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IDENTIFICATION OF THE PROSTHETIC GROUPS OF DIMETHYLAMINE DEHYDROGENASE FROM HYPHOMICROBIUM X

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Summary: Trimethylamine dehydrogenase (TMADH) and dimethylamine dehydrogenase (DMADH) were purified from Hyphomicrobium X. The absorbance spectra of the two enzymes were similar with λmax = 443 nm for TMADH and 440 nm for DMADH. DMADH had an apparent molecular weight of 138,000 daltons and was composed of two subunits of similar molecular weights. DMADH contained 3.91 atoms S and 4.55 atoms Fe per mole of the enzyme. Both DMADH and TMADH contained a covalently bound yellow coenzyme. The coenzyme-peptides obtained from DMADH and TMADH of Hyphomicrobium X by tryptic-chymotryptic digestion were partially purified and found to differ electrophoretically and chromatographically from the coenzyme-peptide obtained similarly from TMADH of bacterium W_3A_1. After digestion with aminopeptidase M the aminoacyl-coenzymes from the three enzymes had identical spectral, electrophoretic and chromatographic properties. DMADH is only the second enzyme yet found to contain 6-S-cysteinyl-FMN as coenzyme. Dissimilarities between the coenzyme-peptides of DMADH and TMADH from either Hyphomicrobium X or bacterium W_3A_1 are consequently located in the peptide component.

The utilization of methylated amines as sole source of carbon by methylotrophic bacteria proceeds by sequential oxidative demethylation to yield finally formaldehyde and ammonia (1). Dehydrogenases catalyzing the oxidative demethylation of trimethylamine and methylamine have been described (2,3). Trimethylamine dehydrogenase (EC 1.5.99.1) from bacterium W_3A_1 (4) was found to contain a novel covalently bound flavin coenzyme, 6-S-cysteinyl-FMN (5,6) and also a tetrameric Fe_4S_4 cluster (7) as redox active groups. The unusual properties of the coenzymes of the methylamine (8,9) and methanol dehydrogenases (10,11,12) have also been described by several workers, but despite a number of attempts at their structural elucidation (8,9,12,13,14) the chemical nature of these coenzymes is uncertain.

Until recently the oxidative demethylation of dimethylamine by methylotrophic bacteria was thought to depend exclusively on the presence of a secondary amine monooxygenase system in these organisms (15). In recent years, however, methylotrophs capable of anaerobic growth on dimethylamine

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as carbon source were isolated (16) and further studies showed the presence of a dimethylamine dehydrogenase as distinct from trimethylamine dehydrogenase (17) in Hyphomicrobium X. It was, therefore, of interest to assess whether this new dehydrogenase contains a coenzyme similar to that found in any of the other unusual dehydrogenases of methylotrophic bacteria.

METHODS

Hyphomicrobium X was a gift from Dr W. Harder of the University of Groningen, Holland and was grown by batch culture under anaerobic conditions using 0.2% w/v dimethylamine as sole source of carbon in the mineral base of Duine, et al. (12) with 0.5% KNO_3 w/v as electron acceptor. Bacterium W_3A_1 was cultured as described (2,4). The trimethylamine and dimethylamine dehydrogenases from Hyphomicrobium X were partially purified as described (17). Further purification of trimethylamine dehydrogenase was affected by gel chromatography on Agarose A 0.5. Gel chromatography was, however, not effective in removing traces of trimethylamine dehydrogenase from the dimethylamine dehydrogenase and consequently the latter was rechromatographed on DEAE cellulose to yield a dimethylamine dehydrogenase preparation containing no detectable trimethylamine dehydrogenase activity. The dehydrogenases from Hyphomicrobium X were assayed as described by Colby and Zatman (18), but 35 mM sodium barbital, pH 7.7, was used as buffer instead of pyrophosphate. Trimethylamine dehydrogenase from bacterium W_3A_1 was purified and assayed as described (19). Non-heme iron, acid labile sulfur and protein were determined by published procedures (20, 21, 22). Dimethylamine dehydrogenase was dialyzed for 12 h against 1.0 mM EDTA before the second DEAE cellulose chromatographic step and again for 5 h followed by 12 h against distilled water prior to analysis for iron in order to remove "adventitious" iron from this enzyme. The apparent molecular weights of dimethylamine dehydrogenase and its subunits were determined as described by Andrews (23) and Weber and Osborne (24), respectively. The purification of the coenzyme peptides from the dehydrogenases and performic acid oxidation of the coenzyme moieties was performed according to published procedures (5). Other methods are given in the figure legends or are included in the Tables.

RESULTS AND DISCUSSION

The purified trimethylamine and dimethylamine dehydrogenases from Hyphomicrobium X had specific activities of 0.36 and 0.20 umoles min^{-1} mg^{-1} when assayed in 87 mM pyrophosphate buffer. These low specific activities as compared to the values reported for the trimethylamine dehydrogenases from bacterium 4B6 (2) and W_3A_1 (19) may in part be ascribed to non-competitive inhibition by pyrophosphate, with the amine as variable substrate, of both enzymes from Hyphomicrobium X. When assayed in 35 mM sodium barbital, pH 7.7, specific activities of 0.60 and 0.41 umoles min^{-1} mg^{-1} were found for trimethylamine and dimethylamine dehydrogenase of the denitrifying organism,

respectively. Slight inhibition by pyrophosphate, apparently of a competitive nature with respect to trimethylamine, was also observed for the trimethylamine dehydrogenase from bacterium W_3A_1.

The molecular weight of dimethylamine dehydrogenase was estimated to be about 138,000 daltons by gel chromatography on Agarose 0.5. SDS-polyacrylamide gel electrophoresis in 7.5% gels containing 0.25% methylenebisacrylamide showed the presence of a single major band with molecular weight of about 75-80,000 daltons for both the trimethylamine and dimethylamine dehydrogenases from Hyphomicrobium X. Only minor contaminants could be detected on the gels. Since the two enzymes could not be separated by gel chromatography these results suggest that they are each composed of two subunits of very similar molecular weights. The optical spectra of dimethylamine dehydrogenase in the oxidized and substrate reduced form (Fig 1) was almost identical to the corresponding spectra for the trimethylamine dehydrogenases from either organism. However in view of the close similarity between the spectral properties of various 8, α-substituted flavins, this observation cannot be regarded as conclusive evidence for the identity of the prosthetic groups in the three enzymes.

Analysis of dimethylamine dehydrogenase for iron and acid labile sulfur showed the presence of 3.9 moles S and 4.55 moles Fe per mole of the enzyme. In the calculation of these values a molar extinction coefficient of 29 mM^{-1} cm^{-1} at 440 nm, as for trimethylamine dehydrogenase from bacterium W_3A_1, was assumed. Protein determinations by both the Lowry and biuret procedures suggested this value for the molar extinction of dimethylamine dehydrogenase to be approximately correct. Moreover, the enzyme was titrated anaerobically to completion, as observed at 440 nm, by the addition of 0.93 moles of dimethylamine if the value given above for the molar extinction coefficient is assumed.

The coenzyme peptides obtained by tryptic-chymotryptic digestion from dimethylamine dehydrogenase and from the trimethylamine dehydrogenases of

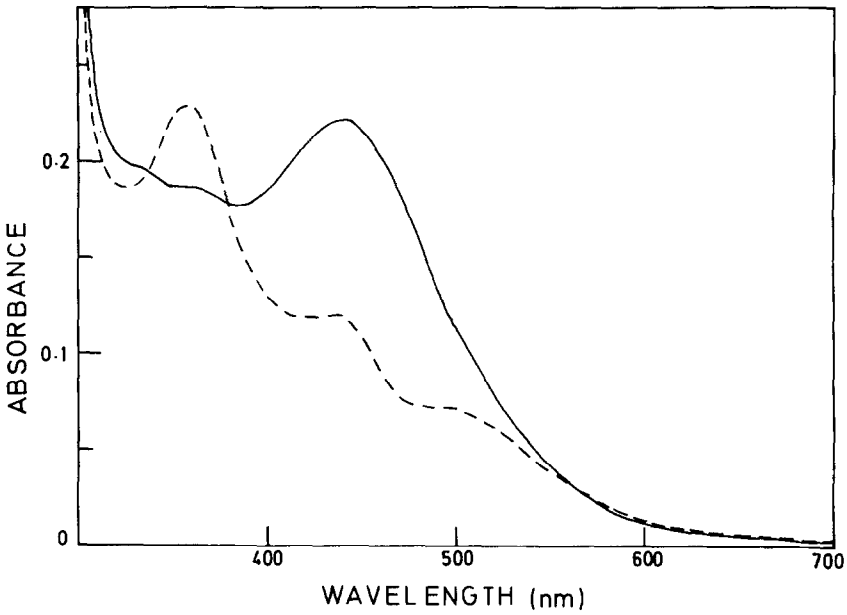

Fig 1 Spectra of dimethylamine dehydrogenase from Hyphomicrobium X in the oxidized (—) form and after addition of dimethylammonium hydrochloride to a final concentration of 6 mM. (- - -) The slight shoulder at 365 nm in the spectrum of the oxidized enzyme suggests the presence of a small amount of the reduced form.

Hyphomicrobium X and bacterium W_3A_1 were partially purified and found to have indistinguishable spectral properties in the visible region. Moreover, performic acid oxidation of the coenzyme peptides from the Hyphomicrobium enzymes resulted in the appearance of fluorescence, the excitation spectra of which was identical with that of 6-S-cysteinesulfonyl-FMN, with emission measured at 525 nm. Nonetheless, the electrophoretic and thin-layer chromatographic properties of the coenzyme peptides from the three enzymes were found to be dissimilar (Table 1 and 2). These differences could not be ascribed to identity of either of the coenzyme peptides from the dehydrogenases of Hyphomicrobium X as a FAD derivative, since incubation with alkaline phosphatase and subsequent high voltage paper electrophoresis indicated the

TABLE 1

THE EFFECT OF ALKALINE PHOSPHATASE[a] AND AMINOPEPTIDASE M[b] ON THE ELECTROPHORETIC PROPERTIES OF THE TRYPTIC-CHYMOTRYPTIC COENZYME-PEPTIDES FROM THE TRIMETHYLAMINE DEHYDROGENASE FROM HYPHOMICROBIUM X (HX-TMADH) AND BACTERIUM W_3A_1 (W_3A_1-TMADH) AND FROM THE DIMETHYLAMINE DEHYDROGENASE FROM HYPHOMICROBIUM X (HX-DMADH)

Treatment of coenzyme-peptide	Electrophoretic mobility relative to FMN (+1.0) at pH 5.0[c,d]		
	W_3A_1-TMADH	HX-TMADH	HX-DMADH
None	0.376	0.248	0.652
Alkaline phosphatase	-	-0.248	-0.028
Aminopeptidase M	0.99	0.98	0.99

a. 1-2 nmol coenzyme-peptide was incubated for 1 h at room temperature with 2 µg calf intestinal alkaline phosphatase in 100 mM ammonium carbonate-bicarbonate buffer, pH 9.0 in a final volume of 24 µl.
b. 5-10 nmole coenzyme-peptide was incubated for 16 h at room temperature with 10 µg aminopeptidase M in 0.1 M N-ethylmorpholinium acetate, pH 8.0 in a final volume of 130 µl.
c. Electrophoresis was performed on Whatman 3 M paper using 125 mM pyridinium acetate, pH 5.0 as electrolyte for 1.5 h at 50 V/cm.
d. The coenzyme moiety was located due to the appearance of characteristic blue-green-fluorescence upon irradiation.

presence of a phosphate monoester, as in FMN, in all three cases. Moreover, the chromophoric groups derived from the three enzymes showed identical chromatographic and electrophoretic behaviour after treatment with aminopeptidase M, indicating that the difference in properties of the coenzyme peptides must be ascribed to differences in the amino acid sequence of the peptides in the vicinity of the active site of these enzymes. The data summarized in Tables 1 and 2, therefore, constitute convincing evidence that dimethylamine dehydrogenase is the second representative of a group of dehydrogenases in which flavin is attached to the peptide backbone as the 6-S-cysteinyl derivative of FMN.

The occurrence of 6-S-cysteinyl-FMN as coenzyme in both trimethylamine and dimethylamine dehydrogenase suggest that attachment of flavin as the 6-thioether to proteins can hardly be fortuitous, but is likely to be of mechanistic significance in terms of catalytic events at the active sites of these enzymes.

TABLE 2

EFFECT OF AMINOPEPTIDASE M ON THE THIN-LAYER CHROMATOGRAPHIC PROPERTIES OF THE TRYPTIC-CHYMOTRYPTIC PEPTIDES FROM THE TRIMETHYLAMINE DEHYDROGENASES FROM HYPHOMICROBIUM X (HX-TMADH) AND BACTERIUM W_3A_1 (W_3A_1-TMADH) AND THE DIMETHYLAMINE DEHYDROGENASE FROM HYPHOMICROBIUM X (HX-DMADH)

System	Rf-values[b]					
	Untreated			After digestion with Amino-peptidase M[c]		
	W_3A_1-TMADH	HX-TMADH	HX-DMADH	W_3A_1-TMADH	HX-TMADH	HX-DMADH
1	0.32	0.19	0.25	0.26	0.25	0.25
2	0.58	0.58	0.46	0.46	0.45	0.45
3	0.30	0.16	0.085	0.14	0.14	0.14
4	0.13	0.08	0.05	0.070	0.070	0.070

a. The systems used were:

1. Methylethylketone / pyridine / water / acetic acid (15 : 10 : 10 : 1 v/v), silica gel as adsorbent.
2. n-Butanol / pyridine / water (1 : 1 : 1 v/v), silica gel as adsorbent.
3. n-Butanol / acetic acid / water (4 : 2 : 2 v/v), cellulose as adsorbent.
4. Methylethylketone / pyridine / water / acetic acid (50 : 25 : 25 : 3 v/v), cellulose as adsorbent.

b. Detection of the coenzyme moiety was described in Table 1.
c. Conditions for digestion as described in Table 1.

Moreover, since both enzymes are synthesized by a single organism a problem of relevance to the biosynthetic mechanism of covalent attachment of the flavin to the peptide backbone in such enzymes presents itself. Evidence has recently been presented for the occurrence of precursor apoenzyme molecules of D-6-hydroxynicotine oxidase (25), which implies that biosynthesis of the protein-flavin linkage is a post-translational event not taking place on the ribosomes. If indeed the trimethylamine and dimethylamine dehydrogenases from Hyphomicrobium X are found to have dissimilar amino acid sequences in proximity to the site of attachment of the flavin, this would pose a serious recognition problem if the same enzyme system were to be responsible for covalent attachment of the flavin to both enzymes. This point is currently

being investigated in greater detail by sequence analysis of the tryptic and chymotryptic coenzyme peptides from these two enzymes.

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