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The Prosthetic Group of Methanol Dehydrogenase

PURIFICATION AND SOME OF ITS PROPERTIES

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Methanol dehydrogenases isolated from bacteria belonging to different classes of methylotrophs contain the same prosthetic group. A procedure for its purification from whole cells is given. The reduced and oxidized form of the enzyme from *Hyphomicrobium* X and those of the isolated prosthetic group are compared and it is concluded that the latter indeed functions in the enzyme. Further evidence is presented that the prosthetic group is not a pterine or lumazine derivative, but a water-soluble nitrogen-containing quinone.

Methanol dehydrogenase [alcohol–(acceptor) oxidoreductase, EC 1.1.99.8] is an unusual enzyme because it is neither an NAD(P)-dependent nor a flavin-containing dehydrogenase. It contains a chromophoric group, giving the enzyme an absorption maximum at 340–350nm. Under denaturing conditions a fluorescing compound appears, which has been considered to be the prosthetic group (Anthony & Zatman, 1967*b*). However, the addition of methanol to the enzyme does not change its absorption spectrum. At the same time, reconstitution of the enzyme from apoenzyme and the resolved prosthetic group has failed so far.

The chemical nature of the prosthetic group is also not clear. Anthony & Zatman (1967*b*) extracted it from methanol dehydrogenase of *Pseudomonas* M27. Owing to its spectral properties, they suggested that it was a pteridine derivative and the enzyme was classified as such (Anthony, 1971; Walsh, 1978). In a study by Urushibara *et al.* (1971) it was tentatively identified as neopterin cyclic phosphate. Based on the properties of a photodegradation product of the prosthetic group, Sperl *et al.* (1973) reported it to be a lumazine derivative. On the other hand, Duine *et al.* (1978) concluded from e.s.r. measurements of methanol dehydrogenase from *Hyphomicrobium* X that the prosthetic group had a quinone structure.

Because of this disagreement it was necessary to study other methylotrophic bacteria and to see whether our previous proposal could be further substantiated. Since an oxidized form of methanol dehydrogenase has been obtained (Duine & Frank, 1980), it was possible to compare the spectral properties of the reduced and oxidized form of the prosthetic group with those of the enzyme, to ascertain the involvement of the former in the catalysis by the enzyme.

Abbreviations used: h.p.l.c., high-pressure liquid chromatography; e.n.d.o.r., electron nuclear double resonance.

Materials and Methods

Chemicals

All chemicals were from E. Merck Nederland B.V., Amsterdam, The Netherlands, except for Amberlyst A21 (50–100μm, analytical grade), a weakly basic macroporous anion exchanger, which was from Serva, Heidelberg, Germany, and Seppak C18-silica cartridges, which were from Waters Associates, Etten-Leur, The Netherlands.

Before use, Amberlyst was pretreated. After removal of fines, it was washed with 3vol. of 4M-NaCl followed by 2vol. of water. The ion-exchanger was stored in methanol/water (9:1, v/v).

Growth of the organisms

Methylobacterium organophilum strain xx, LMD 78.41, was obtained from the Laboratory of Microbiology Culture Collection, Delft University of Technology, and was grown in the same way as *Hyphomicrobium* X (Duine *et al.*, 1978). *Rhodopseudomonas acidophila* 10050 was provided by Professor J. R. Quayle, Department of Microbiology, University of Sheffield, Sheffield, U.K., and was cultured as described by Sahm *et al.* (1976). Wet cells of *Paracoccus denitrificans* LMD 22.21, grown on methanol, were a gift from Mr. W. Hazeu, Laboratory of Microbiology, Delft University of Technology. *Methylophilus methylotrophus* was obtained in the form of Pruteen and was a gift from

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ICI, Billingham, U.K. It is noteworthy that this last mentioned material was only used for extraction of the prosthetic group because it no longer contains active methanol dehydrogenase.

Purification of the methanol dehydrogenases

The enzymes were purified by a reported procedure (Duine *et al.*, 1978), which was modified in the case of *Rh. acidophila* and *Methylobacterium organophilum* (J. A. Duine & J. Frank, Jr., unpublished work). Purity was checked by polyacrylamide-gel electrophoresis and, in some cases, the prosthetic group was extracted directly from the active band.

Purification of the prosthetic group

From purified enzyme. A methanol dehydrogenase solution (100ml) containing 14mg of protein/ml was mixed with 900ml of methanol. The resulting suspension was centrifuged and the supernatant was passed through a column (2.5 x 1.2cm) of 5g of pretreated Amberlyst. The column was washed with 10ml of methanol/water (9:1, v/v) and eluted with 15ml of a solution of methanol/water (1:1, v/v) saturated with NaCl (at room temperature). The eluate was freed from methanol in a rotary evaporator under reduced pressure. The remaining solution was stored at 0°C and, usually after one night, the prosthetic group separated from the fluid as a brick-red precipitate.

From whole cells. Wet cells were extracted with 9vol. of methanol and 1 litre of supernatant was passed through a column (2.5 x 1.2cm) of 5g of pretreated Amberlyst. The column was washed with 10 bed volumes of 0.5M-NaCl in methanol/water (1:1, v/v) to remove flavins and other impurities. After that, the prosthetic group was eluted with 15ml of a solution of methanol/water (1:1, v/v) saturated with NaCl. Fractions were analysed by h.p.l.c., pooled and passed through a Seppak C18-silica cartridge to remove impurities. When the cartridge had become completely brown it was replaced by a new one. Methanol was removed from the filtrate as described above and the remaining fluid was adjusted to pH2.0 with conc. HCl. The solution was passed through a clean Seppak C18-silica cartridge, which now retained the prosthetic group. The yellow–orange band was eluted from the cartridge with 12.5mM-KH_2PO_4 (adjusted with conc. H_3PO_4 to pH2.0)/methanol (8:3, v/v). Methanol was evaporated from the eluate and the remaining solution was passed through a new cartridge. After washing with dilute HCl, pH2.0, the prosthetic group was eluted with 2ml of methanol/water (7:3, v/v). The methanol was removed, the solution saturated with NaCl at room temperature and stored at 0°C. The precipitate was dissolved in a small amount of water, the solution adjusted to pH2.0 with dilute HCl and subjected to preparative h.p.l.c. by injecting up to 200µl at a time. The prosthetic group-containing fractions were pooled and freed from phosphate by using a cartridge as already described. The purified prosthetic group was stored in methanol/water (7:3, v/v) at −20°C.

Reduction of the prosthetic group

A solution of the prosthetic group in 500µl of 0.01M-tetrasodium pyrophosphate, brought to pH9.0 with conc. HCl, was transferred to a cuvette and flushed with N_2 for 15min. After recording the u.v.–visible spectrum, 5µl of 0.05% (v/v) 2-mercaptoethanol in water was added. The cuvette was stoppered and the absorption at 310nm was followed with respect to time. When the absorption showed no further increase, the reduction was considered complete. At that point, the fluorescence of the solution had almost disappeared.

Reaction of the prosthetic group with aldehydes/ketones

A solution of the prosthetic group in water was adjusted to pH9.0 with dilute ammonia and aldehyde or ketone was added to 5% (v/v) final concentration. The prosthetic group was completely converted in about 30min, as judged by h.p.l.c.

H.p.l.c. analysis

This was performed with a Waters model 6000 A pump, equipped with an U6K injection block and an analytical µBondapak C18 column, using 12.5mM-KH_2PO_4 (adjusted to pH2.0 with conc. H_3PO_4)/methanol (8:3, v/v) as the eluent. The flow rate was 1.5ml/min at a pressure of 8MPa. The effluent was monitored with a model 440 absorption detector, operating at 254nm.

Analytical methods

The spectra for u.v. and visible absorption were recorded on a Beckman UV 5260 spectrophotometer. E.s.r. measurements were done under the conditions described by Westerling *et al.* (1979). The n.m.r. spectrum of the prosthetic group (0.5mg) in ^2H_2O with 3-(trimethylsilyl)propanesulphonic acid sodium salt as an internal standard was run on a Varian SC 300 spectrometer operating at 300MHz. The i.r. spectrum of 0.3mg of prosthetic group, pressed with KBr to a disc, was recorded with a Hilger and Watts Infrascan spectrometer. Phosphate was determined by the method of Eibl & Lands (1969). Colour tests for quinones were done by the method of Sawicki & Elbert (1960) and Druey & Schmidt (1950).

Results and Discussion

Extraction and purification of the prosthetic group

Although the prosthetic group can be extracted from the enzyme with methanol in a chromatographically pure form, it is unstable on storage (Duine *et al.*, 1978). However, after treatment of the extract with Amberlyst, a stable preparation was obtained. Furthermore, as it was found that Amberlyst has a high adsorption capacity for the prosthetic group and material adsorbed to it remained stable, this isolation step is very well suited for a large-scale extraction from whole cells.

H.p.l.c. was not only used to analyse prosthetic group-containing fractions, but also for the final purification of material extracted from whole cells. Pretreatment of the samples for h.p.l.c. by means of C18-silica cartridges and the precipitation with NaCl protected the h.p.l.c. column from heavy contamination and provided an excellent prepurification. The chromatographically (by h.p.l.c.) pure preparations were also judged pure by n.m.r.

Comparison of the prosthetic groups from methanol dehydrogenases of different organisms

In general, the absorption spectra of methanol dehydrogenases from different methylotrophic bacteria have the same appearance. Although this suggests that the prosthetic groups are also the same, this point was further checked. Some methylotrophs with a normal dehydrogenase [showing the high pH optimum and need for ammonia, as was first described by Anthony & Zatman (1967*a*)], but differing widely in growth habitat and morphology, were chosen. *Hyphomicrobium* X is a facultative methylotrophic budding bacterium and the enzyme has been described previously (Duine *et al.*, 1978). *P. denitrificans* is a facultative chemolithotroph, able to grow autotrophically on H_2 or methanol (Bamforth & Quayle, 1978*a*). *Methylophilus methylotrophus* is an obligate methylotrophic organism growing on methanol (Ghosh & Quayle, 1978).

Besides these organisms, those with a more or less diverging enzyme were also tested. Methanol dehydrogenase from *Rh. acidophila* (a phototroph growing on methanol) with a normal absorption spectrum has somewhat different properties (Bamforth & Quayle, 1978*b*). It was reported that although the absorption spectrum of the enzyme from *Methylobacterium organophilum* (a facultative methylotroph, also growing on multi-carbon substrates like monosaccharides) was normal, the fluorescence characteristics were different from those reported for the prosthetic group by Anthony & Zatman (1967*b*). This led the authors to the conclusion that the prosthetic group may have a different structure (Wolf & Hanson, 1978). It is noteworthy, however, that the fluorescence excitation and emission maxima they give for the enzyme (285–290 and 355–360nm respectively) are characteristic of aromatic amino acids in a protein.

Analysis by h.p.l.c. of the prosthetic groups of methanol dehydrogenase from these organisms revealed that they all co-chromatographed with the purified prosthetic group from *Hyphomicrobium* X (Fig. 1*a*). Moreover, they gave the same products after reaction with carbonyl compounds, as judged by the characteristic change in retention time after the reaction with, for example, acetone (Fig. 1*b*).

For the product from Pruteen, additional confirmation was obtained from the e.s.r. spectrum, which was found to be identical with the known hyperfine-structure spectrum of the prosthetic group from *Hyphomicrobium* X enzyme (Westerling *et al.*, 1979). Comparison of the n.m.r. spectra led to the same conclusion. From these results we conclude that there are no reasons to believe that methanol dehydrogenases have different prosthetic groups.

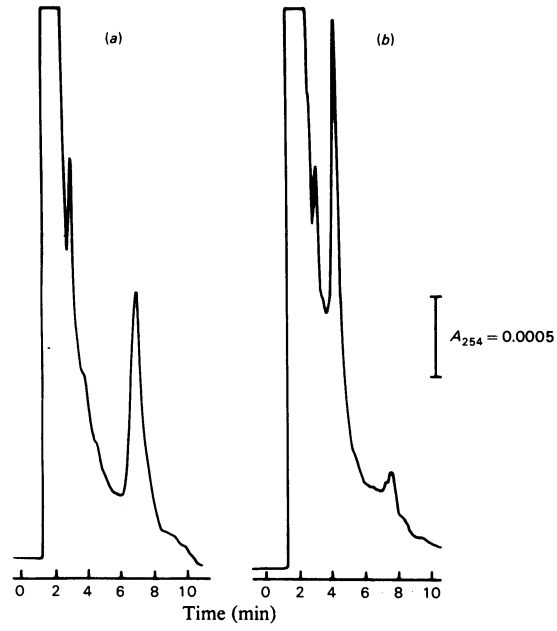

Fig. 1. *H.p.l.c. chromatograms of the prosthetic group from Hyphomicrobium X enzyme and its acetone derivative*

(*a*) A prosthetic group-containing fraction of an extract from whole cells after the Amberlyst purification step. The prosthetic group (10μl injected) has a retention time of 6.8min. (*b*) After reaction of the sample, shown in Fig. 1(*a*), with acetone. A solution of the prosthetic group in water was adjusted to pH9.0 with dilute ammonia and acetone was added to 5% (v/v) final concentration. After 30min, 10μl of the solution was injected. The acetone derivative has a retention time of 4.2min.

Therefore, the conflict in the literature must have other causes.

Anthony & Zatman (1967*b*) purified the prosthetic group from the enzyme of *Pseudomonas* M27 by TEAE-cellulose chromatography. The absorption spectrum of the material they obtained gave a maximum at 250nm, a shoulder at 275nm and a low broad maximum around 350nm. However, the fluorescence-excitation maxima were situated at 255 and 365nm.

Fig. 2 shows the absorption spectrum of the prosthetic group extracted from *Hyphomicrobium* X. As mentioned previously, this preparation no longer exhibits a 'spontaneous' change on storage. Several carbonyl compounds like acetaldehyde, propionaldehyde and acetone were found to react with the prosthetic group. Each time an absorption spectrum (Fig. 3) was obtained identical with the one found after the 'spontaneous' change (Duine *et al.*, 1978). Analysis by h.p.l.c. showed, however, that this spectrum was displayed by chromatographically different products. The retention time of the product with acetone is 4.2min (Fig. 1*b*), whereas the products with acetaldehyde and propionaldehyde have retention times of 3.8 and 9.8min respectively (results not shown). After the reaction, the green fluorescence of the prosthetic group was changed to a more intense blue. The increase in intensity depended on the carbonyl compound used, propionaldehyde being the most effective. These observations suggest an addition type of reaction.

The differences between the maxima of the absorption spectrum of the prosthetic group described in the present paper and the excitation maxima of the one isolated from *Pseudomonas* M27 (Anthony & Zatman, 1967*b*) might be explained by assuming that in the latter case a partial transformation of the type described above had occurred. The reported fluorescence-excitation maxima are then most likely due to the product of transformation, being the dominant fluorescing compound in the mixture. On the other hand, from arguments given below, we consider the substance giving an absorption spectrum as shown in Fig. 2 and a retention time of 6.8min in Fig. 1(*a*) as the real prosthetic group.

The function in methanol dehydrogenase

Reaction of 2-mercaptoethanol with the prosthetic group results in the disappearance of the green fluorescence and at the same time a product is formed with a different spectrum (Fig. 4). Admission of O_2 reverses this spectral change and restores the green fluorescence. These spectral features can

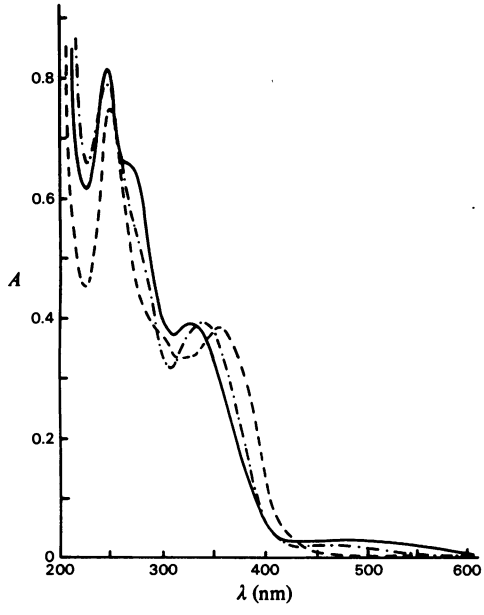

Fig. 2. *Absorption spectra of the purified prosthetic group from Hyphomicrobium X enzyme at different pH values*

–·–·–, Spectrum in 0.05M-KCl solution, titrated with concn. HCl to pH 1.0; ——, spectrum in 0.05M-potassium phosphate buffer, pH 7.0; – – – –, spectrum in 0.05M-KCl, titrated to pH 13.0 with conc. NaOH.

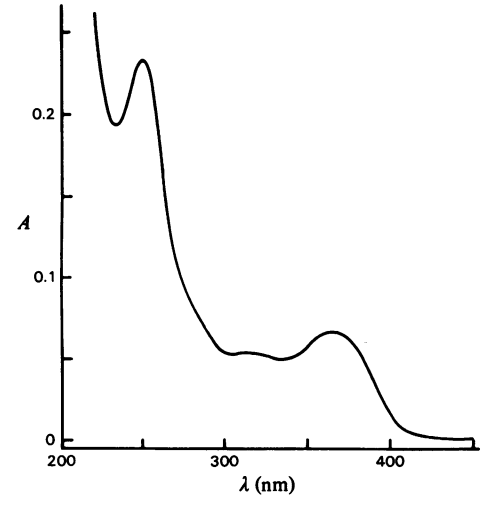

Fig. 3. *Absorption spectrum of an acetone derivative*

After reaction of the purified prosthetic group from *Hypomicrobium* X with acetone, the product was purified by Amberlyst chromatography and adsorbed to a Seppak cartridge. It was eluted with 1ml of methanol/water (7:3, v/v). The methanol was evaporated and the solution was brought to 0.05M-potassium phosphate, pH 7.0.

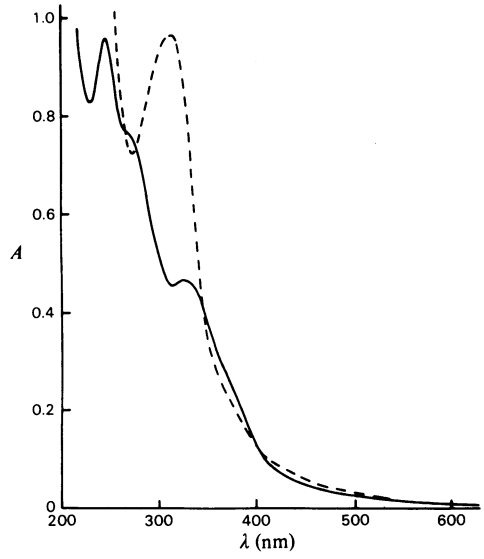

Fig. 4. *Reduction of the purified prosthetic group from Hyphomicrobium X enzyme with 2-mercaptoethanol*
——, Spectrum of the prosthetic group in 0.01 M-tetrasodium pyrophosphate, pH 9.0; ----, spectrum after the addition of 2-mercaptoethanol to 0.005% (v/v) final concentration.

be expected for a quinone. For instance, 1,4-naphthoquinone has an absorption maximum at 250 and 330 nm, coupled with increased absorption at about 330 nm in the reduced state and decreased absorption below 300 nm.

Owing to the presence of 2-mercaptoethanol, the decrease in the region below 300 nm cannot be measured (Fig. 4), but it can be seen on reduction with $NaBH_4$ (Duine *et al.*, 1978). In the latter case, however, some irreversible reduction also occurs.

There is a difference (25 nm) between the absorption maximum of the reduced prosthetic group and the methanol dehydrogenase reduced by substrate, and a still larger shift (about 50 nm) is found for the maximum of an oxidized form of methanol dehydrogenase (Duine & Frank, 1980) and the prosthetic group (Figs. 2 and 4). So the question arises whether there is any relationship between the chromophore in the enzyme and the isolated prosthetic group, although exceptionally large shifts have been reported in the literature e.g. for pyridoxal phosphate (Snell *et al.*, 1968). But on denaturing the oxidized form of methanol dehydrogenase by heat, the normal prosthetic group was obtained and no further compound absorbing above 300 nm was found. Therefore the shift of the maximum to about 400 nm may be due to an interaction of the quinone part of the molecule with amino groups in the protein, an interaction that has been studied with model compounds like chloranil and amino acids by Birks & Slifkin (1963). Of course, other interactions may play a role because a change in the pH (Fig. 2) or the addition of carbonyl compounds (Fig. 3) also shifts the maximum to higher wavelengths.

It is also possible that the prosthetic group has become modified during the extraction and purification. However, reconstitution of activity has been found for an apoenzyme of glucose dehydrogenase (containing the same prosthetic group) from *Acinetobacter calcoaceticus* and the purified prosthetic group (Duine *et al.*, 1980). Moreover, the hyperfine structure of the e.s.r. spectrum of the free radical removed from the enzyme is exactly the same as that induced in the purified prosthetic group by reduction (Westerling *et al.*, 1979).

As no other low-molecular-weight compounds were detected in the enzyme we conclude that the extracted fluorescing compound described in the present paper is in fact the prosthetic group, which functions in the enzyme as the primary hydrogen acceptor.

The nature of the prosthetic group

On the basis of e.s.r. measurements, the prosthetic group is not a pterine or lumazine, but probably a quinone derivative (Duine *et al.*, 1978; Westerling *et al.*, 1979). Strong absorptions in the i.r. spectrum at 1678 and 1710 cm^{-1}, indicating the presence of carbonyl groups, are compatible with such a concept. The n.m.r. spectrum of the prosthetic group in ^2H_2O shows two singlets at 7.14 and 8.16 p.p.m., indicating the presence of two aromatic protons not coupled to each other. The latter result is in agreement with e.s.r. measurements of the prosthetic group (Westerling *et al.*, 1979) and e.n.d.o.r. measurements of the enzyme (De Beer *et al.*, 1979), indicating the presence of three protons (one of which is exchangeable) and two nitrogen atoms in the molecule.

The two colour tests for quinones were positive. Even more can be deduced from the tests, because the 3,4-dimethoxyaniline reagent is claimed (Sawicki & Elbert, 1960) to be specific for inner-ring quinones.

The fact that the prosthetic group is strongly adsorbed to a weakly basic ion-exchanger means that it must be a fairly strong acid. Indeed a pK_a of 2.5 can be estimated from the spectral changes occurring around pH 2.5 (Fig. 2). As no phosphate could be detected, one or more carboxy groups are most probably responsible for this acid character. Around pH 10 another spectral change occurs (Fig. 2), as expected for a nitrogen-containing substance.

From the properties described in the present paper, it can be concluded that the prosthetic group of methanol dehydrogenase is different from any other known cofactor. The presence of nitrogen

atoms and carboxy group(s) can explain its solubility in water. Clearly this compound is not directly comparable with the known quinones active in the electron-transport chain. As large-scale extraction of whole cells is now possible, an important drawback in the elucidation of the structure of the prosthetic group has been removed.

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