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Studies on the active centre of *Rhodotorula gracilis* D-amino acid oxidase and comparison with pig kidney enzyme

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D-Amino acid oxidase (EC 1.4.3.3) from *Rhodotorula gracilis* has been reconstituted with 8-chloro-, 8-mercapto-, 6-hydroxy-, 2-thio-, 5-deaza- and 1-deaza-FAD, and the properties of the resulting complexes have been studied and compared with those of the correspondingly modified pig kidney D-amino acid oxidases. Binding appears to be tight for most analogues, at least as tight as for native FAD ($\sim 10^{-8}$ M). 8-Mercapto- and 6-hydroxy-FAD bind in their *para*- and *ortho*-quinoid forms respectively to yeast D-amino acid oxidase, inferring the presence of a positive charge near the flavin $N_{(1)}$ position, as in the case of the mammalian enzyme. On the other hand, important differences in active-site microenvironment emerge: solvent accessibility to flavin position 8 is drastically restricted in yeast D-amino acid oxidase as indicated by the unreactivity of 8-chloro- and 8-mercapto-FAD enzyme with thiolates and alkylating agents. Significantly different microenvironments are also likely to occur around the flavin positions $N_{(1)}$-$C_{(2)}$ = 0, $N_{(3)}$-H and $N_{(5)}$. This is deduced from the differences in interaction of the two proteins with 1-deaza-FAD, 5-deaza-FAD and 2-thio-FAD and from the properties of the respective complexes. The same *re*-side flavin stereospecificity as shown by the mammalian enzyme was determined for the yeast enzyme using 8-hydroxy-5-deaza-FAD. Thus we can deduce the presence of a similar pattern of functional groups at the active centres of the two enzymes, while the fine tuning of specificity and regulation correlate with environmental differences at specific flavin loci.

INTRODUCTION

Since the early identification of D-amino acid oxidase (EC 1.4.3.3) activity in pig kidney homogenate in 1935 by Krebs [1], and its subsequent purification as one of the very first flavin enzymes in the 1950s by Kubo *et al.* [2], only one further D-amino acid oxidase, that from *Rhodotorula gracilis*, has been purified to homogeneity and studied to some extent [3,4]. This oxidase possesses a remarkably high catalytic activity ($k_{cat.}$ is 43250 min^{-1} for D-alanine at 30 °C [5]) and contains one non-covalently bound FAD per protein monomer of 39 kDa. The yeast enzyme exhibits general characteristics consistent with the properties of the dehydrogenase/oxidase class of flavoproteins [6]. However, the overall properties investigated so far indicate that the yeast enzyme is significantly different from the pig kidney D-amino acid oxidase, which is one of the most thoroughly studied flavin enzymes.

No three-dimensional structure is available for D-amino acid oxidase because of the lack of suitable crystals [7]. In an attempt to compare the two enzymes mentioned and to gain insight into the factors leading to differences and similarities, we have replaced the native cofactor FAD of the *R. gracilis* enzyme with a series of FAD analogues. The properties of several FAD-modified D-amino acid oxidases from pig kidney, which have been prepared to map the active centre, have been reported [6,8]. A discussion on the use of modified flavin cofactors in the study of flavoprotein active centres and of the variety of information which can be obtained by the use of different types of probes has appeared recently [9]. In the present paper we report specifically on some properties of *R. gracilis* D-amino acid oxidase reconstituted with 8-chloro-, 8-mercapto-, 6-hydroxy-, 2-thio-, 5-deaza- and 1-deaza-FAD and compare them with the corresponding modified enzymes from pig kidney. We have also used 8-hydroxy-5-deaza-FAD to determine which side of the flavin reacts with substrate in *R. gracilis* D-amino acid oxidase.

MATERIALS AND METHODS

Enzymes

D-Amino acid oxidase from *R. gracilis* (ATCC 26217) was purified according to [4]. Enzyme isolated following this procedure had a typical A_{274}/A_{455} ratio of 8.2 and a specific activity, when assayed polarographically with D-alanine as substrate [3], of $\sim$ 175 units/mg of enzyme at 37 °C. Apoprotein was prepared by dialysis of purified D-amino acid oxidase against 250 mM-potassium phosphate, pH 7.5, containing 2 M-KBr, 20 % (v/v) glycerol, 0.3 mM-EDTA and 5 mM-2-mercaptoethanol, according to the procedure described in Casalin *et al.* [10]. The apoprotein thus obtained has an $\epsilon_{278}^{0.1\%}$ of 2.14 and showed no catalytic activity when assayed in the absence of exogenous FAD. Pig kidney D-amino acid oxidase and the corresponding 8-mercapto-enzyme were prepared as described in [11].

Preparation of modified flavins

All FAD analogues were prepared from the corresponding riboflavin derivatives by incubation with the flavokinase/FAD synthetase system of *Brevibacterium ammoniagenes* [9,12]. The riboflavin analogues were obtained as reported earlier [9], or as described or reported in the following literature: 8-chloro-FAD, ϵ_{448}, 10600 M^{-1}·cm^{-1} [13]; 8-mercapto-FAD, ϵ_{530} 28600 M^{-1}·cm^{-1} [14,15]; 2-thio-FAD, ϵ_{490} 20800 M^{-1}·cm^{-1} [16]; 6-hydroxy-FAD, ϵ_{323} 19600 M^{-1}·cm^{-1}, ϵ_{427} 22600 M^{-1}·cm^{-1} [17]; 1-deaza-FAD, ϵ_{540} 6800 M^{-1}·cm^{-1} [18]; 5-deaza-FAD, ϵ_{400} 11300 M^{-1}·cm^{-1} [19]; 8-hydroxy-5-deaza-FAD, ϵ_{430} 43600 M^{-1}·cm^{-1} [20]. Modified flavins were quantified at neutral pH using the absorbance coefficients indicated.

Activity measurements

D-Amino acid oxidase activity of reconstituted enzymes was assayed polarographically using D-alanine as substrate at 37 °C

Abbreviation used: MMTS, methylmethanethiolsulphonate.

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and pH 8.5 as described, with the omission, however, of FAD from the assay mixture [3].

Miscellaneous

Absorption spectra were determined with a Kontron Uvikon 860 (Milan, Italy) recording spectrophotometer at 15 °C. For experiments involving reduction of the enzyme, a solution of the latter was placed in a 1 ml cell equipped with side-arms and was made anaerobic by several cycles of evacuation and flushing with purified N_2. Photoreduction, in the presence of EDTA and using 5-deazariboflavin as catalyst, was conducted as described by Massey & Hemmerich [21] at 15 °C. Solvent accessibility of 8-chloro-FAD-reconstituted enzyme was studied using either Na_2S or thiophenol as nucleophiles, and that of 8-mercapto-FAD- and 2-thio-FAD- using methylmethanethiolsulphonate (MMTS) or iodoacetamide [22,16]. Fluorescence measurements were carried out in a Jasco FP-777 spectrofluorometer (Tokyo, Japan); protein fluorescence emission was monitored at 335 nm (excitation at 285 nm), using an excitation slit of 5 nm and an emission slit of 10 nm. Dissociation constants of apoprotein–FAD analogue complexes and of holoenzymes with benzoate were estimated according to Stinson & Holbrook [23] or Benesi & Hildebrand [24].

Determination of the side stereospecificity of flavin

The apoenzyme of general acyl-CoA dehydrogenase from pig liver, a *re*-side-specific flavoenzyme [25], was prepared according to [26], and reconstitution was achieved by incubation of a 52 μM solution of apoprotein in 100 mM-potassium phosphate containing 0.3 mM-EDTA, pH 7.6 (buffer A) with an equivalent amount of 8-hydroxy-5-deaza-FAD at 4 °C for 30 min. Unbound flavin was removed by gel filtration (Sephadex G-25 fine equilibrated with buffer A). Reconstituted 8-hydroxy-5-deaza-FAD–acyl-CoA dehydrogenase was reduced by incubating 7.8 nmol of enzyme in 1 ml of buffer A with 200 μl of 1 M-$NaBCN^3H_3$ at 4 °C for 20 h. Reduced enzyme was chromatographed over a Sephadex G-25 fine column (same buffer) and 8-hydroxy-5-deaza[5-^{3}H]-$FADH_2$ was released, first by heating the protein at 100 °C for 1 min followed by cooling in ice and centrifugation. The labelled analogue was incubated with a 1.2 M excess of apo-D-amino acid oxidase at 4 °C for 4 h, and the reconstituted holoenzyme was isolated by PD10 gel filtration. Re-oxidation was started by addition of 9 μmol each of NH_4^+ and pyruvate to 3.0 ml of enzyme [25], the reaction being followed spectrophotometrically at 405 nm. After completion of the reaction, an aliquot was passed through a PD10 column. Eluate fractions were analysed for radioactivity and protein elution was followed by recording absorption spectra.

RESULTS

8-SH-FAD– and 8-Cl-FAD–D-amino acid oxidase, and solvent accessibility to the flavin position 8

8-Mercapto-FAD binds tightly to the apoprotein of *R. gracilis* D-amino acid oxidase. The process is accompanied by a red shift and an enhanced resolution of the visible absorption band of 8-mercaptoflavin (Fig. 1). The dissociation constant was determined by following the quenching of the protein fluorescence emission [23]; it is approximately 4-fold smaller compared with that induced by the pig kidney enzyme with the same analogue (Table 1).

In contrast with this behaviour, binding of 8-Cl-FAD does not lead to major changes in the absorbance spectrum of the latter enzyme (not shown, Table 1). The K_d obtained by the same method as above (Table 1) is comparable with that for binding of unmodified FAD. This is in agreement with the similar steric requirements of a CH_3- versus a Cl-group as flavin substituents at position 8. Both 8-SH-FAD- and 8-Cl-FAD-reconstituted enzymes are inactive when assayed using the polarographic method. Moreover, addition of the substrate D-alanine (50 mM final concentration) under anaerobic conditions did not lead to a decrease of the oxidized flavin absorption, indicating complete inactivity. Both modified D-amino acid oxidases can be reduced photochemically in the presence of 5-deaza-riboflavin and EDTA (Table 1).

8-Mercapto-FAD–D-amino acid oxidase does not form an $N_{(5)}$ adduct with sulphite (final concentration up to 360 mM), an adduct easily formed by native D-amino acid oxidase (Table 1) and one which is detected spectrally by flavin bleaching [4]; nor does this modified FAD enzyme bind aromatic carboxylic acids such as benzoate, as is shown by the lack of the typical perturbations of the flavin spectrum which usually accompany complex formation [4,27]. At high concentrations of benzoate (0.3 M) the coenzyme is released.

8-SH-FAD–enzyme did not react over a 150 min time period when incubated with MMTS (0.5 mM final concentration) or with iodoacetamide (50 mM final concentration), since the spectrum typical of 8-*S*-alkylated flavin did not appear [14]. This result, indicating restricted accessibility to flavin position 8, was confirmed by the complete unreactivity of 8-Cl-FAD–D-amino acid oxidase with Na_2S (20 mM final concentration) or thiophenol (0.4 mM final concentration).

6-OH-FAD–D-amino acid oxidase and alteration of its p*K*

This holoenzyme has been prepared at pH 7.5 by incubation with excess coenzyme and subsequent gel filtration; it is devoid of measurable catalytic activity, and no reaction with the substrate D-alanine is observed under anaerobic conditions. Its absorbance spectrum is closely related to that of uncomplexed anionic 6-OH-FAD, which has a p*K* of 7.1 [17] (Table 1). This spectrum is not changed at pH values as low as 4.0 indicating that the p*K* of the 6-OH function is lowered by at least three units when in the complex, most probably owing to the same positively charged group(s) affecting 8-mercapto-FAD–D-amino acid oxidase (cf. above). 6-OH-FAD–D-amino acid oxidase was photoreduced in the presence of 5-deaza-riboflavin and EDTA. It does not form complexes with either sulphite or benzoate as shown by the lack of typical spectral changes [4] upon incubation.

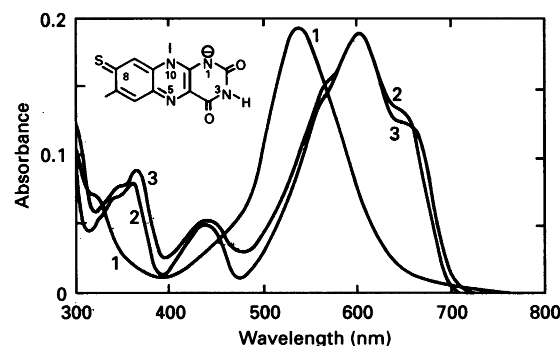

Fig. 1. Spectral changes observed upon binding of 8-mercapto-FAD to apo-D-amino acid oxidase at 20 °C

Traces were obtained in the following conditions: (1) 6.5 μM-8-mercapto-FAD in 100 mM-potassium phosphate buffer, pH 8.0; (2) 6.5 μM yeast 8-mercapto-FAD–D-amino acid oxidase in 50 mM-potassium phosphate buffer, pH 8.0, containing 10% (w/v) glycerol, 0.3 mM-EDTA and 5 mM-2-mercaptoethanol, and (3) 6.5 μM pig kidney 8-mercapto-FAD–D-amino acid oxidase in 20 mM-pyrophosphate, pH 8.5.

Table 1. Correlation of flavin structure and function in yeast and mammalian D-amino acid oxidase

Flavin	Enzyme source	Activity	K_d (M)	$A_{max.}$ (nm)	Spectral characteristics	Benzoate binding	Anthranilate binding K_d (M)	Solvent accessibility to position of substitution	pK_a	Sulphite binding K_d (M)	Specific characteristic	References
FAD	Yeast	175 units/mg	2×10^{-8}	455, 368, 274	$\epsilon_{274}/\epsilon_{455}=8.2$	0.245×10^{-3}	Charge-transfer complex		10.6 $N_{(3)}$-H	1.1×10^{-4}	Red radical	[4]
	Pig kidney	21.3 units/mg	22×10^{-7}	455, 380, 274	$\epsilon_{274}/\epsilon_{455}=9.5$	3×10^{-6}	Charge-transfer complex		9.4 $N_{(3)}$-H	3.5×10^{-3}	Red radical	[27,29, 35,36]
5-Deaza	Yeast	Anaerobic reduction	0.6×10^{-8}	405, 344	Quenching of flavin fluorescence	1.32×10^{-3}	Charge-transfer complex			+	-Re-oxidation by pyruvate and ammonia -*re*-face stereospecificity -$NaBH_4$ reduction	
	Pig kidney	Anaerobic reduction	$3/5\times10^{-6}$	403, 340	Enhancement of flavin fluorescence	4×10^{-6}	Charge-transfer complex				-Re-oxidation with pyruvate and ammonia -*re*-face stereospecificity	[25]
1-Deaza	Yeast	70/80% of native enzyme	1.2×10^{-8}	539, 370		+	Charge-transfer complex?			+		
	Pig kidney	100% of native enzyme	5.6×10^{-6}	540		1×10^{-4}	Charge-transfer complex?			0.9		[19]
2-Thio	Yeast	No turnover, but anaerobic reduction	$<4\times10^{-7}$	495, 317		5.4×10^{-3}		–	8.9 $N_{(3)}$-H			
	Pig kidney			500		+			8.8 $N_{(3)}$-H			[37]
6-Hydroxy	Yeast	–	n.d.	613, 434, 325	Benzoquinoid form	–			<4.0 (6)-OH	–		
	Pig kidney				Benzoquinoid form							[16]
8-Mercapto	Yeast	–	0.8×10^{-8}	597, 437, 358	*p*-Quinoid form	– Release of flavin above 0.3 M		–	<4.0 (8)-SH			
	Pig kidney	–	3×10^{-8}	595, 432, 366, 342	*p*-Quinoid form	–						[9,15,20]
8-Chloro	Yeast	–	1.1×10^{-8}	452, 365		–		–				
	Pig kidney	–		450, 370		+		+				[22]

Abbreviation: n.d., not determined. + denotes complex formation as deduced from spectral perturbations observed (no K_d available), and – indicates no effect observed.

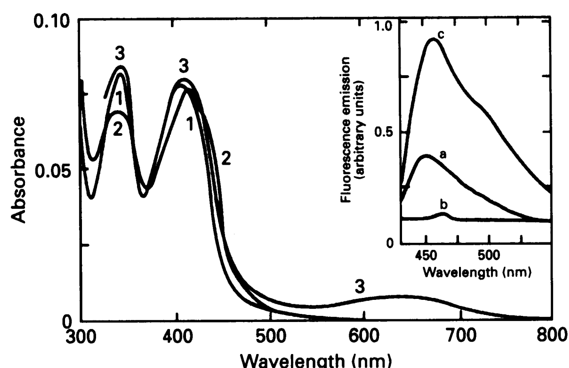

Fig. 2. Spectral effects induced by benzoate and anthranilate on binding to yeast 5-deaza-FAD–D-amino acid oxidase at 15 °C

Experimental conditions: 50 mM-potassium phosphate buffer, pH 8.0, 10% (w/v) glycerol, 0.3 mM-EDTA and 5 mM-2-mercaptoethanol. Trace 1 shows 6.3 μM 5-deaza-FAD–D-amino acid oxidase alone, trace 2 after addition of 11 mM-sodium benzoate and trace 3 after addition of 7.5 mM-sodium anthranilate. Inset: fluorescence emission spectra of: (*a*) 0.56 μM yeast 5-deaza-FAD–D-amino acid oxidase; (*b*) same sample after 30 min incubation with 10 mM-D-alanine; (*c*) 5-deaza-FAD extracted by boiling and centrifugation of the holoenzyme for 1 min. Excitation at 400 nm, 15 °C.

5-Deaza-FAD–D-amino acid oxidase: lack of turnover activity

5-Deaza-FAD binds to yeast apo-D-amino acid oxidase with a K_d similar to that of unmodified FAD. This contrasts with the binding to the enzyme from pig kidney where the K_d for binding of the 5-deaza-analogue is approximately 20-fold higher when compared with normal FAD. This suggests that the environment around the flavin position 5 is significantly different in the two enzymes (Table 1). While the absorption spectrum of the complex (Fig. 2) is similar to that of the pig kidney 5-deaza-FAD–enzyme [19], the fluorescence properties are substantially different. The *R. gracilis* 5-deaza-FAD–D-amino acid oxidase enzyme shows a 45% decrease in flavin fluorescence emission at 463 nm as compared with free 5-deaza-FAD. The reduced form of the 5-deaza-FAD-reconstituted enzyme, obtained from reaction with D-alanine (Fig. 2), is not fluorescent.

The 5-deaza-FAD–D-amino acid oxidase does not catalyse the oxygen-dependent oxidation of D-alanine; the reconstituted enzyme reacts, however, rapidly with D-alanine ($t_{\frac{1}{2}} < 1$ min) and slowly with D-proline ($t_{\frac{1}{2}} = 2$ h). This concurs with a disappearance of the spectrum of the oxidized 5-deazaflavin and formation of a chromophore which most probably has the 1,5-dihydrodeazaflavin structure [28]. No reduction at all was observed with D-glutamate as substrate (native enzyme also does not react), indicating an unchanged substrate specificity. Full reversal of the process, i.e. re-oxidation, was obtained in the presence of pyruvate and ammonia (100 mM and 50 mM final concentration respectively), and was followed by an increase of the absorbance at 405 nm (not shown). This re-oxidation is probably coupled with formation of D-alanine. In agreement with the lack of catalytic activity referred to above, no effect of oxygen on the reduced 5-deaza-FAD enzyme was observed.

5-Deaza-FAD yeast D-amino acid oxidase binds benzoate; this is shown by the appearance of a shoulder in the absorption spectrum at 440 nm, and a shift of the absorption maximum from 405 to 415 nm (Fig. 2). From these changes a K_d value of ~ 1.3 mM was estimated at pH 8.0. Anthranilate also binds, yielding a new absorption in the 630 nm region, which is typically attributable to a charge-transfer interaction [27]. The 5-deaza-FAD–enzyme reacts with sulphite in a process leading to essentially complete bleaching of the spectrum of the oxidized form. From the spectral properties of the product [29] it is assumed that sulphite forms an $N_{(5)}$ covalent adduct ((Table 1) as is the case with normal enzyme [4]. A stable, reduced form of the enzyme was also obtained after reaction with $NaBH_4$ followed by gel filtration. This species is spectrally similar to that obtained upon reaction with substrate (cf. above), and is reconverted into oxidized 5-deaza-D-amino acid oxidase by pyruvate plus ammonia.

1-Deaza-FAD–D-amino acid oxidase: binding of the cofactor and other properties

The properties of holoprotein reconstituted with this analogue are reported in Table 1. The analogue binds to the yeast apoenzyme with a K_d similar to that of normal FAD; of interest is the comparison with what is found with pig kidney D-amino acid oxidase, where 1-deaza-FAD binds much more weakly than normal FAD, and K_d for the modified flavin is approximately 500-fold lower than the corresponding value for yeast enzyme [12]. The activity with alanine and methionine is reduced by approximately 20% as compared with native enzyme, while with D-proline the enzyme is catalytically inactive, although the cofactor is reduced by this amino acid under anaerobic conditions. 1-Deaza-FAD–D-amino acid oxidase binds benzoate, as is reflected by the perturbation of the absorption spectrum, but does not yield a particular long-wavelength charge-transfer band with anthranilate. The charge-transfer absorption could, however, be obscured by the 1-deaza-FAD peak at 540 nm, as proposed for the pig kidney D-amino acid oxidase [12].

2-Thio-FAD–D-amino acid oxidase and solvent accessibility to position 2

The binding constant of 2-thio-FAD cannot be determined reliably by following the protein fluorescence-emission changes. This is probably owing to oxidation of the analogue under the experimental conditions [30]. An estimated value of this constant was obtained by following the spectral changes of the chromophore (Table 1). The pK of 2-thio-FAD for deprotonation at position N(3)-H in the D-amino acid oxidase complex was estimated by following the spectral changes at 490 nm in the pH range 7–10. The obtained value of 8.9 is approximately 1 unit lower compared with that of free 2-thio-FAD [16] and corresponds to that observed with the pig kidney 2-thio-FAD–enzyme (pK_a ~ 8.8, [16]). 2-Thio-FAD–D-amino acid oxidase is catalytically inactive when assayed polarographically with D-alanine acting as substrate. However, it is reduced by the same substrate under anaerobic conditions. The binding of benzoate to the 2-thio-FAD–enzyme at pH 7.5 was monitored by the increase of the absorbance at 490 nm, and is much weaker than that to native enzyme (Table 1). No reaction whatsoever was observed with MMTS (0.125–2.0 mM final concentration) [16] upon incubation at pH 7.5, indicating blockage of access to this position from solvent.

Stereospecificity of flavin reactivity

Chirally C(5)-labelled 8-OH-5-deaza[5-^{3}H]FADH$_2$ was obtained by NaBCN^{3}H$_3$, reduction of the analogue 8-OH-5-deaza-FAD bound to medium-chain acyl-CoA dehydrogenase [20], and was reconstituted with apo-D-amino acid oxidase as described in the Materials and methods section. Transfer of labelled hydrogen to iminopyruvate, obtained by addition of ammonia and pyruvate, was followed spectrophotometrically. Protein and small molecules were then separated by filtration, and the radioactivity was counted in the fractions. Approximately 8.5% of the ^{3}H label was found to co-elute with the protein and ~ 90% with the small-molecule fractions, showing that the label was specifically removed from the same side of the flavin ring to which it had

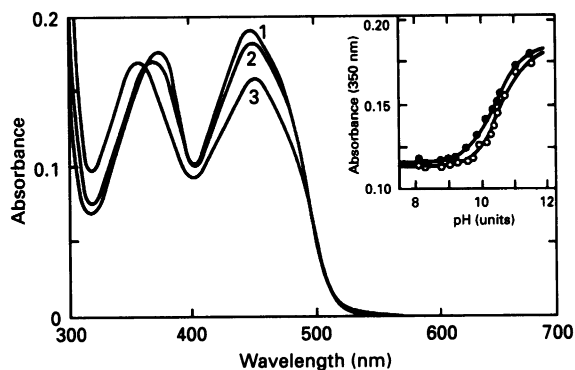

Fig. 3. Spectral dependence of 14.5 μM yeast D-amino acid oxidase on pH at 15 °C

Experimental conditions: 10 mM-potassium phosphate, 10% (w/v) glycerol, 2 mM-EDTA and 5 mM-2-mercaptoethanol at (1), pH 7.5; (2), pH 10.0 and (3), pH 10.6. Inset: pK_a determination of the $N_{(3)}$-H of FAD in yeast D-amino acid oxidase (○) and free FAD (●).

been transferred by $NaBCN^3H_3$. This is compatible with the label being released specifically from the *re*-side of the flavin ring. Note that this is the same side which is reactive in acyl-CoA dehydrogenase and D-amino acid oxidase from pig kidney [26].

Flavin ionization constants

A characteristic of pig kidney D-amino acid oxidase is a lowering of the $N_{(3)}$-H p*K* from ~ 10 (free FAD) to ~ 9.4 [27]. A shift of similar magnitude is observed with the yeast enzyme; however, this occurs in the opposite direction to ~ 10.6, as shown by the pH dependence of the absorption spectra depicted in Fig. 3. This p*K* is lowered somewhat (to ~ 10.4) in the presence of benzoate, while in the case of the mammalian enzyme benzoate induces a shift to 10.9 [27].

DISCUSSION

Several points emerge from the comparison of the behaviour of yeast enzyme reconstituted with various FAD analogues with that of the pig kidney enzyme. As for the native D-amino acid oxidase the binding of most analogues is much tighter with the yeast D-amino acid oxidase, this is reflected by an approximately 10-fold lower K_d (see Table 1). Notable exceptions are 1-deaza-FAD and 5-deaza-FAD which bind better to the yeast enzyme by a factor of ~ 500–600. In the case of 1-deaza-FAD this suggests a much tighter packing and consequently much stricter steric requirements around this position in the case of the mammalian enzyme. This is reminiscent of the cases of flavocytochrome b_2, where this analogue does not bind [31], compared to lactate oxidase, for example, where binding is efficient [32], and where a similar conclusion was reached. In the case of 5-deaza-FAD, an analogy to the flavin dependent α-hydroxycarboxylate oxidase family [31,32] is again likely. With the latter the three-dimensional structure shows an α-hydrogen bridge between the flavin $N_{(5)}$ and a protein backbone [31,32], a bridge clearly not possible in the case of 5-deaza-FAD. This suggests that such an interaction, if present in yeast enzyme, is much less significant than in the case of the mammalian D-amino acid oxidase.

In general the spectral effects and perturbations observed upon binding of the analogues follow the same trend observed with the mammalian enzyme, suggesting a similar pattern of functional groups at the active centre. In particular, binding of the 8-mercapto- and the 6-hydroxy-FAD analogues induces a stabilization of their *para*- and *ortho*-quinoid (canonical) forms respectively. This indicates the presence of a (partial) positive charge near the flavin $N_{(1)}$–$C_{(2)}$=O positions in the yeast enzyme also. This type of functionality, assumed to be important in affecting the flavin redox potential [9], appears to be present in various oxidases such as lactate oxidase, glycollate oxidase [32] or flavocytochrome b_2 [31], and might be an arginine residue in pig kidney D-amino acid oxidase [33]. The observation of a significantly weaker binding of benzoate by the yeast enzyme (100–1000-fold, Table 1) and the less intense long-wavelength charge-transfer band in the presence of anthranilate are difficult to rationalize in the absence of a three-dimensional structure. Since the corresponding differences in K_m for most substrates is much smaller [5], benzoate binding might simply reflect minor changes of groups at the active site, which are involved in governing specificity.

While solvent accessibility to the flavin position $C_{(2)}$=O is similarly restricted in the yeast and mammalian enzymes, indicating comparable environments, the accessibility to the 8 position is drastically lowered in the yeast protein. While this clearly reflects a major structural diversity, a molecular interpretation or a correlation with specific differences in the properties of the two enzymes is not possible at present.

The differences in p*K*-shifts at the position $N_{(3)}$-H which were observed between the yeast and mammalian enzyme also appear to be significant. In the first case there is an increase (to p*K* 10.6 from 10.02 for free FAD), while in the second a decrease to 9.4 is observed (Table 1). This should be discussed in the context of sulphite binding, which is tighter in the case of the yeast enzyme and could parallel a catalytic event involving the attack of a carbanion at the $N_{(5)}$ flavin position. This also correlates with a more positive redox potential by 50 mV and 15 mV for the first and the second electron transfer respectively (M. S. Pilone, P. Casalin & M. Stankovich, unpublished work), as compared with the mammalian enzyme [34]. An increase of redox potential should reflect a situation in which the anionic form of reduced flavin is preferentially stabilized compared with the free form (p*K* lowering). This concept has been verified within the class of α-hydroxyacid oxidases [31,32], and with the mammalian enzyme, where the p*K* of $N_{(3)}$-H is lowered. In the present case of the two related D-amino acid oxidases, the occurrence of two apparently opposite effects can only be interpreted by assuming very different microenvironments affecting the properties of the flavin positions $N_{(1)}$–$C_{(2)}$=O and $N_{(3)}$-H.

In conclusion, the present results suggest that the basic catalytic machinery operating at the active site is closely similar in the two enzymes. However, there are substantial differences such as solvent accessibility, and tightness of binding of substrate and cofactors. These probably reflect the ways in which substrate specificity and oxygen reactivity are regulated differently in the two proteins.

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