First publ. in: Flavins and flavoproteins 1990 : proceedings of the tenth International Symposium, Como, Italy, July 15 - 20, 1990, pp. 175-179

FAD ANALOGUES AS ACTIVE SITE PROBES OF *Rhodotorula gracilis* D-AMINO ACID OXIDASE

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Introduction

The wide range of chemical reactivity exhibited by flavoprotein enzymes is determined fundamentally by the nature of the protein moiety. Studies on the interactions of the isoalloxazine nucleus of flavin with the enzyme are therefore important in understanding this reactivity; flavin analogues are extremely useful probes for elucidating structure-function relationships (1, 2). We have used a number of modified flavins and explored their interactions with the active center of D-amino acid oxidase from the yeast *Rhodotorula gracilis*. The enzyme contains one FAD per protein monomer of 39k and a E_{274}/E_{455} ratio of 8.2 characterises pure holoenzyme (3). A stable and reconstitutable apoprotein has been recently obtained: the binding constant of FAD was 2.0×10^{-8}M (4), one order of magnitude lower than the corresponding value for pig kidney DAAO.

Results and Discussion

8-SH-FAD was bound by the apoprotein of *Rhodotorula* DAAO and the paraquinoid flavin form was stabilized (Fig.1); K_d for

the coenzyme binding, determined by the quenching of protein fluorescence, was 0.75×10^{-8}M. The 8-SH-FAD-reconstituted enzyme, as the 8-Cl-FAD-derivative, was inactive but it was reduced by the 5-deaza-riboflavin photochemical system. No N(5) adduct with sulfite ions (up to 360 mM sulfite final concentration) nor complex with aromatic carboxylic acids such as benzoate was observed.

Position 8 of the flavin appeared to be buried and not accessible to solvent as determined by the reactivity of the 8-SH-FAD-DAAO with methylmethanethiolsulfonate and iodoacetamide and of 8-Cl-FAD-DAAO with Na_2S and thiophenol.

Apo-D-amino acid oxidase has been reconstituted with 6-OH-FAD and also in this case the anionic form of the flavin was highly favoured (Fig. 2); the pK of 6-OH-FAD-DAAO was shifted to pH values below 4.0, much lower than the corresponding pk

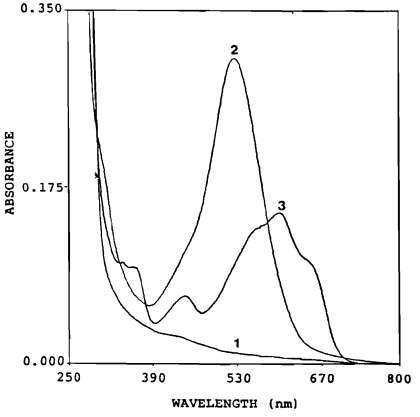

Fig.1. Spectral changes accompanying the binding of 8-SH-FAD to apo-DAAO. The experiment was carried out in 50 mM KPi pH 8.0 containing 10% glycerol, 0.3 mM EDTA and 5 mM 2-mercaptoethanol, 15°C.
(1) 15.8 μM apoprotein of *Rhodotorula* DAAO
(2) 10.56μM 8-SH-FAD
(3) 5 μM reconstituted 8-SH-FAD-DAAO

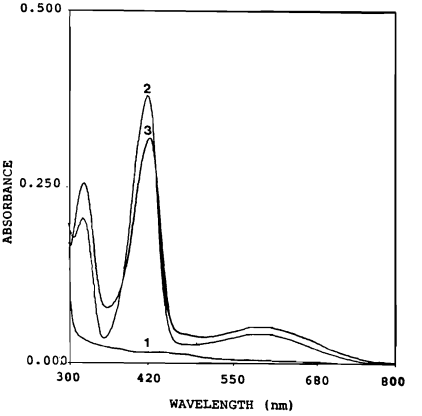

Fig.2. Spectral properties of yeast 6-OH-FAD-DAAO at 15°C.
(1) 15.4 μM yeast apo-DAAO in 250 mM KPi pH 7.5 containing 20% glycerol, 0.3 mM EDTA and 5 mM 2-mercaptoethanol
(2) 16.77 μM 6-OH-FAD in 100 mM KPi pH 7.5
(3) 13.3 μM reconstituted 6-OH-FAD-DAAO at pH 7.5. Identical spectra were obtained changing the pH value up to 4.0.

value for free flavin. 6-OH-FAD-DAAO was photoreduced in the presence of 5-deaza-riboflavin and EDTA and did not give rise to sulfite or benzoate complex.

The 8-SH-FAD-, the 8-Cl-FAD- and the 6-OH-FAD-reconstituted proteins could not be reduced by the substrate.

A K_d of 0.6×10^{-8}M was determined for binding of 5-deaza-FAD to DAAO; flavin fluorescence exhibited a 45% decrease as compared to the free flavin upon binding of this FAD analogue to the apoprotein. The reconstituted enzyme was reduced rapidly by D-alanine ($t_{1/2}$<1 min) but slowly by D-proline ($t_{1/2}$=2 hrs) and it was not reduced by D-glutamate (as the native DAAO). 5-deaza-FAD-DAAO formed a complex with both benzoate and anthranilate, the latter giving the typical charge-transfer absorption bands as the native enzyme did (Fig. 3).

Fig. 4 shows the spectrum of 1-deaza-FAD-DAAO (curve 1). The latter was active with D-alanine as substrate with a K_m of

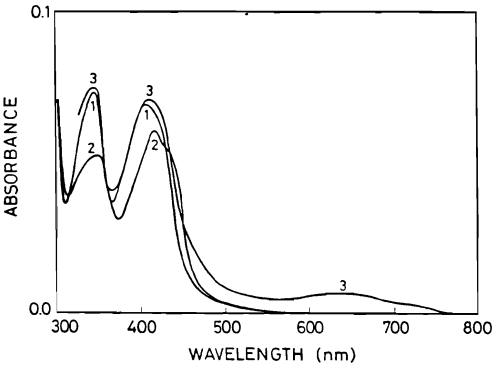

Fig.3. Spectral effects of benzoate and of anthranilate on 5-deaza-FAD-DAAO at 15°C.
(1) 5.7 μM 5-deaza-FAD-DAAO in 50 mM KPi pH 8.0 containing 10% glycerol and 5 mM 2-mercaptoethanol
(2) after addition of 11 mM sodium benzoate
(3) after addition of 7.5 mM sodium anthranilate

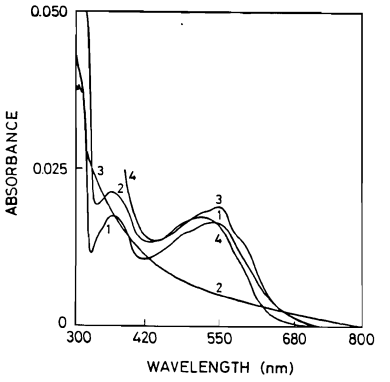

Fig.4. Spectral properties of yeast 1-deaza-FAD-DAAO at 15°C.
(1) 2.4 μM 1-deaza-FAD-DAAO in 50 mM KPi pH 8.0 containing 10% glycerol and 0.3 mM EDTA
(2) 5 min after addition of 40 mM D-proline in anaerobic conditions
(3) 1-deaza-FAD-DAAO after addition of 5 mM sodium benzoate
(4) 1-deaza-FAD-DAAO after addition of 13 mM sodium anthranilate

0.55 mM (versus a K_m of 0.83 for native DAAO); V_{max} was 70% with respect to native enzyme. With D-methionine as substrate the kinetic parameters were: K_m=3.23 mM (0.18 mM for the native enzyme) and V_{max} was 80% with respect to the value for the native DAAO. 1-deaza-FAD was not active with D-proline as substrate, but could be reduced by the amino acid in anaerobiosis (curve 2). Titration of 1-deaza-FAD-DAAO with benzoate gave the typical resolution of the spectrum (curve 3); no distinct long wavelength charge-transfer bands were observed with anthranilate (curve 4).

In conclusion, in both mammalian and yeast reconstituted enzymes the anionic forms of 8-SH-FAD and of 6-OH-FAD are being strongly stabilized indicating the presence of a positive charge/dipole near the flavin locus N(1)-C(2)=O. This correlates with the stabilization of the red (anionic) radical and with the ease of formation of the sulfite adduct to position N(5) (3).

On the other hand substantial differences emerge: with the yeast 8-SH-FAD-reconstituted enzyme there is no reactivity with electrophiles: this flavin region is inaccessible to solvent. With 1-deaza- or 5-deaza-FAD-reconstituted yeast DAAO the reactivity with substrates is significantly different.

In toto, the enzymes from pig kidney and from yeast, although they most probably do share the basic chemical mechanism, appear to be substantially different with respect to environment of the active site.

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