YEAST METHANOL OXIDASES: AN UNUSUAL TYPE OF FLAVOPROTEIN

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1. Introduction

Biological oxidation implies two modes of alcohol dehydrogenation, namely the nicotinamide-dependent mode splitting the substrate CH-bond into hydride plus carbocation [1] and the flavin-dependent mode splitting it into proton plus carbanion [2,3]. In general, flavin-dependent dehydrogenation of alcohol requires activation of the substrate CH-bond by an adjacent carbonyl group, which favors the initial proton abstraction. But there is a group of exceptional flavoenzymes dehydrogenating primary alcohols without such activation, e.g., glycerol phosphate dehydrogenase [4] and the flavin-dependent methanol oxidases [5–8], which may require a specific form of substrate preactivation.

By comparative studies with flavoproteins, 3 mair types of flavin activities (dehydrogenation, electron transfer and dioxygen activation) have been distinguished leading, by combination, to the further distinction of 5 flavoenzyme classes with characteristic experimental parameters [9]:

Table 1

Flavoprotein type	Radical stability	Radical color	O_2-affinity	Product of O_2-reduction	Sulfite adduct
I. Transhydrogenases	Low	–	Low	O_2^-	None
II. Dehydrogenases oxidases	Kin.	Red	High	H_2O_2	Strong
III. Dehydrogenases oxygenases	Low	–	High	$H_2O+[O]$	None
IV. Dehydrogenases e^--transferases	Stable	Blue	Low	O_2^-	None
V. Pure e^--transferases	Very stable	Blue	Low	O_2^-	None

Here, we insert the FAD containing methanol oxidase of *Candida boidinii* into this scheme and to check the validity and variability of this concept.

2. Materials and methods

Methanol oxidase from *Candida boidinii* was isolated according to [8] and flavodoxin from *Peptostreptococcus elsdenii* as in [10]. 5-Ethylriboflavin radical was prepared according to [11] and 5-deazaflavin-3-sulfonate as in [12]. Nitrogen and argon used for anaerobiosis were of 99.999% purity. Deaeration was carried out in a Thunberg cell either by evacuating and flushing with inert gas, or by evacuation alone, or by the addition of glucose oxidase [13] and glucose to the enzyme solution.

Routinely, a mixture of 10^{-4} M Lumiflavin and 10^{-1} M EDTA in the sidearm of the cell was illuminated under deaerated conditions; persistent quenching of the flavin fluorescence indicated the absence of oxygen.

Anaerobic photochemical reductions were performed in the presence of 10^{-6} M lumiflavin as in [14], or with 10^{-6} M 5-deazaflavin-3-sulfonate, according to [15]. A light source as described in [16] was used.

Radical concentrations were determined from double integration of EPR-signals with a Hewlett Packard desk calculator 9280, equipped with a digitizer.

Flavodoxin radical, which was prepared according to [10] (at −150°C) and 5-ethylriboflavin radical (at 25°C) were used as calibration standards, the concentrations being determined from the absorption, assuming $\epsilon_{580}=4000\ M^{-1}\cdot cm^{-1}$ and $\epsilon_{575}=3600\ M^{-1}\cdot cm^{-1}$, respectively.

EPR spectra at −150°C were recorded on a Bruker B-ER 420 X band instrument (equipped with a variable modulation frequency unit, a microwave frequency counter and a B-H 12 NMR oscillator). The samples were frozen in quartz tubes in liquid nitrogen.

EPR-spectra at 25°C (quartz flat cell) were recorded on a Varian instrument E-109-S. Optical absorption spectra were recorded on a Cary 118 instrument. For activity measurements a model 53 Oxygen Monitor System (YSI Co., Yellow Springs OH) was used. The standard activity assay contained 0.1 M methanol in 0.1 M sodium phosphate buffer (pH 7.5) with 3 ml final vol. at 25°C. Titration of the enzyme with Na_2SO_3 was performed as in [17], at 25°C. The K_d calculation of the enzyme–sulfite complex is based on the assumption, that 28% of the total FAD content of the enzyme are in the radical state and do not react with sulfite. The ϵ_{475} value of the radical is assumed to be 3900 $M^{-1} . cm^{-1}$, as in the case of D-amino acid oxidase [18].

3. Results

The spectra shown in fig.1 indicate the presence of considerable amounts of anionic flavin radical ($EFl^{\cdot -}$) in addition to oxidized flavoquinone (EFl_{ox}) in methanol oxidase. The similarity with known semiquinone spectra [18,19] becomes most pronounced upon addition of NH_2OH (a pseudosubstrate) which is isoelectronic and isosteric to methanol and reduces the enzyme bound Fl_{ox} to the radical by irregular 1 e^--transfer. The radical (cf. insert fig.1) shows in the EPR a linewidth of 1.5 mT, typical for anionic flavoprotein radicals [20]. By calibration with flavodoxin and 5-ethylriboflavin as standard radicals, a semiquinone content of 28% ± 5% of the total FAD is deter-

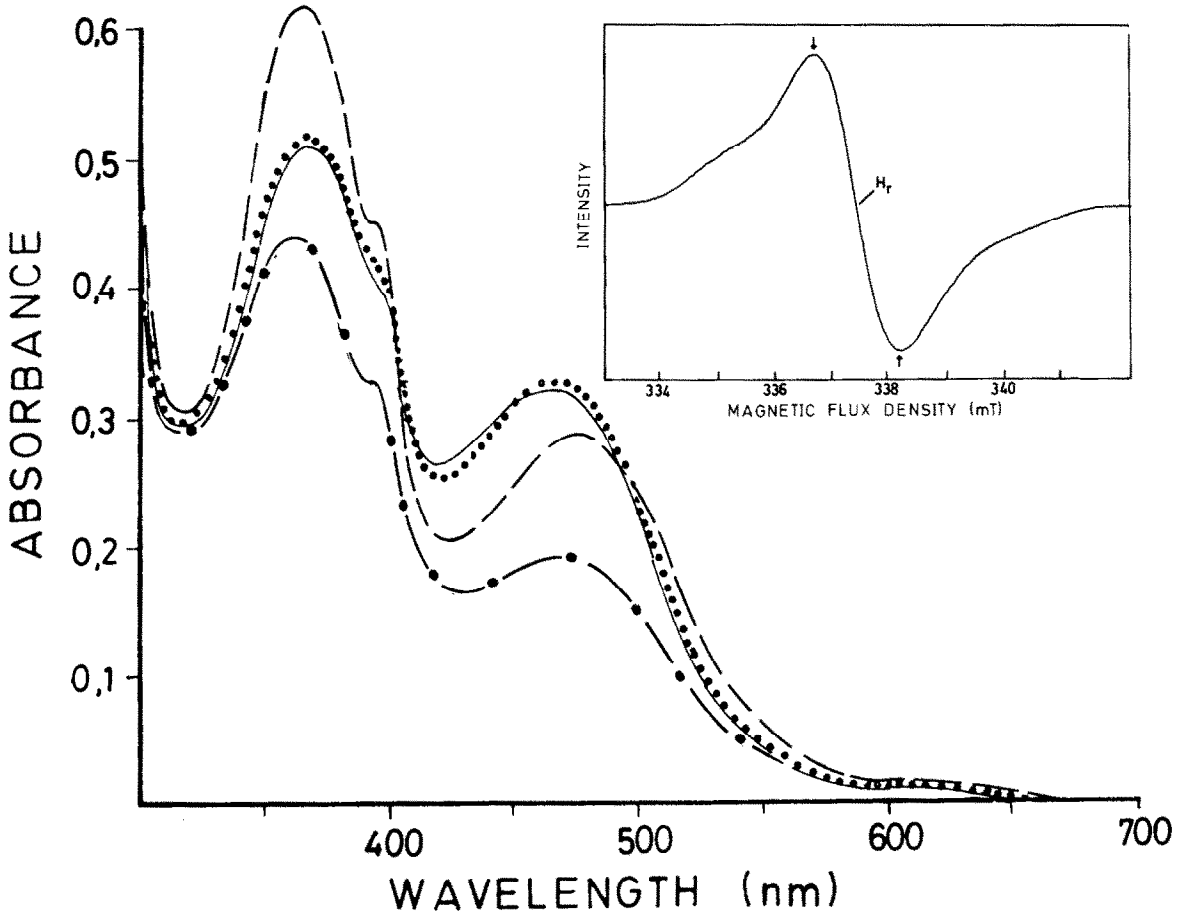

Fig.1. Absorption spectra of methanol oxidase under different conditions. The enzyme concentration was 2.4 × 10^{-5} M in 0.1 M sodium phosphate buffer (pH 7.5) at 25°C: (——) native enzyme; (– –) 20 min after addition of 10^{-3} M NH_2OH; (–.–) 4 h after anaerobization of the enzyme solution; (. . .) immediately after readmission of oxygen. Inset: EPR spectrum of methanol oxidase. The 1.2 × 10^{-4} M enzyme in the same buffer was measured at −154°C, with 100 kHz modulation frequency, 1.0 mT modulation amplitude, 0.2 mW (30 dB) power, 0.2 s time constant, 0.04 mT/s scan rate, 0.46713 GHz microwave frequency. The arrows indicate the line width; H_r is the magnetic field at resonance; g_{eff} = 2.004 ± 0.0005.

Table 2

	Initial state	Reduction method	Reduced state		Reoxidized state		% Residual activity	Activity measured
	(A_1/A_2)		A_1/A_2	$\%A_2$[a]	A_1/A_2	$\%A_2$[a]		
I	1.53	Deazaflavin/ EDTA/light	2.89	57	1.89	93	47	After readmission of air
II	1.54	Lumiflavin EDTA/light	2.87	50	2.07	92	74	After readmission of air
III	1.56	$Na_2S_2O_4$	3.37	52	2.14	82	42	After readmission of air
IV	1.53	$NaCNBH_3$	2.46	50	1.54	84	28	After readmission of air
V	1.54	Deaeration	2.42	57	1.56	101	100	After 24 h under anaerobic conditions and readmission of air
VI	1.50	Methanol (+ O_2)	2.27	52	1.52	103	100 50	5 min after addition of MeOH 1.5 h after addition of MeOH
VII	1.58	Methanol (− O_2)	3.20	39	–	–	100	5 days after addition of MeOH under anaerobic conditions
VIII	1.50	NH_2OH	2.20	83	No reoxidation by O_2		10	30 min after addition of 10^{-3} M NH_2OH

[a] Absorption of initial state is 100%

A_1, optical absorption of MeOx at 370 nm; A_2, optical absorption of MeOx at 470 nm. All experiments except VI and VIII were done under anaerobic conditions

mined, which by addition of NH_2OH is increased 2.5-fold and is not influenced by the presence or absence of oxygen, nor by removal of excess NH_2OH by Sephadex G-100 chromatography over at least 15 h.

The radical is not reducible to the dihydroflavin state ($EFl_{red}H_2$) by any method used (cf. table 2), except to some extent by prolonged substrate turnover (see below). NH_2OH in a 5-fold excess over flavin decreases the enzyme activity by 95%. The inhibitory effect demonstrates, that the radical state is catalytically inactive, as it is common for 2 e^--transferring flavoproteins [18].

Surprisingly, we obtain virtually identical spectra upon deaeration (cf. fig.1) and after the rapid phase of substrate reduction (cf. fig.2B). Both effects are fully reversible with practically no loss of activity. 'Reduction' of the flavin by anaerobiosis can be achieved by either deaeration method used, and is not prevented by the presence of catalase.

We show in fig.2 the course of reduction by methanol. The rapid phase difference spectrum A–B indicates the disappearance of EFl_{ox}. The slow phase difference spectrum B–C, however, closely resembles the optical spectra of anionic flavoprotein radicals and EPR quantitation shows, that in state C the residual $EFl^{\cdot -}$ content is only ~10%, compared to the initial 28% of total FAD. However, the residual absorption of state C at 475 nm can not result exclusively from 10% $EFl^{\cdot -}$ with an assumed ϵ_{475}=3900 $M^{-1} \cdot cm^{-1}$. Thus, we conclude, that either part of the radical must be EPR silent, or that $EFl_{red}H_2$ contributes to the absorption.

The slow phase of substrate reduction (steady O_2-turnover) is accompanied by loss of activity, while in the presence of methanol, under anaerobic conditions (i.e., no turnover), activity is not diminished for ≥24 h.

Titration of the enzyme-bound flavin with sulfite [21] shows $K_d \sim 3 \times 10^{-4}$ M for the enzyme–ligand complex, which is high but still consistent with the values for known flavoprotein oxidases [17]. The spectrum of the sulfite complex is virtually the same, as is observed after the rapid phase of reduction.

Azide, an inhibitor of the enzyme, causes a broadening of the 465 nm absorption band, so that a marked increase of absorption in the 500–650 nm region is observed, while the content of $EFl^{\cdot -}$ is not significantly altered by this agent. Dialysis *vs* azide-free buffer restored the initial spectrum. Very similar effects of azide have been first observed with alcohol oxidase from *Poria contigua* [22].

In denaturation experiments (heat, 4% SDS, 2.5% trichloroacetic acid) all flavin is liberated as Fl_{ox} (cf.

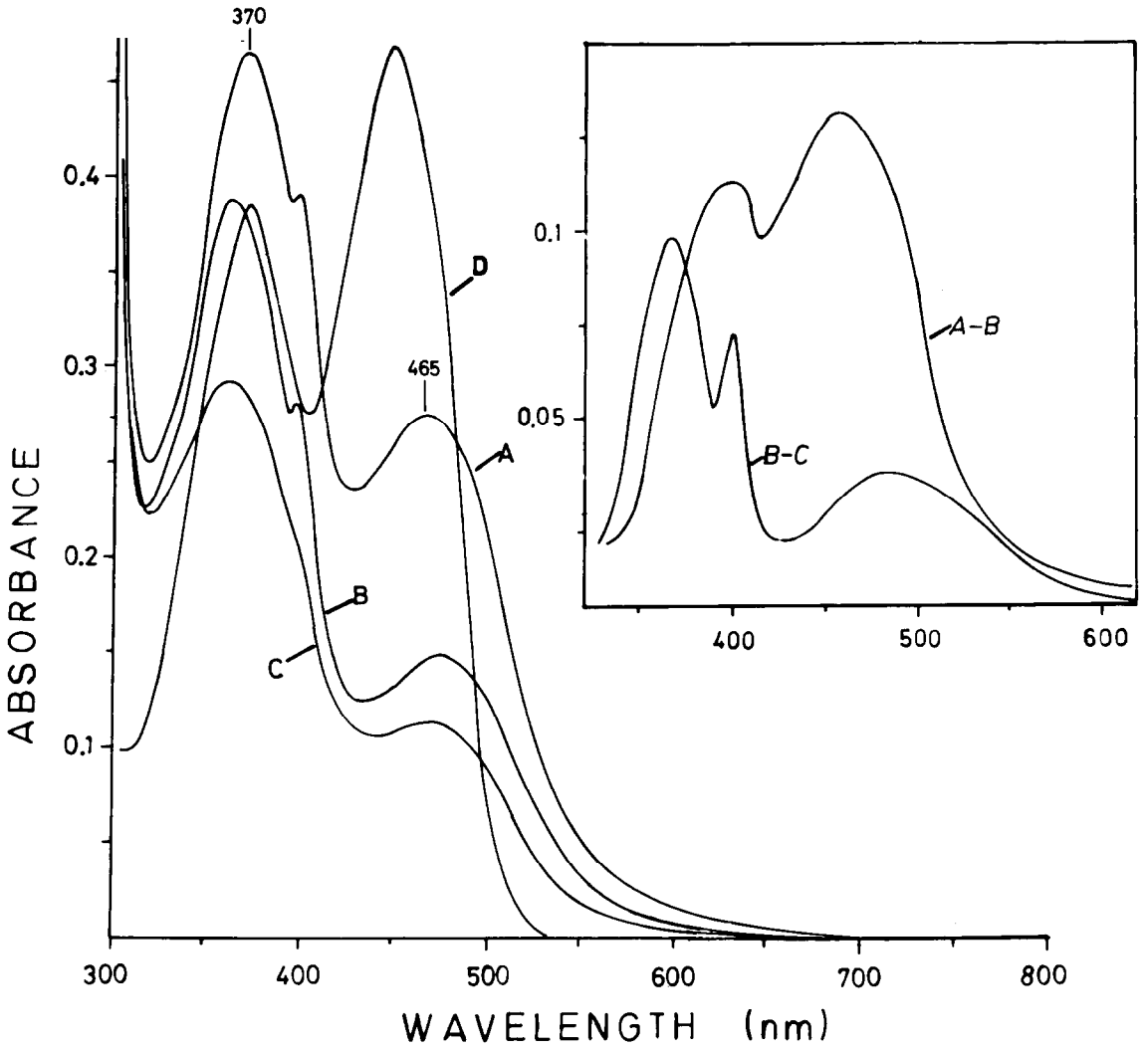

Fig.2. Absorption spectra of methanol oxidase in the presence of methanol. The enzyme was 3.8 × 10^{-5} M in 0.1 M sodium phosphate buffer (pH 7.5) at 25°C; (A) native enzyme; (B) 5 min after addition of 0.1 M methanol (rapid phase of reduction); (C) after 5 h incubation with 0.1 M methanol (slow phase of reduction); (D) after denaturation with 2.5% trichloroacetic acid and neutralisation. Inset: Difference spectra between curves A–B and B–C.

fig.2) and a flavin content of 6 FAD/octamer can be determined. Assuming ϵ_{475}=3900 $M^{-1} . cm^{-1}$ for the 28% radical in the initial state, the resulting extinction coefficient of EFl_{ox} at 475 nm would be as low as 8530 $M^{-1} . cm^{-1}$.

4. Discussion

To gain an insight into the catalytic mechanism we developed earlier a scheme of 4 questions concerning:

1. The redox stoichiometry (1 e^- or 2 e^-);
2. The already mentioned C–H bond cleavage mode (hydride or carbanion);
3. The geometry of the flavin–substrate bond in the Michaelis complex (charge-transfer π or covalent σ);
4. The site of substrate addition at the flavin chromophore [9].

From these results, the redox stoichiometry appears to be 2 e^-, since the well-defined and irregularly stable radical is inactive and apparently serves as a means of activity regulation in a unique and still

mechanistically unexplained way. Full (re)activation of the radical for 2 e^--transfer hitherto could not be accomplished by any means, but perhaps can be in the course of further study.

Though nothing can be said from the present experiments with respect to question 2, we have advanced good arguments opposing hydride transfer in any flavoprotein [23].

Concerning question 3, we can exclude, with the present simple alcoholic substrates, a π-interaction with the flavin; if we postulate a σ-interaction or covalent carbanion catalysis, position N(5) is to be favored as site of substrate action [3], which is known in the case of sulfite–flavin complexation [21], and in the case of covalent adduct formation by formaldehyde with free reduced flavin [24].

Since the stoichiometry of the methanol oxidase reaction is 2 e^-, H_2O_2 and not superoxide is the first product. Very surprising is the finding that, in the present case, this reaction is reversible, according to the formal equation:

$$Fl_{ox} + H_2O_2 \rightleftharpoons H_2Fl_{red} + O_2$$

This equilibrium is shifted to the right-hand-side upon deaeration of the system with concomitant and reversible loss of flavin absorption at 465 nm. This equation requires, that at least one equivalent of peroxide is always present and tightly bound in the enzyme: Thus, it is unreactive with catalase. Upon complete removal of oxygen it finally reduces the flavin.

We can only speculate, whether this tight and only slowly reversible binding of peroxide is of covalent nature or favored by apoenzyme induced deprotonation of peroxide. The thus complexed OOH^- could then be replaced by azide, which leads to inhibition of the O_2-activation process.

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References

[1] Dunn, M. F., Schack, P., Koerber, S. C., Au, A. M.-J., Saliman, G. and Morris, R. G. (1977) in: Pyridine Nucleotide-Dependent Dehydrogenases (Sund, H. ed) pp. 206–221, Walter de Gruyter, Berlin.
[2] Walsh, C. T., Schonbrunn, A. and Abeles, R. H. (1971) J. Biol. Chem. 246, 6855–6866.
[3] Ghisla, S. and Massey, V. (1980) J. Biol. Chem. 255, 5688–5696.
[4] Dawson, A. P. and Thorne, C. J. R. (1969) Biochem. J. 114, 35–40.
[5] Kato, N., Omori, Y., Tani, Y. and Ogata, K. (1976) Eur. J. Biochem. 64, 341–350.
[6] Tani, Y., Miya, T. and Ogata, K. (1972) Agric. Biol. Chem. 36, 76–83.
[7] Tani, Y., Miya, T., Nishikawa, H. and Ogata, K. (1972) Agric. Biol. Chem. 36, 68–75.
[8] Sahm, H. and Wagner, F. (1973) Eur. J. Biochem. 36, 250–256.
[9] Hemmerich, P. and Massey, V. (1979) in: Oxidases and Related Redox Systems; Proc. 3rd Int. Symp. (King, T. E. et al. eds) Academic Press, New York, in press.
[10] Mayhew, S. G. and Massey, V. (1969) J. Biol. Chem. 244, 794–802.
[11] Michel, H. (1981) PhD Thesis, Konstanz.
[12] Blankenhorn, G. (1976) Eur. J. Biochem. 67, 67–80.
[13] Swoboda, B. E. P. (1969) Biochim. Biophys. Acta 175, 365–379.
[14] Massey, V., Stankovich, M. and Hemmerich, P. (1978) Biochemistry 17, 1–8.
[15] Massey, V. and Hemmerich, P. (1978) Biochemistry 17, 9–17.
[16] Gladys, M. and Knappe, W.-R. (1974) Chem. Ber. 107, 3658–3673.
[17] Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G. and Faust, G. P. (1969) J. Biol. Chem. 244, 3999–4006.
[18] Massey, V. and Palmer, G. (1966) Biochemistry 5, 3181–3189.
[19] Ehrenberg, A., Müller, F. and Hemmerich, P. (1967) Eur. J. Biochem. 2, 286–293.
[20] Palmer, G., Müller, F. and Massey, V. (1971) in: Flavins and Flavoproteins (Kamin, H. ed) pp. 123–137, University Park Press, Baltimore MD.
[21] Müller, F. and Massey, V. (1969) J. Biol. Chem. 244, 4007–4016.
[22] Bringer, S. (1980) PhD Thesis, Düsseldorf.
[23] Hemmerich, P. (1976) in: Progress in the Chemistry of Organic Natural Products (Herz, W. et al. eds) pp. 451–527, Springer-Verlag, Heidelberg, New York.
[24] Blankenhorn, G., Ghisla, S. and Hemmerich, P. (1972) Z. Naturforsch. 27b, 1038–1040.