ON THE PRESENCE OF A NOVEL COVALENTLY BOUND OXIDATION-REDUCTION COFACTOR, IRON AND LABILE SULFUR IN TRIMETHYLAMINE DEHYDROGENASE *

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Summary: Trimethylamine dehydrogenase from a facultative methylotroph contains 4 g atoms each of Fe and S and an unknown, covalently bound, yellow coenzyme. The absorbance of the enzyme in the visible range (λ_{max}= 445 nm) is extensively bleached by dithionite. Reduction by substrate causes less extensive bleaching and the appearance of a three banded spectrum which may be representative of a free radical form. Denaturation liberates the Fe-S center(s) but not the organic coenzyme. The latter is covalently linked to the protein via an amino acid residue and is solubilized on proteolytic digestion in the form of the peptide. The coenzyme-peptide has been purified to a constant ratio of amino acid to coenzyme. The oxidized and reduced forms show maximal absorbance at 437 nm and 380 nm, respectively. Based on dithionite titrations its molar absorbance at 437 nm is 12,300 in the oxidized and 4000 in the dithionite reduced form. The cofactor is very labile to photolysis giving rise to several products the predominant one of which shows fluorescence excitation and emission maxima at 394 and 500 nm, respectively. After cleavage of the hydrolyzable amino acids in HCl, the compound consumed 3 moles of periodate. Digestion with aminopeptidase M yields a compound with a single amino acid and ~1 mole of organic P present. Acid phosphatase, but not nucleotide pyrophosphatase affects its mobility. These findings suggest that the coenzyme-peptide is isolated in the form of a mononucleotide, containing a 5-carbon alcohol. The physical and chemical properties of the compound do not agree with those of known flavin or pyridoxine derivatives but are not incompatible with a covalently linked pteridine (lumazine) derivative, although no proof for such a structure is so far available.

Trimethylamine dehydrogenase, an enzyme from methylotropic bacteria, catalyzes the first step in the oxidative demethylation of trimethylamine with PMS as the electron acceptor:

$$(CH_3)_3 N + H_2O + PMS \rightleftharpoons (CH_3)_2 NH + CH_2O + PMSH_2$$

The electrophoretically homogeneous enzyme from Bacterium W3A1 has a molecular weight of 146,800 daltons and contains iron and acid-labile sulfur as well as a yellow prosthetic group covalently bound to the enzyme (1).

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Our interest in trimethylamine dehydrogenase was further stimulated by mainly two observations: (i) the unusual yellow coenzyme could thus far not be identified as any of the known flavin derivatives and (ii) the addition of substrate to the enzyme gave rise to a complex pattern of EPR signals presumably due to the interaction between a free radical which appears during catalysis and an iron-sulfur center. This complex EPR behavior is not observed when the enzyme is reduced by dithionite, which generates a signal with g= 1.94 characteristic of many iron-sulfur proteins (2).

The present communication shows that the dehydrogenase isolated by one of us (1) contains 4 g atoms each of non-heme Fe and labile S as well as a covalently bound organic cofactor of a novel type.

MATERIALS AND METHODS

A culture of Bacterium W3A1 was the kind gift of Dr. L.J. Zatman, University of Reading, England. Trimethylamine dehydrogenase was purified as described (1). Total iron content was determined as per De Bogart and Beinert (3), labile sulfur by the method of Lowenberg et al. (4), protein by the Lowry method (5) and phosphate according to Bartlett (6).

The peptide-bound coenzyme was liberated from the protein and purified by a procedure to be described in detail elsewhere. The principal steps were precipitation of the enzyme with 5% (w/v) trichloroacetic acid to remove Fe and labile S, digestion of the residue with trypsin and chymotrypsin to solubilize the coenzyme-peptide, purification by adsorption on Florisil, DEAE-cellulose chromatography using a linear pyridine-acetate gradient, and by preparative thin-layer chromatography. The coenzyme-peptide from the DEAE-cellulose column was digested with aminopeptidase M. Periodate titrations of acid hydrolyzed coenzyme were performed according to the method of Dixon and Lipkin (7).

RESULTS AND DISCUSSION

The visible spectrum of trimethylamine dehydrogenase in the oxidized form and after

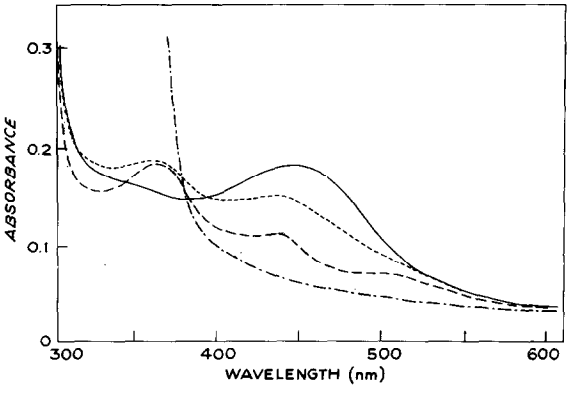

Fig. 1: Effect of trimethylamine (1 mM)(— —), solid sodium dithionite (-•-•), solid sodium borohydride (---) and 8 M urea (••••••) on the spectrum on oxidized trimethylamine dehydrogenase (—) all in 75 mM sodium pyrophosphate, pH 7.7.

reduction with substrate, dithionite, or sodium borohydride is presented in Fig. 1. Although the absorbance band at about 445 nm in the oxidized enzyme resembles a Schiff base between pyridoxal phosphate and an amino group in the enzyme, the prosthetic group does not appear to be a vitamin B_6 derivative, since neither the absorbance spectrum of the enzyme nor of the purified coenzyme-peptide showed the variations with pH characteristic of the pyridoxylidene form of such derivatives and because the borohydride reduced form of the enzyme retained full catalytic activity.

In 8 M urea or 3.85 M guanidine hydrochloride pronounced bleaching of the enzyme occurs and a new spectrum with absorbance maximum at 435 nm appeared (Fig. 1). This phenomenon may be ascribed to the presence of Fe and labile S in addition to an unknown yellow prosthetic group. After dialysis of the enzyme against 1 mM EDTA and rechromatography on DEAE-cellulose a constant ratio of Fe and labile S and of activity to protein in the active fractions (Fig. 2) was found. Based on a molecular weight of 147000 the Fe and labile S to protein ratio was 4:4:1 within experimental error in all the fractions. Since denaturing agents do not liberate the organic cofactor from the enzyme but do remove the Fe and labile S, it seems clear that the partial bleaching of the color of the enzyme in urea and in guanidine solutions represents the contributions of the Fe-S center(s) to the total color of the enzyme.

Examination of the spectral changes shown in Fig. 1 reveals that reduction by dithionite bleaches a significant part of the color in the visible range attributable to both the organic cofactor and the Fe-S center(s). Reduction by substrate causes less extensive bleaching and the appearance of a red color in concentrated solutions. The three-banded spectrum found is

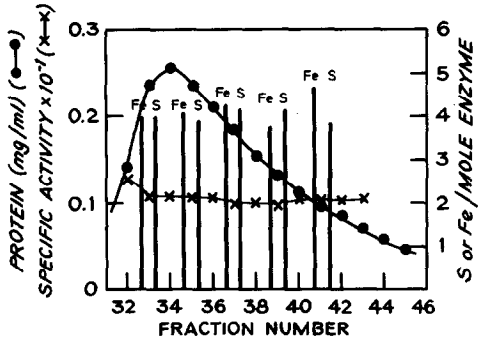

Fig. 2: Chromatography of trimethylamine dehydrogenase on DEAE-cellulose using a linear salt gradient of 0-0.5 M NaCl in 0.05 M potassium phosphate, pH 7.4. Protein concentration (●—●) was determined by the Lowry method. Adjacent fractions were pooled, repeatedly concentrated by ultrafiltration to remove phosphate ions and then analyzed for iron and sulfur. Specific activity is expressed as μmole DCIP reduced per minute per mg enzyme protein.

strongly reminiscent of a lumazine free radical (8). An explanation of this behavior has come from EPR experiments in collaboration with Dr. H. Beinert, to be published elsewhere (2), showing that while dithionite causes the appearance of a typical reduced ferredoxin-type Fe-S EPR signal, reduction by trimethylamine yields an unique EPR spectrum, indicating a very strong spin coupling between an organic free radical and a reduced Fe-S center. Thus the absorption spectrum seen after adding substrate (Fig. 1) may be that of the radical form of the coenzyme which interacts with a reduced Fe-S center.

The conclusion that trimethylamine dehydrogenase contains in addition to Fe and labile S, an organic cofactor in covalent linkage to the protein is based on the following evidence. (a) The chromophore remaining after urea or guanidine treatment is protein-bound, because it accompanies the protein in gel exclusion on Sephadex G-50. (b) The chromophore is precipitated with the protein by trichloroacetic acid. On proteolytic digestion of the precipitate it is solubilized, yielding a spectrum close to that of the urea-denatured enzyme (Fig. 1). (c) On purification of the coenzyme-peptide liberated by trypsin-chymotrypsin, amino acids accompany the chromophore. After elution of the peptide from DEAE-cellulose, on thin-layer chromatography in several solvent systems a constant ratio of about 11 moles of amino acids per mole coenzyme was obtained. (d) The Rf values of the purified chromophore on thin-layer chromatography and its mobility on high voltage electrophoresis changed dramatically on treatment with aminopeptidase M.

One of the most characteristic features of the yellow coenzyme is its photolability. The coenzyme-peptide is only weakly fluorescent, but on irradiation, even in the fluorimeter, a fluorescent product appears. This property is convenient for the detection of the chromophore on chromatograms, but also makes it essential to perform all operations in subdued light. Although only one fluorescent compound could be detected by thin-layer chromatography of the irradiated coenzyme-peptide, different lines of evidence indicate that the photoreaction of this chromophore is complex, probably giving rise to more than one product, only one of which is fluorescent. First, the conversion of the coenzyme to its photoproducts was nearly, but not completely, isosbestic. Second, the absorbance maximum of the final photoproduct (Fig. 3) at 410 nm did not coincide with the fluorescence excitation maximum at 394 nm. Further evidence for the complexity of the reaction comes from anaerobic dithionite titrations.

Dithionite titrations of the coenzyme-peptide proceed with an isosbestic point at 325 nm and the reduced coenzyme shows a well defined peak at 380 nm (Fig. 3) in contrast to the hydroquinone form of reduced flavins which generally have an ill-defined shoulder in this region. Assuming that the coenzyme is a two electron acceptor a molar extinction coefficient of 12.3 $mM^{-1}cm^{-1}$ at 437 nm may be calculated (Inset of Fig. 3). The reduced coenzyme was

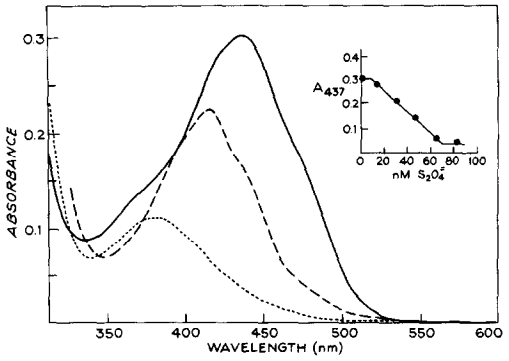

Fig. 3: Absorbance spectrum of trimethylamine dehydrogenase coenzyme-peptide in 88 mM sodium pyrophosphate, pH 7.7 (—), after irradiation for 18 min at a distance of 8 cm from two 15 W daylight fluorescent lamps (— — —) and after reduction by anaerobic dithionite titration (---). The course of anaerobic dithionite titration is shown in the inset.

rapidly reoxidized by atmospheric oxygen. The products obtained by irradiation of the coenzyme consumed only about half the expected dithionite titer as compared to the parent compound. Moreover, this titration was not isosbestic, suggesting again that the photoreaction yields more than one product. The assumption that the parent compound is a two electron acceptor was substantiated by the finding that the molar extinction coefficient of the coenzyme calculated on the basis of the recovery of absorbance in the tryptic-chymotryptic digest was only about 15 $mM^{-1}cm^{-1}$, in reasonable agreement with the value obtained by dithionite titration of the more purified DEAE-cellulose eluate.

The presence of a polyhydroxyalkyl side chain attached by an acid stable linkage to the chromophore was suggested by the periodate consumption of acid hydrolyzed coenzyme-peptide. Treatment with 6 N HCl at 95°C resulted in the appearance of several green fluorescent components on thin-layer chromatography, the relative proportions of which was apparently unaffected by hydrolysis for periods longer than 5 h at this temperature. The coenzyme-peptide was therefore hydrolyzed in 6 N HCl for 5 h at 95°C, extracted into benzyl alcohol and chromatographed on Silica Gel H using 1-butanol-acetic acid-water (20:3:7 v/v) as solvent. The main fluorescent component rapidly consumed 2.9 moles of periodate per mole of the coenzyme (based on the molar extinction coefficient derived from dithionite titration); an almost identical value was obtained when riboflavin was used as a standard under similar conditions.

The product obtained by aminopeptidase M digestion of the coenzyme-peptide was purified by preparative thin-layer chromatography. Ninhydrin analysis indicated the presence

of 1.4 and 2.6 moles of ninhydrin positive material per mole coenzyme before and after hydrolysis in vacuo in 6 N HCl for 16 h at 100°C, respectively. The increase in the ninhydrin reactive material on hydrolysis in 6 N HCl probably resulted from the breakdown of a heterocyclic ring. Small amounts of several amino acids are, for instance, invariably formed from the flavin ring system under these conditions (9). Thus, aminopeptidase digestion probably converts the coenzyme-peptide to an amino acyl coenzyme.

The total P content of the amino acyl coenzyme was found to be 1.3 g atoms per mole. The phosphorylated nature of the amino acyl coenzyme was further established by treatment of the amino acyl coenzyme with acid phosphatase (Table I), whereas the electrophoretic mobility of the coenzyme-peptide or the amino acyl coenzyme at pH 5.0 was unaffected by treatment with pyrophosphatase.

Treatment of the coenzyme-peptide with aminopeptidase M markedly increased the electrophoretic mobility of the yellow chromophore and the amino acyl coenzyme has a mobility similar to FMN. Acid phosphatase digestion converts the amino acyl coenzyme to a neutral compound, which, since it is ninhydrin positive, must be a zwitterion at pH 5.0. This observation suggests that the phosphorylated amino acyl coenzyme has a molecular size similar to FMN and implies that the actual chromophore is smaller than the isoalloxazine ring system (10).

The spectral, fluorescent, and chemical properties of the coenzyme summarized here distinguish it from known flavin derivatives, including covalently bound flavins and flavins

TABLE I

EFFECT OF DIGESTION WITH ACID PHOSPHATASE[a] OR AMINO PEPTIDASE M[b] ON THE ELECTROPHORETIC MIGRATION OF TRIMETHYLAMINE DEHYDROGENASE AMINO ACYL COENZYME AND COENZYME-PEPTIDE, RESPECTIVELY.

Treatment of coenzyme-peptide	Electrophoretic mobility relative to FMN (+ 1.0) at pH 5.0[c]
None	0.295
Amino peptidase M digested	0.985
Amino peptidase M digestion; then acid phosphatase	0.0

[a] 1nm amino acyl coenzyme was incubated for 16 h with 50 µg potato acid phosphatase (Sigma, Grade II) at 38°C in 150 nm ammonium acetate, pH 4.8 in a final volume of 65 µl.

[b] 150nm coenzyme-peptide was incubated for 24 h at 38°C with 50 µg aminopeptidase M in 1 ml 0.1 M N-ethylmorpholine acetate, pH 8.0. The pH was readjusted at intervals.

[c] Electrophoresis was performed on Whatman 3 M paper using 125 mM pyridine acetate, pH 5.0 as electrolyte for 2 h at 40 V/cm.

hydroxylated in the benzene ring (10). The properties of the coenzyme, in particular its pronounced photolability and the presence of a well-resolved absorbance band after reduction by dithionite (Fig. 3) suggest that the coenzyme may be a phosphorylated pteridine derivative which is reduced by dithionite to the dihydro form. It appears to contain a five-carbon aldityl side chain attached to the heterocyclic system, as is found in several naturally occurring lumazine derivatives, the latter being covalently linked to a peptide chain via a functional group other than the α-amino or carboxyl group. If these suggestions prove to be correct, trimethylamine dehydrogenase may be the first instance of an oxidoreductase containing covalently linked pteridine.

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