Purification and some properties of alcohol oxidase from alkane-grown *Candida tropicalis*

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Alcohol oxidase was purified to homogeneity from membrane fractions obtained from alkane-grown *Candida tropicalis*. The enzyme appears to be a dimer of equal-sized subunits of M_r 70000. The purified enzyme is photosensitive and contains flavin-type material which is released by a combination of boiling and proteolytic digestion. The identity of the flavin material is not yet known, but it is not FMN, FAD or riboflavin. The enzyme is most active with dodecan-1-ol, but other long-chain alcohols are also attacked. The enzyme shows a weak, but significant activity towards long-chain aldehydes. Detailed kinetic studies with decan-1-ol as substrate suggest a group-transfer (Ping-Pong)-type mechanism of catalysis.

INTRODUCTION

Work from this laboratory has shown that growth of the yeast *Candida tropicalis* on long-chain alkane substrates induces the synthesis of a long-chain-specific alcohol oxidase (Kemp *et al.*, 1988). The enzyme presumably catalyses one of the steps in the pathway in which alkanes undergo monoterminial oxidation and are converted into long-chain fatty acids before β -oxidation. Our early work established that the enzyme is membrane-bound, but no attempt was made to purify it. Later work showed that the enzyme in crude preparations is light-sensitive: activity was completely lost in a few minutes on exposure to a blue light source (Kemp *et al.*, 1990). It was not, of course, clear whether this lability was due to an intrinsic property of the enzyme or to the generation of harmful radicals in the membranes under bright illumination. Other work established that freeze-dried membrane preparations could be suspended in organic solvents (e.g. octane), with the enzyme still showing good activity (Kemp *et al.*, 1991). As oxygen is the electron acceptor in the oxidation reaction, this result suggested that the enzyme might have a commercial application in the synthesis of long-chain aldehydes.

The results referred to above indicated that purification and further characterization of the enzyme was necessary. Certainly questions about the mechanism of action of the enzyme, the prosthetic group involved in catalysis and the light-sensitivity of the enzyme could not be resolved by using crude enzyme preparations. We have now developed a reliable purification method, and this, and some observations on the properties of the purified preparations, are presented here.

MATERIALS AND METHODS

Materials

Long-chain alcohols, diols, aldehydes, ω -hydroxy-fatty acids and cholic acid were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. NAD^+ was from Boehringer Mannheim UK, Lewes, Sussex, U.K. 2,2'-Azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), catalase, peroxidase, glucose oxidase and CHAPS were from Sigma Chemical Co., Poole, Dorset, U.K. Bincinchoninic acid reagent was from Pierce Europe B.V., BA Oud Beijerland, The Netherlands.

Yeast growth conditions

Candida tropicalis was grown up in 100 ml starter cultures on an orbital shaker at 30 °C on a medium containing (g/l) yeast extract 5.0, malt extract 20.0 and glucose 5.0. After 2 days, 10 ml

portions of this culture were inoculated into each of six 3-litre conical flasks containing 500 ml of minimal salts medium, which contained (g/l) KH_2PO_4 , 7.0; Na_2HPO_4 , 2.0; MgSO_4 , 1.5; yeast extract, 1.5; CaCl_2 , 0.1; FeCl_2 , 0.008; ZnSO_4 , 0.0001; diammonium tartrate, 15; in distilled water at pH 6.5. The carbon source was hexadecane (1 %). The cultures were incubated on an orbital shaker for 50 h at 30 °C before harvesting.

Preparation of microsomal membranes

The procedure was that described by Kemp *et al.* (1988) with only minor alterations. These were that the harvested cells were washed in 50 mM-Hepes buffer, pH 7.5, and that the membrane fraction, obtained after cell breakage in the French pressure cell and differential centrifugation, was washed with 50 mM-Hepes buffer, pH 8.0, containing 0.15 M-KCl. The latter step removed some weakly associated protein before the membranes were stored as a suspension in 50 mM-Hepes buffer, pH 8.0, in liquid N_2 .

Enzyme activity and protein assays

Alcohol oxidase-catalysed reactions were routinely performed spectrophotometrically at 405 nm in coupled assays with ABTS and peroxidase as described by Kemp *et al.* (1988), but modified by using 20 mM-glycine/NaOH buffer, pH 9.0. Occasionally enzyme activity was measured directly by using an oxygen electrode (Rank Brothers, Cambridge, U.K.). The conditions for this assay were as for the spectrophotometric assay, but ABTS and peroxidase were omitted. The spectrophotometric assay is based on the methanol oxidase assay of Haywood & Large (1981). The absorption coefficient for the radical cation of ABTS was taken to be $\epsilon = 18400 \text{ M}^{-1} \cdot \text{cm}^{-1}$ (Werner *et al.*, 1970) with 2 mol of radical cation being formed per mol of substrate oxidized.

Substrates were prepared as solutions in acetone or in 1,4-dioxan. A 20 μl portion of substrate solution was added per 3 ml assay volume via a calibrated glass micro-syringe. For assays where the oxygen concentration was varied, the range of concentrations was obtained by mixing appropriate volumes of assay mixture previously bubbled with N_2 or O_2 for 10 min. The bubbling was done in graduated glass syringes held vertically in a retort stand. After gassing, the syringes were capped either with a rubber seal or by mounting the needle. Liquid could then be transferred from one syringe to another under positive pressure. The final mixture was transferred to an N_2 -filled 3 ml cuvette through a narrow sleeve in the cap. The reaction was then

initiated by adding enzyme and substrate through the same capillary from calibrated micro-syringes.

Protein determinations were by the method of Smith *et al.* (1985), using bincinchonic acid.

Purity of substrates

The purity of long-chain alcohols and aldehydes was assessed by g.l.c. at 140 or 160 °C by using a Pye-Unicam PU 4500 chromatograph fitted with a 1% diethyleneglycol succinate column. All samples, except dodecane-1,12-diol (which was dissolved in ethanol), were dissolved in n-hexane.

Purification of the enzyme

All steps were carried out at 0–4 °C and, because of the light-sensitivity of the enzyme (Kemp *et al.*, 1990), extracts were protected from the light.

The microsomal membranes from 3 litres of culture medium were suspended in 100 ml of 50 mM-Hepes buffer, pH 8.0, containing 1% (w/v) sodium cholate. The suspension was then immediately supplemented by addition of 2 ml of 50 mM-phenylmethanesulphonyl fluoride. After a few minutes (NH₄)₂SO₄ (240 g/l) was added to the membrane suspension, and the precipitated material was removed by centrifugation and discarded. Extraction for a longer period did not yield significantly more solubilized activity, and exposed the enzyme to a greater risk of proteolysis. More (NH₄)₂SO₄ (55 g/l) was added, and the precipitated material was then collected by centrifugation and dissolved in 10 mM-NaHCO₃ buffer, pH 9.2, containing 0.5% (w/v) CHAPS. Phenylmethanesulphonyl fluoride was added to the solution to give a final concentration of 1 mM, and the fraction was then dialysed for 2 h against 10 mM-NaHCO₃ buffer, pH 9.2, containing 0.5% CHAPS.

The dialysed fraction was supplemented with 1 mM-phenylmethanesulphonyl fluoride and applied to a column of QAE-cellulose (2.2 cm × 24 cm) equilibrated with 10 mM-NaHCO₃ buffer, pH 9.2, containing 0.5% CHAPS. The column was developed with a linear gradient formed from 140 ml of equilibrating buffer and an equal volume of the same buffer containing 0.5 M-NaCl. The fractions containing enzyme activity emerged at about half-way through the gradient and were collected together and stored in liquid N₂ overnight.

The preparation was thawed out rapidly, phenylmethanesulphonyl fluoride was added to a concentration of 1 mM, and the enzyme was then dialysed for 4 h against 50 mM-Hepes buffer, pH 8.0, with frequent changes of the dialysis buffer. Note that the detergent was omitted at this point. After dialysis, the small orange-yellow precipitate, which contained little enzyme activity, was removed by centrifugation and discarded. This precipitated material was non-fluorescent. This is an important point, because it shows that the green fluorescence associated with the purified enzyme (see below) was not due to contamination with a residue of this material. Portions of calcium phosphate suspension (30 mg/ml) were now added to adsorb the enzyme from the supernatant fraction. The process was monitored by assay after each addition and was halted after 95% of the enzyme had been adsorbed. (In one representative preparation the 48 ml enzyme fraction required 15 mg of calcium phosphate). The small gel-protein pellet was observed to be pale yellow. The calcium phosphate-enzyme pellet was washed successively with 3 ml volumes of 0.1 M-sodium phosphate buffer, pH 7.6, and 50 mM-Hepes buffer, pH 8.0, and then the enzyme was eluted by washing with 1 ml volumes of 0.5 M-Hepes buffer, pH 8.0, containing 5% (w/v) (NH₄)₂SO₄ and 0.5% CHAPS. The first-eluted fraction contained about 50% of the total enzyme recovered from the gel-protein pellet and was pale yellow. It proved possible to elute the enzyme from the calcium phosphate without

including detergent in the eluting buffer. However, in this case the overall yield from the step was decreased to about 50% of that when detergent was present.

Gel filtration

Experiments were conducted on a 30 cm × 1.5 cm column of Sephacryl S-300 SF (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM-sodium phosphate buffer, pH 7.6, containing 0.5% sodium cholate. Samples (0.2 ml) of proteins (approx. 1.5 mg/ml.) were applied to the column. The column was connected to an LKB h.p.l.c. apparatus (LKB, Bromma, Sweden). The proteins used to calibrate the column were β -amylase (M_r = 200000), yeast alcohol dehydrogenase (M_r = 150000), BSA (M_r = 66000), ovalbumin (M_r = 45000) and cytochrome *c* (M_r = 12400).

Chromatography of flavin samples

T.l.c. Samples were run at room temperature on 20 cm × 20 cm 100 μ m-thick layers of Whatman Gel 300 DEAE-cellulose plates. The moving phase was 4% (w/v) Na₂HPO₄.

H.p.l.c. Samples were run on a Spherisorb S 50 D52 column (25 cm × 4.6 cm) with the solvent methanol/2 M-ammonium acetate (7:3, v/v).

RESULTS AND DISCUSSION

Homogeneity, stability and M_r of the purified enzyme

The results of a typical purification procedure are shown in Table 1. Only 1–2 mg quantities of enzyme are obtained from the standard 3-litre culture of yeast. The purified enzyme is essentially homogeneous as judged by SDS/PAGE (Fig. 1). It is noteworthy that, although detergent is essential to solubilize the enzyme from microsomal membranes, in the last stages of purification the detergent can be removed and the enzyme remain in solution and fully active at concentrations of approx. 0.5 mg/ml. This suggests that in its natural environment the enzyme makes extensive contact with the aqueous phase.

It is essential that the purification procedure is carried out rapidly, that the samples are kept at 0–4 °C and that the proteinase inhibitor phenylmethanesulphonyl fluoride is added to extracts in the early stages of purification. In our initial attempts to purify the enzyme it became clear that considerable proteinase activity contaminated our extracts. Even highly purified extracts, if left at 25 °C overnight, showed only very low- M_r material on subsequent SDS/PAGE. The inhibitor has been shown to be effective against the residual proteinase activity, but the effects are apparently not permanent. Repeated treatments are necessary, and it is not until the final dialysis and calcium phosphate step that proteinase activity in the preparation is lost. It is unfortunate that alcohol oxidase is quite unstable below pH 7.5 (see below). The proteinase(s) in the membranes is much less active at pH 6.0 than at pH 8.0 (about 4.5-fold at 25 °C), and if the oxidase was stable it would be possible to exploit this characteristic and carry out the purification of the oxidase in a more acidic environment.

The final pure product is fairly stable. If kept on ice and in the dark there is no loss of activity in the course of the working day. As a routine we store the enzyme overnight or for longer periods in liquid N₂. The SDS/PAGE behaviour (Fig. 1) of the purified enzyme is not materially affected by incubation of the enzyme at 25 °C overnight, although most of the enzyme activity is lost by this treatment. The initial solubilization of the enzyme is performed with 1% cholate, but 0.2% CHAPS can be used if so desired (higher concentrations of CHAPS yield less enzyme in the supernatant phase). However, CHAPS is considerably more expensive than cholate, and we use the latter where possible. Cholate has, however, proved unsuitable in the ion-exchange

Table 1. Purification of alcohol oxidase from microsomal membranes of *Candida tropicalis*

A unit of enzyme activity catalyses the oxidation of 1 nmol of decan-1-ol/min in the assay. The membranes were obtained from 3 litres of culture medium after growth for 50 h.

Step	Volume (ml)	Activity (units)	Protein (mg)	Sp. activity (units/mg)	Yield (%)
Solubilization	110	160000	120	1330	100
(NH ₄) ₂ SO ₄ precipitation	15	120000	30	4000	75
QAE-cellulose chromatography	40	65000	4.6	14130	41
Ca ₃ PO ₄ adsorption	3.0	42000	1.4	30000	26

Fig. 1. Densitometer scans of gels obtained on SDS/PAGE of alcohol oxidase

Electrophoresis was conducted at 100 V for 2 h in mini-slab gels as described by Hames (1981), with a 10% acrylamide resolving gel and a discontinuous buffer system. Scans of the gels were made with a Joyce-Loebl Chromoscan 3 densitometer. (a) Alcohol oxidase. The M_r standards are (b) BSA (M_r 66000), (c) ovalbumin (M_r 45000) and (d) carbonic anhydrase (M_r = 29000). The gels were immersed for 25 min in a fixing reagent (methanol/acetic acid/water, 40:7:53, by vol.) and then stained for approx. 2 h with Coomassie Blue (0.25%) in the fixing reagent. The gels were destained by immersion in several changes of fixing reagent.

step, presumably because of the affinity of the resin for the negatively charged detergent. For this and subsequent steps we use 0.5% CHAPS.

SDS/PAGE of several preparations of the enzyme and comparisons with marker proteins of known M_r show that the subunit M_r of the enzyme is in the range 68000–72000. Preparations of enzyme obtained without the addition of phenylmethanesulphonyl fluoride were always quite unstable, as described above. They also showed double or triple close-running bands on SDS/PAGE, with the highest polypeptide M_r in the region of 62000. All this suggests that these preparations had been degraded by endogenous proteinases.

An examination of the purified enzyme by gel filtration on a calibrated column of Sephacryl S-300 showed that the sample ran as a single symmetrical peak of protein and activity. As gel filtration was not used as part of the purification procedure, this

constitutes further evidence of homogeneity. The M_r of the enzyme was estimated to be 145000. This seems to show that the enzyme is a dimer.

Purified enzyme preparations apparently contain traces of catalase. When assays of enzyme activity were made by the oxygen electrode, rather than the coupled assay method described in the Materials and methods section, the measured rates were decreased by only 1.6-fold (and not 2-fold) when catalase was included in the assay. There must have been some breakdown of H₂O₂ in the absence of added catalase. The fact that catalase is an extremely active enzyme means, however, that the level of protein contamination from this source must be extremely low.

The enzyme prosthetic group

The flavoprotein nature of the methanol (alcohol) oxidase of *Candida boidinii* (Sahm & Wagner, 1973) and *Pischia pastoris* (Patel *et al.*, 1981) suggests that the alcohol oxidase described here might also be a flavoprotein. Certainly, purified enzyme preparations at a concentration of 1 mg/ml or more are pale yellow in colour. The absorption spectrum of one sample of enzyme is shown in Fig. 2(a). The spectrum shows absorption in the 450 nm region, but does not have the three-banded appearance of the typical flavoprotein. The peak at 410 nm has been found in several preparations of the enzyme. As shown in Fig. 2(a), reduction of the enzyme with sodium dithionite bleached the colour (instantaneously) and abolished the 410 nm peak. Also, continued exposure to air resulted in the re-appearance of colour, but the original spectrum was not completely restored, the 410 nm peak being no longer evident.

Enzyme preparations exhibit a very weak green fluorescence. The fluorescence emission spectrum excited at 450 nm is characteristic of flavin (results not shown), but the excitation spectrum observed at 520 nm (Fig. 2b) is not. On boiling the enzyme for 2 min, the fluorescence (450 nm/520 nm) became about 2-fold more intense, but boiling for a further 4 min did not alter the fluorescence any further. Interestingly, the enzyme retained 12% of activity after 2 min at 100 °C, although all activity was lost after 4 min. Incubation of the heat-treated material (i.e. supernatant and any precipitated protein from 1 mg of purified enzyme) with Pronase (50 µg) for 2 h at 30 °C generated a further large increase in fluorescence (about 6-fold), and this resulted in both excitation (Fig. 2b) and emission spectra now showing the characteristic shape expected for flavin compounds. After further incubation overnight with Pronase, the fluorescence declined and the experiment was not continued. The implication of these results is that the enzyme preparations do contain flavin and that it is probably covalently bound, since treatment with a proteolytic enzyme is needed to release it. Clearly the fluorescence of the enzyme-bound flavin is severely quenched.

The flavin material released after 4 min boiling and Pronase treatment was separated from any remaining solids by centrifugation and examined by t.l.c. and h.p.l.c. On DEAE-cellulose

Fig. 2. Absorption and fluorescence spectra of alcohol oxidase samples

(a) Absorption spectrum of a sample of enzyme (1.3 mg/ml) in 50 mM-Hepes buffer, pH 8.0, +0.5% CHAPS before and after addition of sodium dithionite (approx. 1 mg added to the 1 ml sample). (b) Fluorescence excitation spectra of native alcohol oxidase in 50 mM-Hepes buffer, pH 8.0, +0.5% CHAPS and after treatment by boiling for 4 min and digestion of the boiled material with 50 μ g of Pronase for 2 h at 30 °C. The emission monochromator was set at 520 nm.

t.l.c. plates run in 4% Na_2HPO_4 , riboflavin, FMN and FAD were well separated. The flavin obtained from alcohol oxidase ran nearest to FAD, but had a slightly lower R_f value. Examination by h.p.l.c. provided a similar result. The retention times for riboflavin, FMN and FAD were 5.5, 4.25 and 2.3 min respectively, and that for the enzyme flavin was 2.5 min. Again the sample behaved most like FAD, but was still clearly different from it. Treatment of the 0.2 ml sample at room temperature with 50 μ g of *Naja naja* phosphodiesterase (Mayhew & Wassink, 1980) confirmed that the unknown flavin was not FAD. The sample showed no change in fluorescence at all on contact for 1 h with the phosphodiesterase, whereas treatment of FAD at a similar concentration for 2 min generated an approx. 5-fold increase at 450/520 nm as it was hydrolysed rapidly to FMN.

It appears most likely that the flavin samples obtained from the enzyme are covalently modified and probably have an amino acid or very short peptide substituent. More work is needed to investigate this matter further. If the fluorescence of the liberated flavin is assumed to be the same as that of FMN, it can be calculated that the enzyme sample contained 0.25 mol of flavin/mol of enzyme subunits. However, it is not clear that the sample will have such a high intrinsic fluorescence, and furthermore we do not know if the method we have used to liberate it is quantitative. It will be necessary to purify larger quantities of enzyme and examine different procedures for liberation of the flavin before the number and type of flavin groups attached to the enzyme is established.

We have tested purified enzyme preparations for the presence

of covalently bound quinonoid cofactors, using Nitro Blue Tetrazolium/glycine staining of nitrocellulose electroblots of gels produced by SDS/PAGE by the method of Paz *et al.* (1991). In these tests the single protein band gave no reaction, and there was no reaction elsewhere on the enzyme tracks. Methylamine oxidase from *Candida boidinii*, on the other hand, which is thought to be a pyrroloquinolinequinone-containing enzyme (Large & Haywood, 1990), gave a strong positive reaction. Mixing of crude detergent-solubilized extracts of alcohol oxidase-containing membranes with Nitro Blue Tetrazolium/glycine-staining reagent also gave no reaction, whereas crude extracts from methylamine-grown *Candida boidinii* again gave a strong positive reaction. These experiments provide no evidence for the involvement of quinone-type compounds in the alcohol oxidase of *Candida tropicalis*.

Kinetic studies

When assays of activity of the purified enzyme were performed by direct assay using the oxygen electrode, measured rates were, within 5%, the same as those obtained in the spectrophotometric assay. However, as the latter is much more sensitive, most of the kinetic work has been done by that method. For both assays activity is proportional to enzyme concentration over a limited range. For the spectrophotometric assay this range is 0.02–1.0 ($\Delta A_{405}^1/\text{min}$).

The purified enzyme shows maximal activity at pH 9.0. The activity at pH 7.0 is about 60% of that at pH 9.0. Membrane-bound enzyme shows similar characteristics. Attempts to find alternative electron acceptors to oxygen have been entirely unsuccessful. No activity was observed in assays containing phenazine methosulphate/2,6-dichlorophenol-indophenol, menadione or 2,6-dimethylbenzoquinone/2,6-dichlorophenol-indophenol, potassium ferricyanide, cytochrome *c*, Nitro Blue Tetrazolium or Würster's Blue.

At pH 7.0 and below the enzyme was observed to be unstable in the assays at 25 °C. At pH 6.0, for example, reaction traces were very curved as the activity declined to zero in about 3 mins. At pH 5.5 no activity was detected, presumably because of the increasingly rapid loss of viability. This instability in assays at slightly acid pH values was in marked contrast with the situation at pH 9.0, where the activity was completely stable for at least 10–15 min periods. All subsequent kinetic work has been conducted at pH 9.0.

The oxidation of alcohols by oxygen in the presence of alcohol oxidase is expected to be an essentially irreversible process. This means that the enzyme could be used to provide a quantitative assay method for long-chain alcohol substrates. An evaluation of this possibility is shown in Fig. 3 with dodecan-1-ol as substrate. It is seen that the oxidation is a biphasic process and that the amplitude of the first phase corresponds approximately to the theoretical end-point. Analysis by g.l.c. showed that the dodecan-1-ol sample was 99% pure, so that the second phase of the reaction could not realistically be ascribed to the oxidation of impurities in the substrate sample. When substrate was omitted no reaction whatever was observed, thus showing that the slow phase of the reaction was not due to impurities in other reagents. A possible explanation of the results is that the aldehyde produced in the initial phase of the oxidation is further oxidized to the corresponding acid. In this connection it is noteworthy that horse liver alcohol dehydrogenase is able to catalyse the oxidation of acetaldehyde (Dalziel & Dickinson, 1965).

That the alcohol oxidase preparations can catalyse the oxidation of long-chain aldehydes was shown by adding dodecan-1-al instead of dodecan-1-ol (Fig. 3). About 50% of the added material was oxidized in a rather slow reaction. A similar result was obtained when either tetradecan-1-al or decan-1-al was used

Fig. 3. Oxidation of dodecan-1-ol and dodecan-1-al by alcohol oxidase

Assays were conducted spectrophotometrically at 405 nm and 25 °C as described in the text. The reactions were initiated by addition of 140 units of enzyme activity (in 0.02 ml) to the 3 ml reaction mixtures. The assays contained 13.0 μ M substrate, and the theoretical end-point is indicated by the horizontal dashed line drawn across the graph. The points are added merely to help identify the curves.

Table 2. Substrate specificity of alcohol oxidase

Assays were conducted with air-saturated solutions at pH 9.0 in glycine/NaOH buffer at 25 °C. The substrate concentration was 67 μ M. Substrates (except decan-1-ol, which was dissolved in acetone) were prepared as solutions in 1,4-dioxan; 0.02 ml portions of the substrate solutions were added to 3 ml reaction mixtures.

Substrate	Relative rate (%)	$V_{\max, \text{app.}}$ (s^{-1})	$K_{\text{m, app.}}$ (μ M)
Octan-1-ol	34	11	> 1000
Decan-1-ol	70	25	31
Dodecan-1-ol	100	30	6.1
Tetradecan-1-ol	75	12	1.5
Hexadecan-1-ol	19	5	5.0
Octadecan-1-ol	12		
Decane-1,10-diol	2		
Dodecane-1,12-diol	39		
Hexadecane-1,16-diol	19		
12-Hydroxydodecanoic acid	6		
16-Hydroxyhexadecanoic acid	15		
Decan-1-al	1.3		
Dodecan-1-al	2.1		
Tetradecan-1-al	3.2		
Dodecan-2-ol	20		
Dodecan-4-ol	0		
Dodecan-5-ol	0		

instead. Again no reaction was observed in the absence of added substrate. All the aldehydes were very pure (approx. 98%) on analysis by g.l.c., and in any case the traces of impurity that were seen did not correspond to the long-chain alcohols which are substrates. The full extent of these very slow reactions with aldehydes was probably not realized. The green colour produced in the peroxidase-based assay was bleached gradually, but ultimately completely, by a secondary process which we have not

Fig. 4. Variation of the specific initial rate of decan-1-ol oxidation by oxygen at 25 °C and pH 9.0 catalysed by alcohol oxidase

Assays were performed as described in the text. The substrate was prepared as solutions in acetone, with 0.02 ml being added to the 3 ml reaction mixtures. Specific rates were calculated by using a subunit M_r of 70000 (see the text). (a) Primary double-reciprocal plots at various oxygen concentrations: $\blacktriangle$, 1.48 mM; $\triangle$, 0.31 mM; $\circ$, 0.15 mM; $\bullet$, 0.074 mM. (b) Secondary plots of the intercepts ($\bullet$) and slopes ($\circ$) from (a) versus the reciprocal of the oxygen concentration.

investigated, but which seemed to vary with different batches of peroxidase. In the present case the bleaching process occurred with a $\Delta A_{405}/\text{min}$ about one-third of that seen in the initial phase of oxidation of dodecan-1-al. Thus the reaction records of the oxidation of the long-chain aldehydes were a balance between two competing processes, and the full extent of the aldehyde oxidation reactions and the measured rates of these processes were probably significantly under-estimated.

It appears that alcohol oxidase has a small, but definite, activity towards long-chain aldehydes. Presumably it is the hydrated (*gem*-diol) forms of the aldehydes which are acted upon. An alternative explanation of the results is that the enzyme preparation was contaminated with a trace of a previously unreported aldehyde oxidase. This, however, is unlikely. Alcohol oxidase-containing membrane preparations showed about the same relative rate towards decan-1-ol and decan-1-al (100:1) as the purified enzyme (Table 2).

The results of a study of the substrate specificity of the purified enzyme are shown in Table 2. Under standard conditions (67 μM substrate, 25 °C, pH 9.0) the preferred substrates are C_{12} and C_{14} alkan-1-ols. Ethanol is not attacked at all even at very high concentrations (0.5 M) and with large enzyme concentrations. Long-chain alkanediols and ω -hydroxy fatty acids are also acted on, but at slower rates than the alkan-1-ols of the same chain length. The specificity towards secondary alcohols seems to be quite restricted, with dodecan-2-ol being a fairly active substrate and dodecan-4-ol and dodecan-5-ol being completely inactive. When dodecan-2-ol was used as a substrate (99% pure by g.l.c.) in an experiment analogous to that of Fig. 3, the reaction was apparently complete when about 40% of the substrate was used up. This reaction was completed within 8 min, and is not likely to be seriously compromised by the slow bleaching reaction described above. The result suggests, not surprisingly, that only one isomer of dodecan-2-ol is acted on. Which isomer that is remains to be elucidated. As indicated above, long-chain aldehydes are poor substrates, though recorded rates are probably under-estimates. When dodecane-1,12-diol was used in an experiment analogous to those of Fig. 3, the reaction proceeded significantly past the theoretical point for total oxidation of the alcohol groups. Thus, not only are both hydroxyl groups attacked, but the aldehyde groups are also available for oxidation. However, these additional processes would lead to the formation of 12-hydroxydodecanoic acid and dodecanoic acid semi-aldehyde. The results of Table 2 show that 12-hydroxydodecanoic acid is a rather poor substrate, and one suspects that dodecanoic acid semi-aldehyde would also be a very poor substrate.

More detailed kinetic studies have been conducted with some substrates, as indicated in Table 2. Lineweaver-Burk plots for octan-1-ol, decan-1-ol, dodecan-1-ol, tetradecan-1-ol and hexadecan-1-ol were linear, and apparent V_{max} and K_m values were obtained at pH 9.0 and 25 °C in air-saturated buffer. For dodecan-2-ol and dodecane-1,12-diol the plots were markedly convex towards the abscissa. We have not investigated reactions with these substrates any further.

Detailed kinetic studies of the oxidation of decanol in which the substrate and oxygen concentrations were varied independently were carried out at pH 9.0 and 25 °C. The results are shown in Fig. 4. The parallel lines of the primary plot indicates that a group-transfer (Ping-Pong) mechanism is in operation. This is a common situation with flavoprotein oxidases, and was therefore not unexpected. The kinetic coefficients obtained from this analysis were $V_{\text{max}} = 32 \text{ s}^{-1}$, $K_m(\text{O}_2) = 0.061 \text{ mM}$ and $K_m(\text{decanol}) = 0.042 \text{ mM}$.

Light-sensitivity of the purified enzyme

In early work with microsomal enzyme preparations it was established that the enzyme activity was unstable in blue light (Kemp *et al.*, 1990). It was not clear then if this was a rather peculiar property of the enzyme itself or if it was due to the generation of harmful agents (possibly oxygen radicals) by other component(s) of the membranes. The fact that the aldehyde dehydrogenase of the membranes was unaffected by irradiation suggested that the effect was specific and not simply due to the production of agents having a generally damaging effect. We have now repeated the experiments with purified alcohol oxidase. Fig. 5 shows that the purified enzyme is inactivated at about the same rate as when the enzyme is resident in natural membranes. The slightly slower rate of inactivation in the membrane preparation is probably not significant. It could easily be due to the greater turbidity of the membrane suspension and the less effective illumination of the sample because of scatter. The photo-sensitivity is then a property of the enzyme itself. In contrast, the FAD-containing alcohol (methanol) oxidase from *Pischia*

Fig. 5. Photo-inactivation of alcohol oxidase

Samples containing enzyme activity at a concentration of approx. 400 units/ml were incubated at 25 °C in 50 mM-Hepes buffer pH 8.0 in 1 cm path-length cells held in a thermostatically controlled spectrophotometer cell holder. The beam from the 50 W xenon lamp was attenuated by passing through a 1 cm cuvette containing water and a blue photographic filter (maximum transmission = 70% at 470 nm) before entering the enzyme compartment: ○, purified enzyme; ▲, membrane preparation.

pastoris and glucose oxidase from *Aspergillus niger* were completely stable for up to 30 min under the same conditions which produced inactivation of the alcohol oxidase of *Candida tropicalis*. It was also found that yeast and liver alcohol dehydrogenases were stable in similar illumination experiments. The yeast enzyme was inactivated over a 20 min period when approx. 10 μM -FMN was included in the illuminated mixture. On the other hand, the *Pischia pastoris* alcohol oxidase was not sensitized to light by including 10 μM -FMN.

At present we have no further information about the problem of light-sensitivity. It is, however, noteworthy that tryptic/chymotryptic digestion of trimethylamine dehydrogenase from the facultative methylotrophic bacterium W3A1 liberates a photosensitive dodecapeptide containing 6-S-cysteinylriboflavin (Steenkamp *et al.*, 1978a,b). With purified preparations of alcohol oxidase available, it should be possible to analyse enzyme samples after irradiation and determine what molecular events are responsible for the loss of activity. Such studies should provide useful information about the enzyme mechanism.

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REFERENCES

- Dalziel, K. & Dickinson, F. M. (1965) *Nature* (London) **206**, 255–257

- Hames, B. D. (1981) in *Gel Electrophoresis of Proteins: A Practical Approach* (Hames, B. D. & Rickwood, D., eds.), pp. 1–91, IRL Press, Oxford
- Haywood, G. W. & Large, P. J. (1981) *Biochem. J.* **199**, 187–201
- Kemp, G. D., Dickinson, F. M. & Ratledge, C. (1988) *Appl. Microbiol. Biotechnol.* **29**, 370–374
- Kemp, G. D., Dickinson, F. M. & Ratledge, C. (1990) *Appl. Microbiol. Biotechnol.* **32**, 461–464
- Kemp, G. D., Dickinson, F. M. & Ratledge, C. (1991) *Appl. Microbiol. Biotechnol.* **34**, 441–445
- Large, P. J. & Haywood, G. W. (1990) *Methods Enzymol.* **188**, 427–435
- Mayhew, S. G. & Wassink, J. H. (1980) *Methods Enzymol.* **66**, 217–220
- Patel, R. N., Hou, C. T., Laskin, A. I. & Derelenko, P. (1981) *Arch. Biochem. Biophys.* **210**, 481–488
- Paz, M. A., Fluckiger, R., Boak, A., Kagan, H. M. & Gallop, P. M. (1991) *J. Biol. Chem.* **266**, 689–692
- Sahm, H. & Wagner, F. (1973) *Eur. J. Biochem.* **36**, 250–256
- Smith, P., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, F. K., Goeke, N. M., Olson, R. J. & Klenk, D. C. (1985) *Anal. Biochem.* **150**, 76–85
- Steenkamp, D. J., Kenney, W. C. & Singer, T. P. (1978a) *J. Biol. Chem.* **253**, 2812–2817
- Steenkamp, D. J., McIntire, W. & Singer, T. P. (1978b) *J. Biol. Chem.* **253**, 2818–2824
- Werner, W., Rey, H.-G. & Wielinger, H. (1970) *Z. Anal. Chem.* **252**, 224–228

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