Archives of Biochemistry and Biophysics 493 (2010) 37–52

Contents lists available at ScienceDirect

Archives of Biochemistry and Biophysics

journal homepage: www.elsevier.com/locate/yabbi

Flavin-containing heme enzymes

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ARTICLE INFO

Article history:
Received 22 September 2009
and in revised form 13 October 2009
Available online 19 October 2009

Keywords:
Flavin
Heme
Flavocytochrome
Mechanism
Structure
Electron transfer
Dehydrogenase
Monooxygenase
Dioxygenase

ABSTRACT

There are many examples of oxidative enzymes containing both flavin and heme prosthetic groups that carry out the oxidation of their substrate. For the purpose of this article we have chosen five systems. Two of these, the L-lactate dehydrogenase flavocytochrome b_2 and cellobiose dehydrogenase, carry out the catalytic chemistry at the flavin group. In contrast, the remaining three require activation of dioxygen at the heme group in order to accomplish substrate oxidation, these being flavohemoglobin, a nitric oxide dioxygenase, and the mono-oxygenases nitric oxide synthase and flavocytochrome P450 BM3, which functions as a fatty acid hydroxylase. In the light of recent advances we will describe the structures of these enzymes, some of which share significant homology. We will also discuss their diverse and sometimes controversial catalytic mechanisms, and consider electron transfer processes between the redox cofactors in order to provide an overview of this fascinating set of enzymes.

Introduction

Oxidative flavin-containing heme enzymes may be considered as a loose grouping of proteins that catalyse the dehydrogenation, mono-oxygenation or dioxygenation of substrates. There are several examples of these in the literature and each type of reaction catalysed will be discussed here in terms of the structure and mechanism of the enzyme(s) involved. Enzymes which contain both flavin and heme as cofactors are generally termed flavocytochromes. However, this description encompasses not only enzymes which consist of a single polypeptide, but also multisubunit enzyme complexes such as flavocytochrome b_{558}, the phagocyte NADPH (nicotinamide adenine dinucleotide phosphate) oxidase [1], and flavocytochrome *c* sulfide dehydrogenase from *Allochromatium vinosum* [2]. For the purposes of this review we will limit discussion to enzymes which contain heme and flavin cofactors bound within a single polypeptide. More specifically we will cover the dehydrogenases flavocytochrome b_2 and cellobiose dehydrogenase, flavohemoglobin, which has nitric oxide dioxygenase activity, and the mono-oxygenases NOS (nitric oxide synthase) and flavocytochrome P450 BM3. While many of these enzymes have been previously reviewed in depth, this article aims to concisely describe their structures, mechanisms and electron transfer processes in light of recent advances.

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doi:10.1016/j.abb.2009.10.005

Flavocytochrome b_2

Physiological role

Flavocytochromes b_2 are 2-hydroxyacid dehydrogenases located in the inter-membrane space of yeast mitochondria, where they couple substrate oxidation to cytochrome *c* reduction. Examples of flavocytochromes b_2 include L-lactate dehydrogenases from *Saccharomyces cerevisiae* and *Hansenula anomala* [3] and L-mandelate dehydrogenase from *Rhodotorula graminis* [4]. Of these the *S. cerevisiae* enzyme is by far the most studied, and will therefore be the one referred to here.

As a respiratory enzyme, flavocytochrome b_2 production is induced by the presence of oxygen and L-lactate. The pyruvate produced as a result of L-lactate oxidation is utilised by the Krebs cycle, but flavocytochrome b_2 also forms part of a short respiratory electron transport chain which results in one ATP (adenosine triphosphate) molecule being produced for every L-lactate consumed.

Structure

The crystal structure of flavocytochrome b_2 was first published in 1990 to a resolution of 2.4 Å (PDB (protein data bank) ID 1FCB [5]), but since then a total of 10 models (of full-length enzyme) have been deposited to the Protein Data Bank (www.pdb.org). The enzyme is homotetrameric with subunit molecular mass of 57.5 kDa. Each protomer consists of two functionally-distinct

domains. The N-terminal cytochrome domain consists of the first 100 amino acids and binds the *b*-type heme group, while the larger C-terminal flavodehydrogenase domain (~400 residues) binds FMN (flavin mononucleotide). The two domains are linked by a short 'hinge' peptide [6] and at the C-terminus there is a 'tail' which is intimately involved in tetramer integrity. The subunit structure of flavocytochrome b_2 is shown in Fig. 1. The importance of the other published structures will be discussed in the relevant sections below.

The mechanism of substrate oxidation

The oxidation of L-lactate to pyruvate is accompanied by the reduction of FMN to the hydroquinone form. The precise mechanism by which this occurs has been discussed and reviewed extensively [7–10], and has been proposed to occur *via* either a carbanion intermediate or *via* direct hydride transfer from the substrate to the flavin (Fig. 2). Early experiments on the related enzymes D-amino acid oxidase [11,12], lactate oxidase [13] and flavocytochrome b_2 itself [14,15] were suggestive of a carbanion intermediate in the catalytic cycle. In order for such a species to be formed during catalysis there is a requirement for deprotonation of the substrate α-carbon by an active site base [7]. This would be followed by the transfer of the equivalent of two electrons to the flavin, perhaps with the formation of a covalent adduct between the carbanion and flavin N5 [9]. However, with the advent of the crystal structure of D-amino acid oxidase it has been shown that there is no appropriately-positioned active site base to effect substrate deprotonation [16,17]. In light of this finding it would appear that the afore-mentioned experiments, which were considered to be diagnostic of carbanion formation during catalysis, must be called into question. This doubt raised over formation of a carbanion in D-amino acid oxidase is compounded by work carried out on L-lactate oxidase. Yorita et al. [18] studied the activity of this enzyme with a number of *para*-substituted L-mandelates, and found that the mechanism of the enzyme involved the development of little charge in the transition state. Clearly this is not consistent with the formation of a carbanion intermediate, but would be compatible with a hydride transfer or other synchronous mechanism wherein any developing charge is simultaneously neutralised by other events.

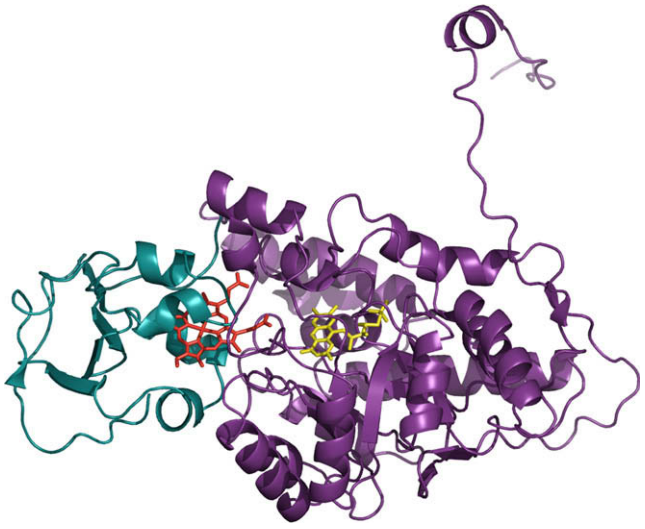

Fig. 1. The subunit structure of flavocytochrome b_2 (PDB ID 1FCB [5]). The heme domain is shown in blue with the heme in red, and the FMN-binding domain is shown in purple with FMN in yellow. All crystal structure figures were generated using Pymol [176].

In the case of flavocytochrome b_2, and unlike D-amino acid oxidase, there is a residue (histidine 373) appropriately positioned to act as an active site base. However, while it is conceivable that this may act to deprotonate the substrate α-carbon to form a carbanion intermediate, it is equally likely that the substrate α-hydroxyl group would be deprotonated, with concomitant transfer of the α-hydrogen to N5 of FMN as a hydride (Fig. 2). The operation of a hydride transfer mechanism in flavocytochrome b_2 has been further supported by a series of recent publications. Much of this work has involved the Y254F mutant flavocytochrome b_2. This mutant enzyme has a V_{max} value some 28-fold lower than that of the wild-type enzyme ($13.2 \pm 0.4\ s^{-1}$ cf. $372 \pm 0.4\ s^{-1}$ [19]), and a slightly raised L-lactate K_m value (0.28 ± 0.02 mM cf. 0.16 ± 0.02 mM [19]), in line with previously reported data [20,21]. These data show that Tyr254, and specifically its hydroxyl group, is important for catalysis, with a role proposed for Tyr254 in hydrogen-bonding and stabilising the transition state [21]. In terms of the two mechanistic possibilities the role of Tyr254 would differ; in a carbanion mechanism Tyr254 would be expected to aid electron transfer from the carbanion to the flavin by deprotonating the substrate α-hydroxyl group, while in a hydride transfer mechanism the role of the residue would be in orienting the substrate correctly by hydrogen-bonding. However, the very minimal effects of the substitution of Tyr254 upon the pH profile of the enzyme [22] would indicate that the residue does not act as a base, in line with its proposed function in a hydride transfer mechanism. Sobrado and Fitzpatrick [22] carried out a set of kinetic isotope effect (KIE)[1] experiments on the Y254F mutant of flavocytochrome b_2. The results of these studies indicate that in the Y254F enzyme the cleavage of the α-CH and α-OH bonds occurs in a concerted manner, consistent with hydride transfer. However, while these bonds appear to be cleaved in a stepwise manner in the wild-type enzyme, this is explained by the formation of a deprotonated lactate alkoxide intermediate which is transiently stabilised by Tyr254 before it collapses as it transfers the α-H as a hydride to the flavin.

Further support for the operation of a hydride transfer mechanism in flavocytochrome b_2 has come from recent structural insights. In the crystal structure of the wild-type enzyme [5] pyruvate is observed at the active site where the carboxylate moiety interacts with Arg376 and Tyr143, the α-keto oxygen hydrogen bonds with His373 and Tyr254, and the pyruvate methyl group is in van der Waals contact with the side chains of Ala198 and Leu230. The stereochemistry of L-lactate dictates that for operation of either a carbanion or hydride transfer mechanism a different substrate binding mode is necessary, in terms of whether the α-CH or α-OH atom is best positioned for abstraction by His373, the proposed active site base. However, although the product binding mode gives no definitive indication as to the structure of the Michaelis complex, structural evidence obtained for mutant forms of the enzyme and for the related enzyme (*S*)-mandelate dehydrogenase is illuminating. In the case of (*S*)-mandelate dehydrogenase the structure of the enzyme has been obtained in complex with a substrate, (*S*)-2-hydroxyoctanoate, which is bound in the appropriate orientation for hydride transfer (PDB ID 2A85 [23]). Further to this, in an attempt to re-engineer the substrate selectivity of flavocytochrome b_2 towards longer chain 2-hydroxy acids and the aromatic substrate L-mandelate, Daff et al. [24] and Sinclair et al. [25] used mutant forms of flavocytochrome b_2 where Ala198 and/or

[1] *Abbreviations used:* KIE, kinetic isotope effect; CDH, cellobiose dehydrogenase; GMC, glucose–methanol–choline; CBQ, cellobiose quinone oxidoreductase; CBO, cellobiose oxidase; FNR, ferredoxin reductase; NOS, nitric oxide synthases; CaM, calmodulin; CPR, cytochrome P450 reductase; Hb, hemoglobin; Mb, myoglobin; trHbs, truncated hemoglobins; flavoHbs, flavohemoglobins.