic analysis [2,3] revealed that the 394 amino acids of PHBH fold into a two domain structure, characterised by an FAD-binding domain, which provides the cofactor binding site, and an interface domain, which forms the contact region between the two subunits of the dimeric enzyme (Fig. 1a). More recently, other two flavin-dependent bacterial enzymes, cholesterol oxidase (COX) from *Brevibacterium sterolicum* [5] and glucose oxidase (GAL) from *Aspergillus niger* [6], have been shown to possess a three-dimensional structure resembling PHBH. Furthermore, such a 'PHBH fold' has been detected also in the mammalian D-amino acid oxidase (DAAO) [7,8], which is considered the paradigm of flavin-dependent oxidases due to the wealth of studies performed on this protein. COX, GAL and

Abbreviations: *p*-hydroxybenzoate hydroxylase, PHBH; Cholesterol oxidase, COX; Glucose oxidase, GAL; D-amino acid oxidase, DAAO; GDP-dissociation inhibitor, GDI; Flavin-adenine dinucleotide, FAD

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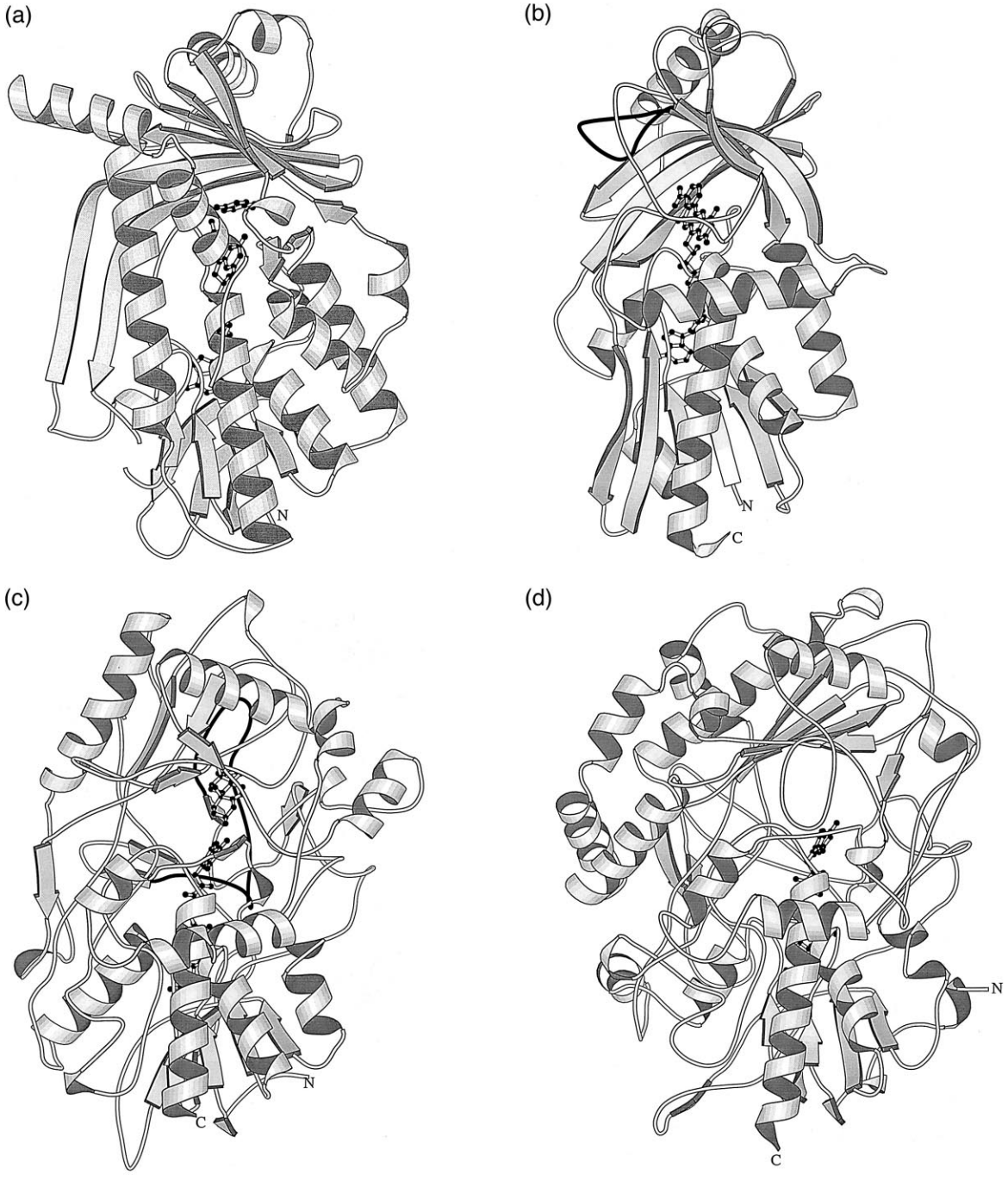

Fig. 1.

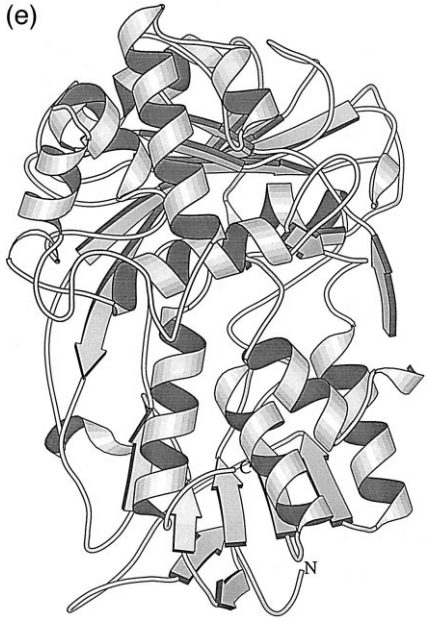

Fig. 1.(*continued*) Ribbon representation [4] of the subunit of: (a) PHBH in complex with *p*-hydroxybenzoate, (b) DAAO in complex with benzoate, (c) COX in complex with dihydroisoandrosterone, (d) GAL, and (e) GDI. In this orientation, the upper and lower portions of the proteins correspond to the interface and FAD-binding domains, respectively. The letters 'N' and 'C' indicate the N- and C-terminus of the proteins. The 'active site lid', which, in COX and DAAO, controls the active site accessibility is in black. Coordinates were taken from the Protein Data Bank (PDB entry codes: 1PBE, 1KIF, 1COY, 1GAL, 1GND).

DAAO display a structure which is topologically identical to PHBH, even though there is virtually no sequence identity (below 20%).

Similarity between the three-dimensional structure of proteins, which are dissimilar in their primary sequence, is not an unusual observation in structural biology. It is thought that only a limited number ($\sim$1000) of folding topologies exists in nature [9], so that the occurrence of proteins with similar three-dimensional structure and dissimilar amino acid sequence is becoming increasingly frequent. However, the family of PHBH related enzymes is noticeable in that its members share similar overall structure and essentially identical FAD binding site but display different catalytic centres, so that the catalytic residues of these enzymes are not positioned on homologous regions of their structures. To add spice to the story, Schalk et al. [10] have demonstrated that the PHBH fold is not restricted to flavoenzymes. In particular, a GDP-dissociation inhibitor (GDI) specific for RAB GTPases displays a folding topology very similar to that of PHBH. Remarkably, GDI is not an enzyme nor is known to have a flavin cofactor binding site [11], underlining the versatile nature of the PHBH fold, which allows the implementation of

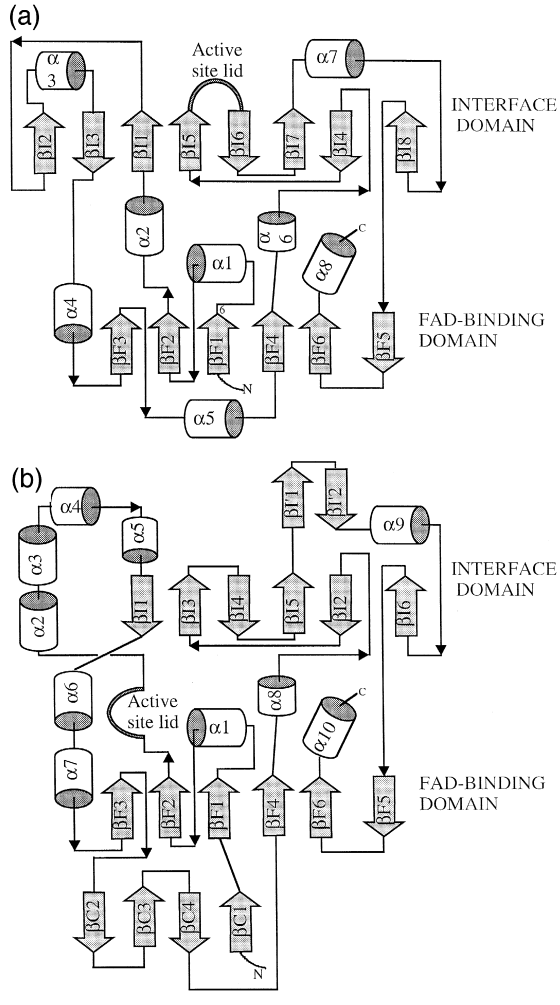

Fig. 2. Topology diagram of the DAAO (a) and COX (b) subunits. α-Helices are labelled by consecutive numbers. β-Strands belonging to the central β-sheets of the FAD-binding and interface domains are labelled by the letters F and I, respectively. The folding topology of PHBH and GDI is similar to that of DAAO, whereas the topology of GAL is closely related to that of COX (see text).

highly diverse functionalities, such as flavin-dependent catalysis and GPTase binding.

1. The PHBH fold

Among the PHBH related proteins, DAAO is the smallest enzyme, since its subunit consists of only 347 amino acids [12]. For this reason, the DAAO three-dimensional structure was chosen as the reference model to carry out the comparison with the other members of the family. DAAO is a widely distributed eukaryotic enzyme, which catalyses the oxidation of several of D-amino acids to the corresponding α-keto acids. DAAO was the second flavoprotein to be uncovered and is one of the most thoroughly investigated flavin-dependent enzymes [12]. The crystallographic analysis has revealed [7,8] that the molecule is comprised of two domains (Fig. 1b and Fig. 2a): the FAD-binding domain consisting of a six stranded β-sheet sandwiched between five α-helices and the interface domain characterised by a eight-stranded β-sheet flanked by three α-helices.

The structure of PHBH (394 residues; Fig. 1a) [3] is clearly similar to that of DAAO. Superposition by means of the program DALI [13] indicates a root-mean-square deviation of 3.5 Å for 249 α-carbons (12% sequence identity for the residues used in the superposition). With respect to DAAO, PHBH mainly differs for two features: (i) the replacement of helix α5 of DAAO (Fig. 2a) with a three-stranded β-sheet (the so-called β-meander) [14], and (ii) the presence of two additional helices at the protein C-terminus.

COX catalyses the oxidation and isomerisation of 3β-hydroxy steroids [15]. The enzyme consists of 506 amino acids (Fig. 1c), being about 150 residues bigger than DAAO. Optimal superposition of the COX and DAAO three-dimensional structures results in a root-mean-square deviation of 4.4 Å for 223 Cα pairs (15% sequence identity). The comparison reveals that COX and DAAO have similar FAD-binding domains but differ in their interface domains. Particularly, the central β-sheet of the COX interface domain consists of six rather than eight strands (Fig. 2b), with one strand (βI1) running in the opposite direction with respect to the homologous strand of DAAO (Fig. 2a). Moreover, the interface domain of COX encompasses three additional helices (α2, α3, α4) and an additional two stranded β-sheet (βI′1 and βI′2). These features characterise also the structure of GAL (583 residues; Fig. 1d), a bacterial enzyme which oxidises β-D-glucose to δ-gluconolactone [6]. In fact, the structure of GAL closely resembles that of COX, as indicated by a root-mean-square deviation between the two enzymes of 1.8 Å for 313 Cα pairs. GAL differs from COX only for the presence of three additional α-helices, inserted between strand βI3 and βI4 (see Fig. 2b).

GDI is not a flavoenzyme, but its three-dimensional structure [10] (Fig. 1e) is remarkably similar to that of DAAO. Upon superposition, a root-mean-square deviation of 3.8 Å for 243 Cα atoms (10% sequence identity) is calculated. The only conspicuous variation is provided by an additional helical domain, consisting of about 100 residues, which in GDI are inserted in the loop homologous to the polypeptide segment linking βI3 and α4 in DAAO (Fig. 2a). Such a strong similarity makes it very likely that GDI is evolutionarily related to the family of PHBH-related flavoenzymes.

2. Similar folds but diverse catalytic sites

The main features of FAD binding are essentially identical in all PHBH related enzymes. The extended ADP-ribityl moiety is embedded within the FAD-binding domain, whereas the isoalloxazine ring is located at the interface between the two domains forming each subunit (Fig. 1a–d). Particularly, the C-terminal helix of the FAD-binding domain (α8 in DAAO, α10 in COX; Fig. 1b,c and Fig. 2a,b) points with its N-terminus toward the pyrimidine ring of the flavin group. The interaction with the positive charge associated to the helix dipole is crucial for the modulation of the cofactor redox properties and for the stabilisation of the anionic form of the reduced FAD [16].

Adjacent to the flavin ring, is located the substrate binding site. The nature of the catalytic residues is revealed by the structures of the complexes of DAAO with the inhibitor benzoate [7,17], of COX with dihydroisoandrosterone [15] and of PHBH with the substrate and product of the reaction [3,18]. It is beyond the scope of this review, to analyse in detail

these structures and their implications for the catalytic mechanism. However, it is worth noticing that the binding mode of the active site ligands clearly changes in each enzyme. In DAAO, the benzoate inhibitor is parallel to the flavin ring at a distance of 3.5 Å from the flavin *re* side (Fig. 3). This arrangement supports a mechanism, in which D-amino acid oxidation occurs via a direct hydride transfer from the substrate Cα to the flavin N5 [17]. An hydride transfer step is thought to characterise also the COX reaction. However, in this enzyme, the bulky steroid substrate is positioned above the flavin, such that the C3 carbon atom, which is the site of enzymatic attack, points towards the flavin N5 (Fig. 3). In PHBH, we observe yet another active site geometry: the *p*-hydroxybenzoate substrate rests almost perpendicular to the flavin, bringing the *ortho* carbon of the benzoate ring in the proper position for hydroxylation by the flavin (Fig. 3). The hydroxylation reaction is known to involve a flavin-peroxide adduct, formed after FAD reduction by NADPH (see Ref. [19]). It is remarkable that, after more than two decades of research on PHBH, its NADPH binding site is still unknown [2].

Given the dissimilar substrate binding modes, it is not surprising that the residues crucial for binding and catalysis (Tyr224, Tyr228, Arg283 in DAAO; Tyr201, Arg214, Tyr222, Tyr385 in PHBH; Glu361, His447, Asn485 in COX) are not located on homologous positions. This observation emphasises the versatility of the PHBH fold, which has allowed the development of dissimilar active site architectures in the framework of the same protein scaffold.

3. Gates to control active accessibility

Despite the differences in the substrate binding sites, the complexes of DAAO, COX and PHBH with their respective active site ligands reveal a common feature: in the three proteins, the substrate is buried within the enzyme in an internal cavity. Substrate burying within the protein interior is a recurrent feature in flavin-dependent oxidases and oxidoreductases (see Ref. [20]). Sealing the substrates from the solvent is thought to effect catalysis by protecting labile reaction intermediates [2,17] and by enhancing the strength of the polar interactions, instrumental to substrate activation.

A fascinating observation concerns the mechanism developed by these enzymes to control the active site accessibility. In DAAO, the combination of crystallographic and limited proteolysis studies [17,21] have revealed that loop connecting strands βI5 and βI6 (residues 216–228; Fig. 1b and 2a) is able to switch from a 'closed' conformation, which blocks the access to the active site, to an 'open' structure, which allows substrate binding and product

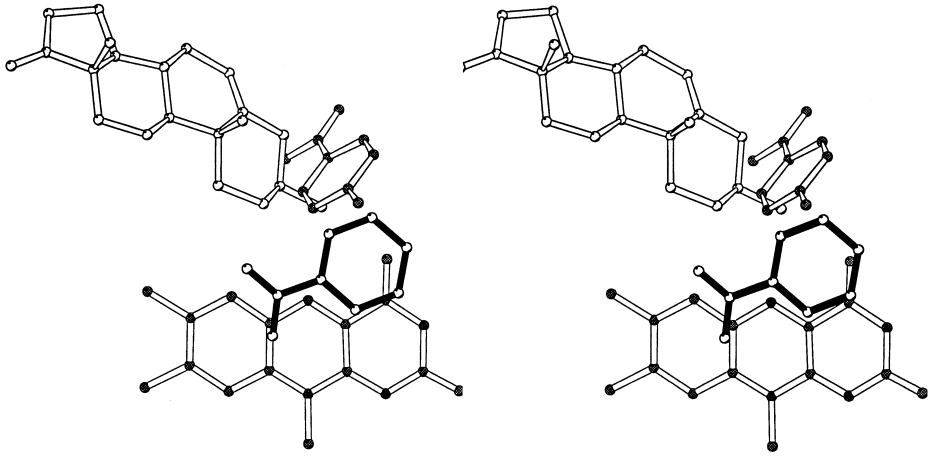

Fig. 3. Comparison between the substrate binding modes in PHBH, DAAO and COX. The stereo picture was generated by superimposing the isoalloxazine ring of the three enzymes. The flavin ring and the *p*-hydroxybenzoate substrate of PHBH are drawn with filled atoms and open bonds, benzoate of DAAO with open atoms and filled bonds, dihydroisoandrosterone of COX with open atoms and open bonds.

release. In COX [15], the crystallographic data indicate that loop 70–90 (linking βF2 to α2; Fig. 1a and 2b) performs a similar function. In the case of PHBH, there is no such 'active site lid' but it is the flavin itself, which acts as the gate. This hypothesis has been suggested on the basis of the complex between PHBH and 2,4-dihydroxybenzoate [22–24]. This structure has revealed the ability of the flavin to adopt, in addition to the standard 'in' position, an 'out' conformation, involved in substrate binding and/or product release. In this context, the native structure of GAL is somewhat exceptional in that its active centre appears to be fully solvent accessible [6]. However, it cannot be ruled out that upon binding of a substrate the accessibility of the active centre may change.

4. The evolutionary history of GDI

The finding that GDI has three-dimensional structure similar to that of the PHBH related flavoenzymes has led to the speculation that GDI may have the ability to bind a flavin cofactor [11]. However, the protein region which, by similarity with PHBH, should form the FAD binding site is, at least partly, filled by protein residues. Therefore, substantial rearrangements would be required in order to bind a flavin compound. Furthermore, soaking GDI crystals in FAD did not result in any additional density, which could be associated to a bound cofactor [10].

The strong structural similarity raises the question of the type of evolutionary relationship of GDI to the other members of the PHBH family. It is possible that GDI has originated from a flavoenzyme, which, in the course of evolution, has developed new biological functions, gradually loosing those directly associated to the flavin cofactor. In this context, it worth noticing that several flavoproteins are known, in which the cofactor has no functional role [25]. Alternatively, the family of PHBH related proteins may have evolved from an ancestor, which, originally, was not a flavoenzyme and from which may have derived proteins with diverse activities, ranging from flavin-dependent catalysis to GTPase binding. If this is the case, it is likely that more proteins will be uncovered, having PHBH topology but lacking a FAD-binding site.

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