Structural and kinetic analyses of the H121A mutant of cholesterol oxidase

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Cholesterol oxidase is a monomeric flavoenzyme that catalyses the oxidation of cholesterol to cholest-5-en-3-one followed by isomerization to cholest-4-en-3-one. The enzyme from Brevibacterium sterolicum contains the FAD cofactor covalently bound to His[12]. It was previously demonstrated that the H121A substitution results in a ≈ 100 mV decrease in the midpoint redox potential and a ≈ 40-fold decrease in turnover number compared to wild-type enzyme [Motteran, Pilone, Molla, Ghisla and Pollegioni (2001) Journal of Biological Chemistry 276, 18024–18030]. A detailed kinetic analysis of the H121A mutant enzyme shows that the decrease in turnover number is largely due to a corresponding decrease in the rate constant of flavin reduction, whilst the re-oxidation reaction is only marginally altered and the isomerization reaction is not affected by the substitution and precedes product dissociation. The X-ray structure of the mutant protein, determined to 1.7 Å resolution (1 Å ≡ 0.1 nm), reveals only minor changes in the overall fold of the protein, namely: two loops have slight movements and a tryptophan residue changes conformation by a rotation of 180° about χ1 compared to the native enzyme. Comparison of the isoalloxazine ring moiety of the FAD cofactor between the structures of the native and mutant proteins shows a change from a non-planar to a planar geometry (resulting in a more tetrahedral-like geometry for NS). This change is proposed to be a major factor contributing to the observed alteration in redox potential. Since a similar distortion of the flavin has not been observed in other covalent flavoproteins, it is proposed to represent a specific mode to facilitate flavin reduction in covalent cholesterol oxidase.

Key words: covalent flavin, flavoprotein, kinetics, protein structure, redox, structure–function relationships.
elucidate the function of covalent flavin linkage in BCO, we have previously performed a functional characterization of a mutant enzyme in which the substitution of His$^{121}$ (corresponding to His$^{69}$ in the mature form) with an alanine prevents formation of the histidyl-FAD bond [18]. The mutant enzyme retains catalytic activity, but with a turnover rate decreased $\approx$40-fold. Stabilization of the flavin semiquinone and binding of sulfate are markedly decreased, and this result correlates with a lower midpoint redox potential ($\approx$ 204 mV compared to $\approx$ 101 mV for wild-type BCO). An increase in midpoint redox potential and in catalytic activity was obtained by reconstitution of BCO apoprotein with the `high potential' flavin analogue 8-chloro-FAD. We thus concluded that the flavin $\alpha$-linkage to a (N1)histidine is a pivotal factor in the modulation of the redox properties of this CO to increase its oxidative power [18].

In order to further probe the function of His$^{121}$ in modulating redox activity for the covalent BCO we have pursued structural studies of the H121A mutant enzyme and performed a detailed investigation of its kinetic properties. The present study demonstrates that the covalent linkage between His$^{121}$ and FAD results in a distortion of the isoalloxazine ring moiety to more closely resemble that expected for the reduced enzyme.

**EXPERIMENTAL**

**Structure solution and refinement**

Wild-type and mutant BCO were a gift from Roche Molecular Biochemicals. Crystals were grown by microseeding in hanging drops over a precipitant solution composed of 10–12% (w/v) PEG 8000, 15% glycerol and 74 mM MnSO$_4$ in 100 mM sodium cacodylate buffer at pH 5.2. Equal volumes of protein at 8.7 mg/ml in 10 mM Hepes (pH 7.0) and precipitant solution were mixed for each drop. The drops were streaked with a cat whisker carrying microseeds [19]. Crystallization trays were incubated at 17°C. Rod-shaped crystals suitable for diffraction were obtained after 2 weeks; the largest crystals measured 0.5 mm $\times$ 0.1 mm $\times$ 0.1 mm.

Crystals used for data collection were transferred to a cryoprotectant solution containing the precipitant where the glycerol concentration was increased to 20%. Individual crystals were flash frozen as propane popsicles and stored in liquid nitrogen until they were used for diffraction experiments.

Diffraction data to 1.7 Å (1 Å $= 0.1$ nm) resolution were obtained from a single crystal on an ADSC CCD Quantum detector at Beamline 5.0.1 at the Advanced Light Source (Lawrence Berkeley Laboratories) and processed with the HKL2000 program suite [20]. The crystal was maintained at 100 K in a nitrogen gas cryostream during data collection. The complete dataset consisted of 182 frames of 1 degree each. The total exposure time was 4 h. Early in the data collection the region of the crystal exposed to the X-ray beam underwent a bleaching from its original yellow colour. This suggested that the enzyme was undergoing reduction in the X-ray beam, however the extent of reduction could not be monitored.

With the exception of the addition of glycerol to the precipitant solution, the crystallization conditions for the H121A mutant protein were similar to that of the wild-type enzyme, crystallized in the P2$_1$ space group [21]. Despite these similarities however, the mutant enzyme crystallized in the C2 space group, with cell dimensions $a = 140.6$ Å, $b = 85.9$ Å, $c = 78.8$ Å and $\beta = 112.5^\circ$. The asymmetric unit of this lattice could hold one or two molecules of a 62 kDa protein corresponding to a Matthews coefficient of 3.5 and 1.8 or a solvent content of 65% or 30% respectively. Both are within the ranges usually observed for globular proteins.

<table>
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<th>Table 1 Data processing and crystallographic refinement statistics</th>
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The structure was solved by molecular replacement using chain A of the wild-type BCO as the search model [10]. The FAD, ligands and water molecules were excluded and, for those side chains modelled in two conformations, only one was chosen. The rotation function was computed using data between 15 and 4 Å resolution with the program CNS [22]. The cross rotation search gave a strong signal, 5.3 times above the second peak in the rotation function. Translation search with this orientation again resulted in one significant signal, seven times the height of the second peak. This confirmed that only one protein molecule was present in the crystal asymmetric unit, in contrast to the wild-type P2$_1$ crystals, which contained two enzyme molecules in the asymmetric unit.

The solution from molecular replacement was used as the starting model for crystallographic refinement. Initially refinement was performed using data to 2.2 Å. FAD was included in the model based on the difference electron density maps. For subsequent refinement, the data was extended to 1.6 Å resolution. Molecular replacement and earlier stages of refinement were carried out using the CNS software suite [22] and modelling was performed with the program O [23]. During the later stages of refinement, the program REFMAC from the CCP4 program suite [24] was used and rebuilding performed with XFIT from the Xtalview package [25]. Side chains in alternate conformation were added and the current model contains 44 residues with alternate conformations. The current model contains 536 amino acid residues, one FAD molecule, 696 water molecules, 2 cadotte molecules, 3 glycerol molecules and two manganese ions. Table 1 gives the data processing and refinement statistics.

**Kinetic measurements**

Rapid kinetic measurements were performed as described previously [12,26] using a BioLogic SFM-300 stopped-flow instrument equipped with a 1-cm path length and interfaced to a J & M diode-array detector at 25°C. All of the kinetic experiments were performed in 50 mM sodium pyrophosphate, (pH 7.5), 1% Thesit and 1% propan-2-ol. Spectra were recorded from the time of mixing until completion of the reaction in the wavelength range 190–700 nm and with a frequency of 1 spectrum/ms. Anaerobiosis of substrate (in the 0.05–0.75 mM range) and enzyme (6–12 µM) solutions was obtained in tonometers by repeated cycles of evacuation and flushing with O$_2$-free argon. Oxygen was scrubbed
from the stopped-flow apparatus by incubating with a dithionite solution for 16 h followed by rinsing with anaerobic buffer. As a further control of absence of oxygen from reaction mixtures, some of the experiments were also performed in the presence of 100 mM glucose, 6–50 nM glucose oxidase and 0.7 μM catalase [27]. For re-oxidation experiments, enzymes were reduced with a 2-fold excess of cholesterol under anaerobic conditions and then reacted with buffer solutions equilibrated with various N₂/O₂ mixtures [12,27].

Rate constants were determined from fitting of traces of absorbance at 446 nm versus time that were extracted from the spectra versus time data sets, as well as by global analysis of the same data sets using Specfit/32 software (Spectrum Software Associates, Chapel Hill, NC, U.S.A.). The same program was used for simulations of the entire spectral traces based on a three-step kinetic model for the reductive half-reaction (see eqn 1 below). Secondary kinetic data were analysed by least squares curve fitting procedures and graphics were generated with KaleidaGraph software (Synergy Software, Reading, PA, U.S.A.). Rates and dissociation constants were estimated as described in [28].

For isomerization experiments, the reaction was followed by monitoring the production of cholest-5-en-3-one from cholest-5-en-3-one spectrophotometrically at 240 nm (Δε = 5.47 mM⁻¹ · cm⁻¹ as measured in the stopped-flow apparatus). For the reaction starting from the reduced form, the enzyme (12.5 nM) was reduced with a 400-fold excess of cholesterol (5 μM) under anaerobic conditions and then reacted in the stopped-flow apparatus with different concentrations of cholest-5-en-3-one (0.025–0.5 mM concentration range); the reaction was followed at 240 nm up to 120 s. The isomerization reaction of the oxidized form was studied similarly, but in the presence of oxygen and in the absence of cholesterol. All concentrations mentioned in these experiments are final ones, i.e., those after mixing.

RESULTS

Kinetic course of the reductive half-reaction

The kinetic mechanism of wild-type BCO with the substrate cholesterol has been studied previously by a combination of steady-state and pre-steady-state approaches [12], and was proposed to consist of a Ping Pong (binary complex) process. The steady-state kinetic behaviour of H121A BCO was studied analogously using the enzyme monitored turnover method and with cholesterol as substrate (see Table 2) [18]. The double-reciprocal (Lineweaver–Burk) plot of the turnover number as a function of substrate concentration for the H121A BCO also gave sets of parallel lines, consistent with a Ping Pong mechanism (results not shown). Enzyme monitored turnover experiments confirmed that the absence of the covalent flavin linkage in BCO induces a large decrease in the turnover number (Table 2) [18]. In the present study, the reductive half-reaction of H121A BCO was studied by reacting the oxidized form anaerobically with various concentrations of cholesterol (0.05–0.75 mM), such that pseudo-first-order conditions were maintained [conditions: 1 mM catalase, 10 μM PMS, 0.025–0.5 mM concentration range]; the rate constant kred was obtained from the dependence of kobs1 from [cholesterol] and k2 corresponds to kobs2 (see Figure 2 and Eqn 1). The rate constants k1 and k−1 are estimated from the simulation of the spectral time courses using Specfit/32. The re-oxidation parameters were obtained from the double reciprocal plot reported in Figure 2(b) inset according to [12].
Where error bars are not shown the data scatter is smaller than the size of the symbols. Inset: The spectra shown are those obtained by deconvolution with Specfit/32. Spectrum 1, oxidized enzyme(s); Spectrum 2, reduced enzyme–product intermediate complex; and Spectrum 3, free reduced enzyme. The residuals are the subtraction of the experimental data points at 446 nm from the traces obtained from fit or simulation procedures.

Figure 1 Time courses of the anaerobic reduction of wild-type (a) and H121A (b) BCO

Anaerobic solutions of enzymes (≈9 μM and ≈6.3 μM for wild-type and H121A BCOs respectively) and 0.7 mM cholesterol were mixed in the stopped-flow instrument, in the presence of 1% propan-2-ol/1% Thesit (pH 7.5) at 25°C. (1) represent the data points at 446 nm. Curve (−−−−−) is the fit for a single exponential decay; curve (−−) is the fit for a double exponential decay; curve (−) is the trace obtained from simulations using Specfit/32 software and based on the sequence of steps of Eqn 1, on known extinction coefficients for the oxidized and reduced enzyme forms (see insets) and on the rate constants reported in Table 2. Insets: The spectra shown are those obtained by deconvolution with Specfit/32. Spectrum 1, oxidized enzyme(s); Spectrum 2, reduced enzyme–product intermediate complex; and Spectrum 3, free reduced enzyme. The residuals are the subtraction of the experimental data points at 446 nm from the traces obtained from fit or simulation procedures.

Figure 2 Dependence of the rates of flavin reduction and re-oxidation for wild-type and mutant enzymes

(a) Dependence of the observed rates of flavin reduction for wild-type BCO from the cholesterol concentration. The observed rate constants are obtained from fits of the absorbance change at 446 nm using a double exponential algorithm as detailed in the text (conditions as detailed in the legend of Figure 1). The data points are: (●) the values of $k_{obs1}$, the rates corresponding to the transformation of species (1) into species (2) (inset of Figure 1a); (○) the values of $k_{obs2}$, the rates corresponding to the transformation of species (2) into species (3) (inset of Figure 1a). Where error bars are not shown the data scatter is smaller than the size of the symbols. (b) Dependence of the rates of flavin re-oxidation for wild-type (●) and H121A BCO (○) from the oxygen concentration. The observed rate constants were obtained from fits of traces reflecting the absorbance increase at 446 nm (the time courses are essentially monophasic). Where error bars are not shown the data scatter is smaller than the size of the symbols. Inset: double reciprocal plot of the same data as reported in the main graph.

(spectrum 3) that of free reduced enzyme. It should be stated that the second (slow) phase can be fitted equally well with a biexponential algorithm such as required for two reactions occurring in parallel, where the slow phase would belong for example to an enzyme species having substantially reduced activity. However, and in contrast to the observation, in such a case a dependence of the rate of $k_{obs2}$ from [substrate] might be expected. This lends support to the scheme of eqn (1), but clearly cannot exclude other kinetic alternatives. We attribute the failure to locate such a phase in previous experiments [12,18] to the superior quality of the data obtained in the present study with a diode-array photometer and to the corresponding global fitting analysis method.

In the case of the H121A BCO mutant, a fit of the experimental traces at 446 nm for the reductive half-reaction based on a biexponential term is required only at high cholesterol concentration (>0.5 mM) where the separation between the rates is relatively large. The maximal rate of flavin reduction estimated at high [cholesterol] is <0.5 s⁻¹, i.e. significantly lower than that observed for the wild-type enzyme. As with wild-type BCO (see Experimental section for details) the spectral courses at different cholesterol concentrations were simulated using Specfit/32 (global analysis) based on the set-up of eqn (1). The trace shown in Figure 1(b) was obtained using the rate constants reported in Table 2; it does not show relevant trends in the residuals, indicating a reasonable correspondence of model and experimental data. The simulation results also show that the decrease in the rate of enzyme reduction ($k_2$) for the H121A mutant enzyme compared to wild-type BCO is accompanied by a decrease in rate constants for substrate binding/dissociation ($k_1$ and $k_{−1}$) and in the rate constant for the second phase of the reductive half-reaction ($k_{−2}$).

Kinetic course of the oxidative half-reaction

The re-oxidation reaction of BCO was studied in the stopped-flow spectrophotometer by following the appearance, in the spectrum, of the oxidized enzyme when the uncomplexed reduced enzyme was treated with varying oxygen concentrations in buffer solutions containing 1% Thesit and 1% propan-2-ol. With up to 21% oxygen saturation, the time courses of flavin re-oxidation are similar for wild-type and H121A BCO, whereas at higher oxygen saturation the reaction is slower for the mutant. For both BCO forms, the rate of re-oxidation shows saturation with increasing oxygen concentration (Figure 2b), in agreement with the inferred presence of an intermediate [12]:

$$E\text{-}\text{FAD}_{\text{red}} \overset{k_{\text{ox}}}{\underset{k_{\text{ox}}}{\rightleftharpoons}} E\text{-}\text{FAD}_{\text{ox}} + O_2 \overset{k_4}{\underset{k_{4}^{-1}}{\rightleftharpoons}} E\text{-}\text{FAD}_{\text{ox}} \sim H_2O_2$$ (2)

The y-axis-intercept of the corresponding double reciprocal plot (inset of Figure 2b) yields $1/k_{\text{ox}}$ and the x-axis-intercept corresponds to $−k_4/k_{\text{ox}}$. These values are only slightly modified by the H121A substitution (Table 2).
**The isomerisation reaction**

The $\Delta 5\rightarrow \Delta 4$–5 isomerisation reaction that is represented by eqn 3:

![Diagram of the isomerisation reaction]

\[
5 \rightarrow 4
\]

(3)

can be followed directly by monitoring the absorbance changes at 240 nm that accompany the formation of cholest-4-en-3-one, the final product. With SCO this reaction is catalysed by both the oxidised and the reduced forms, although at a 2000-fold lower rate for the latter [12,28]. Firstly, and in order to assess whether reduced BCO catalyses the isomerisation reaction, wild-type and H121A enzymes (2 $\mu$M) were reduced anaerobically by the addition of a 25-fold excess of cholesterol. The spectral course of the isomerisation reaction was then followed in a diode array spectrophotometer for up to 30 min upon addition of 50 $\mu$M cholest-5-en-3-one. The extent of absorbance increase at 240 nm was similar for both BCO forms and the spectra collected during the reaction course confirm that the flavin remains in the reduced state during the entire process for both BCO forms. The kinetics of the isomerisation reaction was then studied using the stopped-flow instrument by mixing the oxidized or the reduced forms of wild-type and H121A BCOs with increasing concentrations of cholest-5-en-3-one under the conditions used for the reductive half-reaction (see above). The rates extracted from the 240 nm absorbance versus time traces are plotted in Figure 3 as a function of [cholest-5-en-3-one] (in the 0.025–0.5 mM range). The results summarized in Table 3 show that the rate of the isomerisation reaction is not affected by the presence of the H$^{121}$–flavin covalent bond and by the state of oxidation of the flavin itself. Comparison of the rate constants for the isomerisation reaction (Table 3) with those for the dehydrogenation of cholesterol (Table 2), shows that for H121A BCO the $\Delta 5\rightarrow \Delta 4$–5 rearrangement is not rate-limiting, whereas it is close to the rate of flavin reduction for the wild-type BCO at saturating substrate concentration [12,28].

**Crystallography**

In order to ascertain what structural effects the removal of the covalent linkage has on the enzyme, we undertook a crystallographic study of the mutant protein. Surprisingly, under identical crystallization conditions, the mutant enzyme crystallized in a different space group from that of the wild-type enzyme, suggesting some possible significant changes on the surface of the protein that affect the packing of the molecules in the crystal. Analysis of the structure revealed that the overall fold of the mutant form is identical to the native enzyme. The mutant protein crystallizes with one molecule in the asymmetric unit of the C2 cell whereas the native BCO crystallizes with two molecules in the asymmetric unit of the P2$_1$ cell. Comparison of the packing for the native and mutant structures shows that the non-crystallographic dimer of the native structure becomes a crystallographic dimer in the mutant structure. Identical crystal contacts, utilizing two manganese atoms and an arsenate molecule, are maintained in both crystal forms. A superposition of secondary structure elements between the native and the mutant protein resulted in an rmsd (root mean square deviation) of 0.4 Å. The region of the structure that lies on the side of the dimethylbenzene ring of the FAD makes the most significant movement (in particular the loops consisting of residues 116–128) and appears to open somewhat relative to the wild-type enzyme structure. This is assigned to the absence of the covalent linkage between His$^{121}$ and the FAD molecule. Other significant movements from the native position occur in loops 74–90 and 422–431 (Figures 4A and 4B).

Both the mutant protein crystals and the native crystals rapidly bleach upon exposure to X-rays most likely due to formation of the reduced flavin. Given that both forms of the enzyme undergo bleaching, a direct comparison of the structures is appropriate and relevant as they both reflect the reduced enzyme form. The electron density map at position 121 of the mutant enzyme structure clearly shows the absence of the histidine side-chain and the subsequent loss of a covalent linkage between the protein and C8 of the cofactor (Figure 5A). Furthermore, the electron density features for the FAD cofactor are very clearly delineated (Figure 5B). A comparison of the isosalloxazine ring system of the FAD cofactor for the native and mutant protein structures reveals significant differences (Figure 4C). In the native enzyme the isosalloxazine ring adopts a butterfly twist about the N5 and N10 atoms: in particular the dimethylbenzene ring is twisted significantly away from planarity. This twist is a consequence of the covalent linkage between the 8-methyl group of the dimethylbenzene ring and the ND1 atom of His$^{121}$; the electron density for the H121A mutant structure clearly reveals a planar isosalloxazine ring system (Figure 4C).

Other significant movements in the structure of the mutant enzyme are apparent in three loop regions situated near the dimethylbenzene ring system of the cofactor (77–86, 120–127 and 423–430) (Figure 4B). The loop containing residues 120–127 includes the mutated histidine residue. The largest movement in this loop is the position of a glycine residue directly following position 121, thus redirecting the main chain for the mutant protein structure.
This deviation from the native structure repositions the carbonyl oxygen atom of Ala$^{121}$ to make a hydrogen bond to O3 of the ribityl chain of FAD. In the native structure this main chain oxygen atom is rotated away from the ribityl chain and makes a hydrogen bond with a water molecule which in turn hydrogen bonds to the main chain of Ser$^{81}$. Thus the bound water in the native structure is displaced by the repositioned main chain, and the carbonyl oxygen atom of Ala$^{121}$ in the mutant protein structure rotates to hydrogen bond directly with the ribityl chain of the cofactor (Figure 6). This conformational change in the main chain also results in a repositioning of Cα of Gly$^{122}$ nearer to the C8M carbon of the dimethylbenzene ring of the cofactor (4.6 Å relative to 6.2 Å for the wild-type enzyme). Interestingly, Arg$^{117}$ of this region becomes sensitive to trypsin cleavage in the unfolding intermediate obtained at 2 M urea [30].

A second loop (residues 77–86) behind that of 120–127 also undergoes a conformational shift, placing the main chain region at the tip of the loop approximately 2 Å closer to the dimethylbenzene ring of the cofactor. In addition, the side chain of Trp$^{80}$ undergoes a rotation about $\chi_2$ of 180° compared to the native structure.
Structure and function of cholesterol oxidase H121A mutant enzyme

Figure 5  The 2Fo−Fc electron density map for the H121A mutant protein

The map, contoured at 1.5σ, shows density features for (a) the FAD cofactor and residue 121 and (b) a side view of the isalloxazine ring moiety.

Figure 6  Interactions in the region of residues 121 and 122

Stereo view showing the intramolecular interactions in the region of 121–122 for (a) the native enzyme and (b) the mutant BCO. Hydrogen bonds are depicted as green broken lines.

(Figure 4B). Thus although the indole ring is still positioned perpendicular to the C8M atom of the cofactor, the opposite side of the ring now faces the cofactor compared to the native enzyme. This rotation of the side chain fills up the volume occupied by the histidine residue in the native structure. The side chain of Trp80 is sandwiched between the methyl groups of the flavin cofactor and the side chain of Arg429. The reorientation of Trp80 is correlated with a reorientation of Arg429 such that the guanidinium group π stacks over the aromatic indole ring system. The alterations of the tertiary structure induced by the removal of the flavin-His121 covalent link are reflected by the spectral properties. Thus the tryptophan fluorescence emission maximum is shifted from 331 to 335 nm and increased in intensity in the mutant enzyme. The latter exhibits differences in the near-UV CD spectra such as a higher ellipticity at 285 nm and a lower one at 253 nm [30].

DISCUSSION

In an attempt to understand the factors that govern the redox chemistry of CO we undertook the present detailed kinetic and structural study of a mutant of BCO where the histidine residue
Since the catalytic mechanism proposed for BCO [12] is of the Ping Pong type this requires $k_3$ to be faster than $k_{cat}$, i.e. that release of the final product cholest-4-en-3-one from the reduced enzyme via $k_3$ is not rate-limiting. Consequently the oxidative half-reaction starts from the free reduced enzyme ($k_4$ in eqn 4 and eqn 2). Based on this we conclude that the main effect of the flavin covalent attachment to His$^{121}$ is on the redox potential of the flavin itself that in turn affects the rates of substrate dehydrogenation.

A crystallographic analysis of the mutant protein was carried out to a significantly high resolution to visualize structural details that may be associated with the differences in redox properties of the enzyme. As the mutant protein crystal was exposed to X-rays a rapid bleaching was observed. The identical phenomenon was observed for the native crystals when cryoprotected with glycerol. This photobleaching has been reported for ribonucleotide reductases, where the rate of reduction was highly dependent on the concentration of glycerol, an effective electron hole-trapping agent [31,32]. We have also observed a bleaching in a glycerol concentration dependent fashion for the non-covalent form of CO (P. Lario and A. Vrielink, unpublished work). As this bleaching most likely reflects the presence of the reduced enzyme, a comparison can be made between the native and H121A structures in the reduced enzyme form: a significant difference is observed in the planarity of the isoalloxazine ring system of the cofactor (Figure 4C).

A number of covalently linked flavoenzymes have been structurally characterized recently [10,33–36]. The factors that modulate redox activity and, particularly, the role that the covalent modification may play in the redox activity of the cofactor are a focus of study for this sub-class of enzymes. Of the covalent flavoenzymes where the reduction potentials are known [1,37–40], BCO has the lowest $E_m$. Comparisons of the redox potentials in the presence and absence of the covalent linkage have only been carried out for VAO and BCO [18,37]. In the case of VAO the midpoint redox potential is $+55$ mV for the native enzyme and $-65$ mV for a mutant enzyme where the covalent linkage has been removed (H422A), this corresponding to a decrease of around $110$ mV [37]. Structural analysis of this mutant enzyme shows no changes to the conformation of the flavin ring system. In the case of BCO, the difference of the $E_m$ between covalently and noncovalently bound FAD is of the order of $100$ mV ($-101$ mV for wild-type BCO compared with $-204$ mV for the H121A mutant enzyme). The differences in $E_m$ corresponding to approx. $100$ mV between the two enzymes are very similar and could therefore be attributed to the covalent linkage. It should be recalled that the substitution at position 8e with a histidine residue induces a change in the flavin $E_m$ of $\pm 50$ mV ($-209$ and $-154$ mV for FAD and 8-imidazolylriboflavin respectively) [41,42]. This effect is, however, pH dependent and reflects the state of ionization of the imidazole substituent. Since the latter can be influenced by the protein environment, it is difficult to differentiate between effects originating with the 8e-substitution and effects brought about by the protein environment, indeed these effects will be interconnected, at least to some extent. A quantitative interpretation of the $100$ mV difference in midpoint redox potential in terms of reaction rates (changes in free energy) is not straightforward. To our knowledge within flavoproteins, the most comprehensive treatment of the dependence of the rate(s) of flavin reduction from the redox potential of the flavin cofactor itself is from Yorita et al. [43]. Therein it is shown that with the enzyme lactate oxidase there is a linear dependence on $E_m$ of the flavin coenzyme, although upper limits/plateaus occur that probably correspond to rate control by diffusion. The slopes of the dependence(s) varies markedly depending on the substrate (e.g. $\Delta k/100$ mV $\approx 1000$ for L-lactate and $\approx 15$ for L-mandelate) [43]. No molecular explanation was put forward to rationalize this observation in molecular terms. However, it appears reasonable to assume that electronic effects will not necessarily be translated quantitatively into effects on...
rates. The extent of the effect might depend strongly on the geometry of the transition state(s) and on the mode(s) of orbital reorganization during the redox process(es). In conclusion the effect estimated in the present work appears to be intermediate to those reported by Yorita et al. [43] and might be interpreted assuming that the redox potential is an important contributor, albeit not the sole contributor, to the modulation of the rate of substrate dehydrogenation.

It is the sum of many effects exerted by the microenvironment around the FAD cofactor that modulates its redox potential. VAO has a number of interactions between the isoalloxazine ring system and the protein: a hydrogen bond between the O2 of FAD and the guanidino moiety of Arg60 and an interaction between N5 of the FAD and the carboxylate of Asp70. Mutation of Asp70 to a serine residue significantly decreases the midpoint redox potential of the enzyme [44]. In contrast, for BCO there are relatively few contacts between the isoalloxazine ring system and the protein. Only the pyrimidine N3, O2 and O4 make hydrogen bond contacts to main chain oxygen and nitrogen atoms; N5 has no hydrogen bond partner. This suggests that modulation of the redox potential is substantially different between VAO and BCO. In the latter, the finding of an Eo for the noncovalent flavin corresponding to that of free FAD suggests that the effect of the protein is a minor one. On the other hand, for BCO, the covalent linkage between His31 and the flavin could play a role in modulating the geometry of the isoalloxazine ring and in fixation of the flavin such that the chemistry involving the cofactor becomes more facile, i.e. in decreasing the activation energy needed for reduction of the flavin. Theoretical studies by Miller and Walsh [45] have shown that flavin reduction potentials are dependent on the conformation of the isoalloxazine ring; a planar conformation is energetically preferred for the oxidized species and a bent conformation is preferred for the reduced state. Our structural studies support these theoretical findings. The protein therefore acts to fine-tune the environment around the isoalloxazine moiety in order to optimize the orientation required for orbital overlap during hydride transfer (as made evident by the observed decrease in the rate of flavin reduction following the removal of the flavin covalent link). In the case of BCO, His31 facilitates maintaining the isoalloxazine ring system in a bent conformation more favorable for the reduced enzyme. The absence of this covalent linkage in the native structure, and the rearrangement of the protein environment around the benzene ring of FAD (movement of Trp98 and the loops around the benzene portion), results in the cofactor adopting a less favourable planar conformation in the reduced state. A difference in the flavin geometry for the native and mutant enzymes is not seen for other covalently linked flavoenzymes: it is probably due to the specific FAD-apoprotein interactions that finely modulate the redox properties in different flavoenzymes.

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