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PURIFICATION AND PROPERTIES OF METHANOL DEHYDROGENASE FROM *HYPHOMICROBIUM* X

J.A. DUINE, J. FRANK and J. WESTERLING

Laboratory of Biochemistry, Delft University of Technology, Julianalaan 67, Delft (The Netherlands)

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Summary

(1) A method for the isolation of methanol dehydrogenase (alcohol:(acceptor) oxidoreductase, EC 1.1.99.8) from *Hyphomicrobium* X is decribed. The purified enzyme was resolved by polyacrylamide gel electrophoresis into one main and two minor active bands. Iron and manganese were the only detected metals in the enzyme preparation.

(2) The substrate, methanol, was oxidized to formic acid by a stoichiometric amount of artificial electron acceptor. During the reaction, no free formaldehyde could be detected. Other primary alcohols were oxidized to the corresponding aldehydes and the aldehydes were a poor substrate or no substrate at all.

(3) Some new and efficient one-electron acceptors were found. With these electron acceptors, the enzyme had a high pH optimum and ammonia was still required in the assay system.

(4) ESR spectroscopy showed the presence of an enzyme-bound organic free radical. With X-band ESR the signal had a peak-to-peak linewidth of about 0.7 mT. The signal was further resolved by Q-band ESR and the values $g_{\parallel} = 2.0024$ and $g_{\perp} = 2.0056$ were derived.

(5) Under denaturing conditions the ESR signal and enzymatic activity disappeared at the same time as fluorescence appeared. Enzymatic activity is not restored when extracted cofactor and apoenzyme are brought together under normal conditions. Some properties of the unusual prosthetic group are presented in a preliminary form.

Abbreviations: tricine: *N*-[tris(hydroxymethyl)-methyl]-glycine; DCIP: 2,6-dichlorophenol indophenol. Compounds I and II are defined under Materials.

Introduction

Methanol dehydrogenase (alcohol:(acceptor) oxidoreductase, EC 1.1.99.8), methylamine dehydrogenase [1] and trimethylamine dehydrogenase [2] are enzymes found in methylotrophic bacteria grown on the corresponding substrate. These key enzyme reactions provide one-carbon units for assimilation and also energy by oxidation of one-carbon substrates [3]. They are NAD(P)-independent enzymes and use phenazine methosulphate as the primary electron acceptor.

However, the nature of the coenzyme, the natural electron acceptor and the mechanism of action are not known. For methanol dehydrogenase, these interesting enzymological aspects may have wider importance as this enzyme is present in bacteria, which are under investigation for their use in the production of single-cell protein or metabolites [4—6]. For instance, *Hyphomicrobium* (a facultative methylotrophic bacterium) is a component of a mixed culture growing on methane [7].

Anthony and Zatman [8] were the first to isolate a methanol dehydrogenase and to describe some properties of the enzyme from *Pseudomonas* M 27. The enzymatic activity of this primary alcohol dehydrogenase could only be detected in the presence of ammonia. The assay system was optimal at a pH of 9 while phenazine methosulphate was the only suitable acceptor. From the fluorescence properties of a substance obtained by denaturing the enzyme, methanol dehydrogenase was classified as a pteridine containing enzyme [9]. Based on this suggestion, mechanisms for catalysis were proposed [10].

In the past decade, several similar methanol dehydrogenase have been isolated from bacteria grown on methane or methanol [11—16]. Urushibara et al. [17] reported the prosthetic group to be neopterin cyclic-phosphate but, later, owing to the spectral properties of a photodegradation product, it was concluded to be a lumazine derivative [18].

In order to test the hypotheses about the prosthetic group, we developed a method for the large scale production of enzyme. As reconstitution was not obtained earlier, it was thought important to assess the purity of the enzyme and to detect the occurrence of low molecular weight compounds. Some new properties of the enzyme are reported and it is also compared with previously described preparations.

Materials and Methods

Materials

DCIP, phenazine methosulphate, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, tricine, silicagel type 60 (Merck), 2,2'-azino-di-[3-ethyl benzthiazoline-6-sulphonic acid] (Boehringer), phenazine ethosulphate, Triton X-100 (Sigma), hydroxyapatite type HTP (Biorad), DEAE-cellulose 23 (Whatman), cellulose F (Baker) and Porapak Q (Waters) were commercial products.

The one-electron acceptors compound I and compound II (free radicals) were made by one-electron oxidation of 2,2'-azino-di-[3-ethyl benzthiazoline-6-sulphonic acid] and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine, respectively. For every mmol of 2,2'-azino-di-[3-ethyl benzthiazoline-6-sulphonic acid] or

N,N,N',N'-tetramethyl-*p*-phenylenediamine, half a mmol of aqueous Br_2 was used. Compound II was isolated as the perchlorate salt according to Michaelis et al. [19]. Compound I was precipitated with acetone. For some purposes compound II was purified further by adsorbtion on CM-cellulose and elution with the desired buffer.

Growth of the organism

Hyphomicrobium X was a kind gift from Dr. W. Harder. Cells were grown with aeration in batch culture at 30° C on the medium used by Attwood and Harder [20], but with some modifications. The spore solution contained (per l): 7.8 mg $CuSO_4 \cdot 5\ H_2O$, 10 mg H_3BO_3, 10 mg $MnSO_4 \cdot 4\ H_2O$, 70 mg $ZnSO_4$ and 10 mg MoO_3. The calcium solution contained 0.25 mg $CaCl_2 \cdot 2\ H_2O$/l. The iron solution contained (per l): 3.5 g $FeCl_3 \cdot 6\ H_2O$ and 24.2 g tricine. The mineral salt medium contained (per l): 2.28 g $K_2HPO_4 \cdot 3\ H_2O$, 1.38 g $NaH_2PO_4 \cdot H_2O$, 0.50 g $(NH_4)_2SO_4$, 0.02 g $MgSO_4 \cdot 7\ H_2O$ and was brought to pH 7.0 with a 2 N NaOH solution. The solutions were autoclaved or (for iron solution and methanol) sterilized by filtration. To 1 l of mineral salt medium were added 1 ml of the spore, calcium and iron solutions and 5 ml methanol. The modifications of the additions to the mineral salt medium were essential to obtain colonies on agar plates and a good yield in the fermentor.

In the fermentor, the pH was maintained at 7 with a 25% ammonia solution. During the large scale fermentation (2000 l) the methanol concentration in the culture fluid was measured at regular intervals and readjusted twice to 0.5%. A wet-cell yield of 25 g/l was obtained and the wet cells were frozen and stored at −20° C.

Preparation of the cell extract

1 kg of thawed cell-cake was suspended in 1.4 l 0.1 M Tris · HCl/0.01 M EDTA (pH 7.0). The cells were lysed at room temperature by adding 600 mg lysozyme and the suspension was centrifuged for 10 min at 27 300 × *g*. Supernatants were discarded and the pellets suspended in 1.2 l 0.1 M Tris · HCl/ 0.5% Triton X-100 (pH 9.0). After standing for 30 min at room temperature the suspension was centrifuged as above and the supernatants stored at 4° C. The pellets were re-extracted in the same way. After two extractions, the enzyme activity in the pellets was negligible. The supernatants were combined and used immediately.

Enzyme purification

The precipitate formed from the extracts at 45—85% $(NH_4)_2SO_4$ saturation at room temperature was dissolved in a minimum of water and dialyzed overnight at 4° C against 10 l 0.02 M potassium phosphate pH 7.0 (0.02 M phosphate). The red solution was put on a silicagel column (4 × 20 cm) in 0.02 M phosphate. Cytochromes adhered to the silicagel and methanol dehydrogenase was eluted with 0.02 M phosphate.

Nucleic acids and other impurities were removed on a DEAE-cellulose column (4 × 15 cm) equilibrated with 0.02 M phosphate. The enzyme was eluted with 0.02 M phosphate and adsorbed on a hydroxyapatite column (4 × 10 cm) in 0.02 M phosphate. The column was washed with 0.02 M phos-

phate till no more fluorescing material was seen in the eluate and the enzyme was eluted with 0.1 M potassium phosphate (pH 7.0). The purified enzyme had an absorbance ratio (A_{280nm}/A_{347nm}) of 11 and was used immediately for extraction of the prosthetic group. For other purposes, it was dialyzed overnight against 0.02 M phosphate, concentrated by ultra filtration and stored at −80° C.

Prosthetic group, extraction and modification

Purified enzyme was mixed at room temperature with 9 vols. methanol and the denatured protein was removed by centrifugation. The supernatant was concentrated in a rotary evaporator. Oxidation with periodate and alkaline permanganate was done according to the literature [21,22]. Extraction of the prosthetic group under reducing conditions was performed by adding $NaBH_4$ (0.1%) to the methanol and enzyme solution.

The purity of the prosthetic group and its derivatives was checked by cellulose thin-layer chromatography with *n*-propanol/2% ammonium acetate (1 : 1, v/v).

Enzyme assay

The test was based on the system developed by Anthony and Zatman [8]. A 0.1 M sodium pyrophosphate buffer (pH 9) was used as this gave twice as much activity as the original Tris buffer. Based on our experiments with ammonia (Duine, J.A. and Frank, J., unpublished results), the test buffer contained 50 mM NH_4Cl. For experiments with a pH above 9, a $NaHCO_3$ buffer was employed.

Since phenazine methosulphate and phenazine ethosulphate are coupled to DCIP as end-acceptor, the enzymatic activity is expressed as μmol reduced DCIP/min at 21° C. The test buffer normally contained 1 mM KCN, because this partly suppressed the reoxidation of reduced DCIP. When compound I was the acceptor, KCN could not be used as this destroyed the acceptor. In the experiments with compounds I and II no end-acceptor was used and the measurements were performed at 420 and 612 nm respectively.

Analytical procedures

Methanol, ethanol, cinnamylalcohol, formaldehyde, acetaldehyde and cinnamaldehyde concentrations were estimated by gaschromatography on a Varian aerograph 2100 equipped with a flame ionisation detector. A Porapak Q column of 1.5 m length (internal diameter 2 mm) was used with N_2 as carrier gas, flowing at 30 ml/min. Column temperature was 175° C, injector- and detector temperature 200° C. Compound II was chosen as the electron acceptor because phenazine methosulphate gave decomposition products with the same retention time as methanol. There was a linear relation between peak height and methanol concentration in the working range used. 1 μl aqueous samples were injected directly into the column. Methanol was detected about 1.3 min after injection. Formic acid was determined spectrophotometrically with 2-thiobarbituric acid [23]. Care was taken to prevent the reoxidation of *N,N,N',N'*-tetramethyl-*p*-phenylenediamine by O_2 as this caused the disappearance of formic acid.

Polyacrylamide gel electrophoresis

The experiments were performed as described by Davis [24] in 5% polyacrylamide gels crosslinked with 0.17% bisacrylamide. In some experiments the gels were pre-electrophorized. Protein staining was done with Coomassie Brilliant Blue G 250. Active bands were detected with compound II or phenazine methosulphate/DCIP in 0.1 M sodium pyrophosphate/0.05 M NH_4Cl/0.01 M methanol (pH 9.0). The active bands appeared as white rings on a blue or green background, respectively.

Molecular weight estimation

A rough estimation by gel filtration was performed according to Andrews [25]. Protein was determined by the Lowry procedure with bovine serum albumin as a standard [26].

ESR spectra

The spectra were recorded on a Varian model E4 X-band spectrometer. Solutions and lyophilized enzyme were measured in a standard flat cell and in quartz capillary tubes (internal diameter 3 mm), respectively. Further resolution of the signal was first seen on an uncalibrated Q-band spectrometer at room temperature, but for determining the principal g-factors, a K_a-band (34.660 GHz) superheterodyne spectrometer at 4.2 K with a pill of enzyme powder was used (de Beer, R. and Van Ormondt, D., unpublished results).

Metal analysis

Enzyme was brought into a 0.05 M NH_4Cl/NH_3 (Suprapur, Merck) buffer (pH 9) via a Bio-Gel P-10 column. Total metal analysis was performed in an Ion Microprobe Mass Analyser (MI-TNO Apeldoorn) on gold and on platinum foil. Iron and manganese were quantitatively estimated by atomic absorption spectrophotometry. Appropriate blanks were taken around the column fractions where the enzyme appeared.

Results and Discussion

Enzyme purification

The procedure described was reproducible and convenient for large-scale enzyme production. The stable enzyme was obtained in good yield (Table I). An absorbance ratio (A_{280nm}/A_{347nm}) of 11 was found (Fig. 1), lower than the ratios estimated from published spectra [10,15,16]. The shape of the absorption spectrum is comparable to those found by others.

Although Anthony and Zatman [8] stated that methanol dehydrogenase is a soluble enzyme, our extraction conditions suggest a particle-bound enzyme, a conclusion that holds for methanol dehydrogenases from methane-grown bacteria [16,27].

From the results of polyacrylamide gel electrophoresis (Fig. 2) we deduce that the enzyme is electrophoretically pure because the bands detected with the activity test and the protein stained bands are identical and no other protein stained bands were seen. Moreover, the absorption spectrum of the main electrophoresis band (measured above 300 nm) appeared to be the same as for the purified enzyme.

TABLE I

PURIFICATION OF METHANOL DEHYDROGENASE FROM *HYPHOMICROBIUM* X

The procedure was started with 2 kg cell-cake. Activities were determined at pH 9.0 in 0.1 M sodium pyrophosphate/0.01 M methanol/0.05 M NH_4Cl/0.5 mM phenazine methosulphate/60 μM DCIP at 21°C. A volume of 3 ml of this mixture was pipetted into a cuvette containing 75 μl enzyme solution. One unit of enzyme activity = 1 μmol DCIP reduced · min^{-1}.

Purification step	Volume (l)	Total activity (units) ($\times 10^{-3}$)	Total protein * (g)	Specific activity units/mg	Yield (%)
Extract	4.80	97	—	—	100
Dialysis	0.87	66	21.6	3.1	68
Silicagel eluate	0.87	67	10.9	6.1	68
DEAE-cellulose eluate	0.88	66	9.2	7.2	68
Hydroxyapatite chromatography	0.29	48	5.9	8.1	49

* The first purification step was not tested because Triton X 100 disturbed the protein estimation.

The closely spaced banding pattern, consisting of one major and two minor bands, was reproducible for several batches of enzyme and different electrophoresis conditions. This means that either the enzyme occurs in different forms or it is an artifact of the separation procedure.

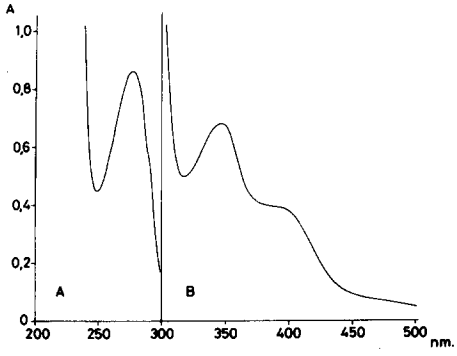

Fig. 1. The absorption spectrum of purified methanol dehydrogenase in 0.02 M potassium phosphate (pH 7.0). (A) Protein concentration 0.9 mg/ml. (B) Protein concentration 8.2 mg/ml.

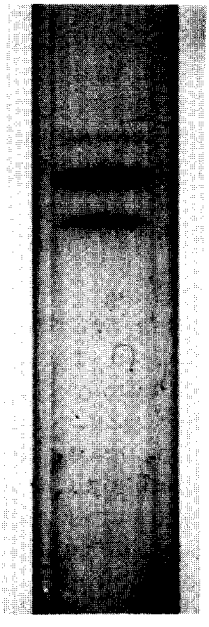

Fig. 2. Polyacrylamide gel electrophoresis of purified methanol dehydrogenase after staining with Coomassie Brilliant Blue G 250. An amount of 110 μg protein was added to the gel and electrophoresis was for 25 min.

Summarizing we conclude that our enzyme preparation, although heterogeneous, was not contaminated by other proteins.

Properties of the enzyme

When the enzyme was subjected to gel filtration with protein markers a molecular weight of 120 000 was found. This is in accordance with values of other methanol dehydrogenases (except the one isolated from *Methylosinus sporium* [16] which has a molecular weight of 60 000).

On total metal analysis of the enzyme, only the presence of iron and manganese was established. Quantitative measurements indicated that only about 1 metal atom is present per 7 enzyme molecules. The metals were also detected when 1 mM EDTA was included in the column buffer, which means that they are firmly bound to the enzyme. Therefore it was not surprising that EDTA had no influence on the enzyme test. Anthony and Zatman [8] reported that metals were absent from their enzyme preparation, although they found an inhibiting effect of EDTA and other chelators in their test [28].

Given the low metal content and the fact that all the protein bands after electrophoresis were enzymatically active we tentatively conclude that metals have no function in the enzyme activity in vitro.

Substrate oxidation in vitro

To correlate the substrate oxidation with the acceptor reduction, the methanol concentration during the reaction was estimated via gaschromatography. It appeared that, at the point where all the acceptor (compound II) was reduced, a quarter of the corresponding molar quantity of methanol was oxidized. This observation can be explained if the methanol is oxidized to formic acid. Indeed formic acid could be detected by the test with thiobarbituric acid. The reaction rate and stoichiometry in the methanol oxidation were not influenced by formaldehyde binding substances like dimedone and semicarbazide. Furthermore, because in the experiment described methanol was present in excess, it is clear that no free formaldehyde is formed and methanol is directly oxidized to formic acid. This behaviour of the enzyme can be explained by the fact that formaldehyde is a good substrate [15].

In contrast, acetaldehyde accumulated when ethanol was the substrate. At the point where all the ethanol was consumed acetaldehyde oxidation was detected but at a much slower rate.

An example of an aldehyde, that is not a substrate, is cinnamaldehyde, although cinnamyl alcohol is a better substrate than ethanol (Duine, J.A. and Frank, J., unpublished results). Sperl et al. [12] have investigated some aldehydes as substrate and concluded that only hydrated aldehydes are a substrate.

The direct oxidation of methanol to formic acid was rather unexpected. In methylotrophic bacteria several formaldehyde oxidizing enzymes are present [3]. Moreover, methanol dehydrogenase has a task to supply formaldehyde for biosynthesis. From the results described here, no explanation can be given how this is regulated in the cell.

Electron acceptors

Several of the usual electron acceptors were tested but, as was already

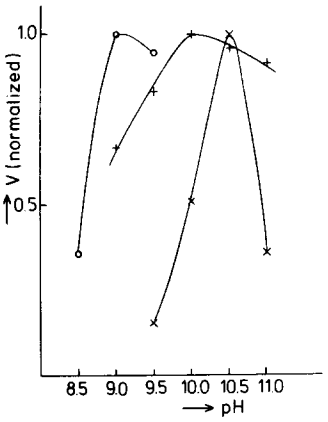

Fig. 3. pH optimum of methanol dehydrogenase with different electron acceptors. Conditions are given under materials and methods and in Table I. (o-----o, phenazine methosulphate; +-----+, compound I; X-----X, compound II). Compound I and II at a concentration of 60 μM and 0.1 mM respectively.

known, only phenazine methosulphate [28] and also phenazine ethosulphate (Fig. 3) sufficed. When phenazine methosulphate was dissolved in the test buffer a large amount of radicals was formed (as found by ESR). This is due to nucleophilic attack at high pH [29]. We therefore considered the possibility that those radicals are the acceptor, rather than phenazine methosulphate itself.

A search was made in the literature for water-soluble stable radicals with a satisfactory redox-potential. Compound II is a positively charged stable radical [19]. Its reduced form is employed as electron donor for cell respiration studies [30] but, to our knowledge, we are the first to show its use as an electron acceptor for methanol dehydrogenase. The same applies to compound I, a radical with an overall negative charge [31]. The reduced form has been advocated as an electron donor for peroxidases [32]. Like phenazine methosulphate these new electron acceptors required ammonia in the test but the enzyme had now a high pH optimum (Fig. 3). The pH optimum with phenazine methosulphate was not exactly definable because above pH 9, decomposition increased considerably and interfered with the test. Compound I and II also decompose at high pH, but not as rapidly as phenazine methosulphate thus, with appropriate blanks, measurements were possible. At pH 11, enzyme denaturation took place.

The results with the free radicals brought us to the question whether electron acceptors, which form stable radicals after one-electron reduction, are active. Indeed, chloranil (tetrachloro-*p*-benzoquinone) appeared to function as an acceptor. After the test mixture had reacted, the absorption spectrum showed the presence of the semiquinone form [33]. No further experiments were undertaken because chloranil is not very soluble.

Considering the results with chloranil, the activity of phenazine methosulphate can also be explained by regarding it as an acceptor with a stable one-electron reduced form. The com- and disproportionation reactions between oxidized, fully reduced and half reduced forms of phenazine methosulphate, phenazine ethosulphate and chloranil makes a statement about their func-

tioning under these conditions impossible. But it is clear that in the case of compound I and II, where the equilibrium lies strongly in favour of their radical state, only one-electron steps are possible. In this light, it is remarkable that a substance like potassium ferricyanide is not an acceptor, although its redox potential and dimensions seem acceptable. A certain degree of lipophilic or aromatic character may be necessary.

ESR spectroscopy

In all stages of the purification an ESR signal, with the same properties as was found for the pure enzyme, accompanied the active enzyme fractions. It appeared that the X-band signal was slightly asymmetric with a peak-to-peak linewidth of 0.7 mT.

Sometimes ESR at a higher microwave frequency, having a better resolving power, can give information about the origin of the asymmetry. From Fig. 4 it is clear that only one radical is present in the enzyme with $g_{//} = 2.0024$ and $g_{\perp} = 2.0056$ and that the asymmetry in the X-band signal is caused by overlapping $g_{//}$ and $g_{\perp}$ lines. From these data we can calculate that $g_{iso} = 2.0045$. On scanning a wide range of the spectrum at room temperature or at 4.2 K no further signals were seen.

The saturation behaviour and the absence of hyperfine structure in the spectrum suggest a protein-bound organic free radical. Methanol addition had no influence neither on the ESR signal nor on the optical absorption spectrum. As we shall prove (Duine, J.A. and Frank J., unpublished results) this phenomenon may be explained by the fact that the enzyme is in the reduced form.

Considering the linewidth and g_{iso} value, it is improbable that the signal can be attributed to a pteridine [36,37] or flavin [34,35] radical, where the spectrum width [34—36] and *g*-value [37,38] are larger. On the other hand these values are in excellent agreement with the data for quinone radicals [39]. In a forthcoming paper we will demonstrate that the signal can be related to the prosthetic group and the mechanism of action of the enzyme.

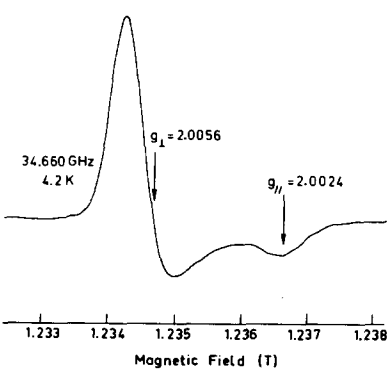

Fig. 4. The ESR-signal of methanol dehydrogenase at K_a-band frequency. Lyophilized enzyme was compressed to a pill. The *g*-values were estimated at the points indicated with arrows.

Properties of the prosthetic group

Enzymatic activity was not influenced by dialysis but was totally destroyed by heat denaturation, buffers with high or low pH and polar liquids such as methanol or dimethyl sulphoxide. In these circumstances a green fluorescence appeared and the ESR signal was lost.

The prosthetic group, isolated by methanol extraction, was chromatographically pure with a fluorescent spot at R_f = 0.33. The spot could also be detected by spraying with reduced Methylene Blue. The absorption spectrum is shown in Fig. 5 and is in the range of the spectrum of the enzyme. As the enzyme was pure and no other low molecular weight compounds were detected we consider this fluorescing compound to be the prosthetic group. On standing at room temperature for 2 days, the extracted substance had completely changed to a blue fluorescing compound with an R_f of 0.63 and a different absorption spectrum (Fig. 5). This process could not be accelerated by oxygen, H_2O_2 or light and we have no explanation for this behaviour.

After oxidation with alkaline permanganate no absorption spectrum could be detected. This shows that the cofactor is not a folate [10] or neopterine cyclic-phosphate [17], because in these cases a distinct spectrum is expected [22]. However, oxidation with periodate produced a vivid blue fluorescing substance with little change of the absorption spectrum and with consumption of periodate. We suggest that the cofactor contains a polyol or sugar moiety which can be oxidized by periodate.

Reduction with $NaBH_4$ under an argon atmosphere gave a non-fluorescing product with the spectrum shown in Fig. 6. Upon admission of oxygen this changed to a blue-green fluorescing substance with a spectrum different from that of the extracted prosthetic group (Fig. 6). This substance was stable and gave an R_f of 0.33 under chromatography.

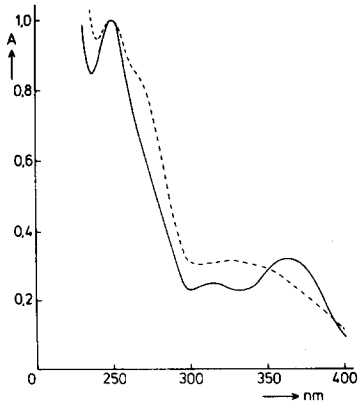

Fig. 5. Absorbance spectrum of the extracted prosthetic group and its change on standing. (- - - - - -, extracted prosthetic group; ———, the same after standing for one day at room temperature).

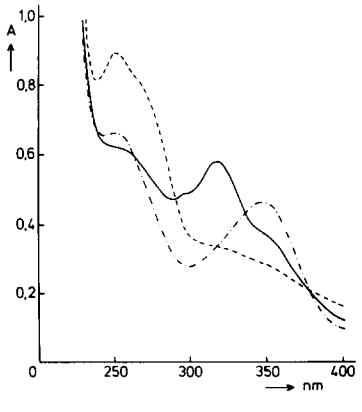

Fig. 6. Reoxidation of the reduced prosthetic group. (- - - - -, extracted prosthetic group; ———, reduced with $NaBH_4$; — · — · —, reoxidation with O_2).

All the results point to a prosthetic group which in its extracted form is in the oxidized state and can be reduced by different substances. Since in the isolated enzyme the prosthetic group is in the reduced form (Duine, J.A. and Frank, J., unpublished results), it is understandable why all trials for reconstitution were unsuccessfull [10]. Therefore further work is necessary to prove the nature and rôle of the extracted fluorescing substance.

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References

1 Eady, R.R. and Large, P.J. (1968) Biochem. J. 106 245—255
2 Colby, J. and Zatman, L.J. (1973) Biochem. J. 132, 101—112
3 Quale, J.R. (1972) Adv. Microb. Physiol. 7, 119—203
4 Sheehan, B.T. and Johnson, M.J. (1971) Appl. Microbiol. 21, 511—515
5 Cooney, C.L. and Levine, D.W. (1972) Adv. Appl. Microbiol. 15, 337—365
6 Sato, K., Ueda, S. and Shimizu, S. (1977) Appl. Environ. Microbiol. 33, 515—521
7 Wilkinson, T.G., Topiwala, H.H. and Hamer, G. (1974) Biotechnol. Bioeng. 16, 41—59
8 Anthony, C. and Zatman, L.J. (1967) Biochem. J. 104, 953—959
9 Anthony, C. (1971) Methods. Enzymol. 18, part B, 803—813
10 Anthony, C. and Zatman, L.J. (1967) Biochem. J. 104, 960—969
11 Heptinstall, J. and Quale, J.R. (1970) Biochem. J. 117, 563—572
12 Sperl, G.T., Forrest, H.S. and Gibson, D.T. (1974) J. Bacteriol. 118, 541—550
13 Mehta, R.J. (1973) J. Microbiol. Serol. 39, 303—312
14 Goldberg, I. (1976) Eur. J. Biochem. 63, 233—240
15 Patel, R.N., Bose, H.R., Mandy, W.J. and Hoare, D.S. (1972) J. Bacteriol. 110, 570—577
16 Patel, R.N. and Felix, A. (1976) J. Bacteriol. 128, 413—424
17 Urushibara, T., Forrest, H.S., Hoare, D.S. and Patel, R.N. (1971) Biochem. J. 125, 141—146
18 Sperl, G.T., Forrest, H.S. and Gibson, D.T. (1973) Bacteriol. Proc. 1973, 151
19 Michaelis, L. and Granick, S. (1943) J. Am. Chem. Soc. 65, 1747—1755
20 Attwood, M.M. and Harder, W. (1972) J. Microbiol. Serol. 38, 369—378
21 Dixon, J.S. and Lipkin, D. (1954) Anal. Chem. 26, 1092
22 Forrest, H.S. (1971) Methods Enzymol. 18, part B, 599—600
23 Barker, S.A. and Somers, P.J. (1966) Carbohydrate Res. 3, 220—224
24 Davis, B.J. (1964) Ann. N.Y. Acad. Sci. 121, 404—427
25 Andrews, P. (1964) Biochem. J. 91, 222—233
26 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
27 Wadzinski, A.M. and Ribbons, D.W. (1975) J. Bacteriol. 122, 1364—1374
28 Anthony, C. and Zatman, L.J. (1965) Biochem. J. 96, 808—812
29 McIlwain, H.J. (1937) J. Chem. Soc. 1937, 1704—1711
30 Jurtshuk, P., Marcucci, O.M. and McQuitty, D.N. (1975) Appl. Microbiol. 30, 951—958
31 Hünig, S., Balli, H., Conrad, H. and Schott, A. (1964) Justus Liebigs Ann. Chem. Bd 676, 36—51
32 Childs, R.E. and Bardsley, W.G. (1975) Biochem. J. 145, 93—103
33 Kawai, K., Shirota, Y., Tsubomura, H. and Mikawa, H. (1972) Bull. Chem. Soc. Japan 45, 77—81
34 Palmer, G., Müller, F. and Massey, V. (1971) in Flavins and Flavoproteins (Kamin, H. ed.), pp. 123—136, University Park Press, Baltimore
35 Westerling, J., Mager, H.I.X. and Berends, W. (1975) Tetrahedron 31, 437—440
36 Westerling, J., Mager, H.I.X. and Berends, W. (1977) Tetrahedron 33, 2587—2594
37 Bobst, A. (1968) Helv. Chim. Acta 51, 607—613
38 Eriksson, L.E.G. and Ehrenberg, A. (1964) Acta Chem. Scand. 18, 1437—1453
39 Hales, B.J. (1975) J. Am. Chem. Soc. 97, 5993—5997