The Natural Flavoprotein Electron Acceptor of Trimethylamine Dehydrogenase*

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SUMMARY

The isolation and partial characterization of a flavoprotein which functions as the electron acceptor of trimethylamine dehydrogenase (EC 1.5.99.7) from a methylotrophic bacterium is described. It has a molecular weight of 77,000 and is composed of two dissimilar subunits. All preparations examined contained only 1 mol of FAD/mol of the flavoprotein. Trimethylamine dehydrogenase, in the presence of trimethylamine or dithionite, reduced the flavoprotein to a stable anion semiquinone form. No evidence for the participation of the fully reduced flavoprotein in catalysis could be obtained.

Studies on the utilization of 1-carbon fragments by methylotrophic bacteria have revealed the presence of three rather unique dehydrogenase enzymes in such organisms (1). These enzymes all use phenazine methosulfate as electron acceptor in vitro and generate formaldehyde as one of the products. When grown on trimethylamine as the carbon source, trimethylamine dehydrogenase, which catalyzes the oxidative demethylation of trimethylamine to dimethylamine and formaldehyde, is induced (2, 3). Although this enzyme is also slowly reduced by dimethylamine (4), the oxidative demethylation of dimethylamine is probably too slow to be physiologically significant and further metabolism of dimethylamine proceeds by mono-oxygenation (5). Recent studies have provided firm evidence for a novel covalently bound flavin, 6-cysteinyl-FMN, as the coenzyme of one of these proteins, trimethylamine dehydrogenase (6, 7). Also of interest is the unusually intense EPR-detectable interaction signal between the flavin and tetrameric iron-sulfur center of trimethylamine dehydrogenase (8) generated on reduction with substrate.

A further development, described below, is the discovery and isolation of a flavoprotein which seems to act as a natural electron acceptor of trimethylamine dehydrogenase.

METHODS

During the purification of trimethylamine dehydrogenase from bacterium W3A1 (6), a yellow component, which may be recognized by its typical flavin absorption, was eluted with a linear sodium chloride gradient at a slightly higher ionic strength (~0.28 M NaCl in 50 mm potassium phosphate, pH 7.2) than the dehydrogenase itself. The fractions containing the flavoprotein were pooled, diluted 4-fold with distilled water to prevent dissociation of the coenzyme at elevated ionic strength, and concentrated by ultratitration. The flavoprotein was applied to a column of Bio-Gel A-1.5 m or Sephadex G-100 and eluted with 50 mm potassium phosphate, pH 7.0. The gel chromatography step was repeated twice to lower the concentration of trimethylamine dehydrogenase to a level where it cannot be detected in catalytic assays in which reduction of the flavoprotein by trimethylamine is observed. A slight further purification of the flavoprotein could be obtained by ethanol fractionation of the semiquinone form of the flavoprotein. Assay mixtures from various experiments in which the flavoprotein had been used as the terminal electron acceptor were pooled and dialyzed against 50 mm potassium phosphate, pH 7.0. Upon subsequent addition of ethanol, while maintaining the temperature between 0°C and -10°C, the semiquinone form of the flavoprotein was precipitated between 50 and 75% (v/v) ethanol. The oxidized form of the flavoprotein was not precipitated, however, even by 90% (v/v) ethanol.

Protein was determined by the Lowry (9) and biuret (10) methods. High voltage electrophoresis was run at 40 V/cm and pH 1.6 (8% (v/v) formic acid) or pH 5.0 (1% pyridine titrated to pH 5.0 with glacial acetic acid). FAD was determined by following the reconstitution of apoglucose oxidase polarographically (11). Gel electrophoresis was performed as described (12). The subunit molecular weights of the flavoprotein were estimated by SDS-polyacrylamide gel electrophoresis (13) and that of the intact protein by gel chromatography on Sephadex G-100 (14).

The concentration of the flavoprotein was in most instances estimated from the absorbance at 438 nm, assuming a molar extinction coefficient of 11,300 liter mol⁻¹ cm⁻¹.

RESULTS AND DISCUSSION

Identification and Oxidation-Reduction Properties of the Electron Acceptor Flavoprotein of Trimethylamine Dehydrogenase—The involvement of the electron acceptor flavoprotein in the metabolism of trimethylamine by bacterium W3A1 was first recognized by the pronounced spectral change in the flavin region which occurred on the addition of trimethylamine (Fig. 1). The absolute dependence of this reaction on the presence of trimethylamine dehydrogenase could readily be demonstrated after removal of traces of the dehydrogenase by gel chromatography, since it is incompletely separated from the flavoprotein by DEAE-cellulose chromatography. Trimethylamine, in the presence of its dehydrogenase, converts the flavoprotein to a species spectrophotometrically indistinguishable from the anionic semiquinone which has been observed in several flavoenzymes (15, 16). The free radical nature of the reaction product was further confirmed by EPR spectroscopy (Fig. 2). The shoulders of the EPR signal shown in Fig. 2 varied in magnitude with the microwave power as had been observed for many flavoprotein radicals (17) and may perhaps be ascribed to anisotropic power saturation of the flavin nucleus (18). Conversion of the flavoprotein to the semiquinone form appeared to be quantitative. When the level of trimethylamine dehydrogenase in the reaction mixture was low enough to allow repetitive scanning of the spectrum during reduction of the flavoprotein to the semiquinone form, the curves were isosbestic, and, assuming ε₄₃₈ = 11.3 for the oxidized flavoprotein at 438 nm, a value of ~16.0 for the semiquinone absorption band at 368 nm was calculated, in good agreement with values reported in the literature for well characterized flavoproteins (16). The stabilization of the...
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Flavoprotein may be the natural electron acceptor of trimethylamine dehydrogenase. Methanol dehydrogenase and methylamine dehydrogenase isolated from trimethylamine grown bacterium W3A1 failed to mediate the reduction of the flavoprotein by methanol in the presence of ammonia or by methylamine, respectively. A reasonable degree of specificity for trimethylamine dehydrogenase is thus indicated.

Characterization of the Flavoprotein—Gel electrophoresis by the method of Clarke (12) showed the presence of only minor contaminants in the flavoprotein preparation after the gel chromatography step (Fig. 4). Also shown in Fig. 4 are polyacrylamide and SDS-polyacrylamide gel electrophoresis patterns of the flavoprotein recovered from alcohol fractionation of the semiquinone form.

The molecular weight of the flavoprotein was approximately 77,000 from chromatography on Sephadex G-100 (15). SDS-polyacrylamide gel electrophoresis (14) showed the presence of two components with molecular weights of 38,000 and 42,000, respectively.

Determining of protein by the biuret (10) and Lowry (9) procedures gave identical values and comparison of the 438 nm absorbance with the protein content showed the presence of 1.08 mol of FAD/mol of flavoprotein. Incubation of the flavoprotein with FAD prior to gel chromatography did not increase the FAD:protein ratio in the eluate fractions.

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trimethylamine dehydrogenase at pH 7.7. The variation of the reaction rate with flavoprotein concentration (Fig. 5a) seems to show simple saturation kinetics with a $K_{m, app}$ of ~6.7 μM and $V_{app} ~2.1$ μM/min/mg for the flavoprotein. Substrate inhibition by trimethylamine in the millimolar range is evident in Fig. 5b. This observation is of interest, since an inhibition of the rate of formation of the spin-coupled form of trimethylamine dehydrogenase at high trimethylamine concentrations has been observed by us in stopped flow kinetic measurements (4).

The semiquinone form of the flavoprotein was not auto-oxidizable, but could be converted back to the oxidized form by treatment with an equimolar amount of ferricyanide, followed by chromatography on Sephadex G-25. However, the reoxidized flavoprotein so obtained was unstable and over a period of a few days gradually lost its electron acceptor activity. The flavoprotein could also couple the oxidation of trimethylamine via trimethylamine dehydrogenase to the reduction of 2,6-dichlorophenolindophenol without the mediation of phenazine methosulfate. The $V_{app}$ and $K_{m, app}$ for the flavoprotein so obtained differed, however, from the values observed when the flavoprotein was used as the electron acceptor, suggesting that reduction of the dye by the half-reduced flavoprotein may be sufficiently rate-limiting to give a variable steady state level of the oxidized flavoprotein during turnover when dichlorophenolindophenol is the terminal electron acceptor, depending on the assay conditions. The flavoprotein did not couple the oxidation of trimethylamine by its dehydrogenase to oxygen, as measured polarographically or to the reduction of equine cytochrome c.

Although the molecular weight of the new electron acceptor flavoprotein is of the same magnitude as that of the electron transfer flavoproteins which had been isolated from various sources (19, 20) and its visible absorption spectrum is rather similar to that of mammalian ETF, this flavoprotein differs from previously isolated electron transfer flavoproteins in several respects. The trimethylamine dehydrogenase electron acceptor flavoprotein contained only 1 mol of FAD/mol of protein and reduction to hydroquinone form either by the dehydrogenase or by dithionite could not be demonstrated. It is, therefore, reasonable to suppose that the flavoprotein couples the oxidized and anionic semiquinone forms during catalysis, rather than between the oxidized and semiquinone forms. Moreover, in contrast to the Peptostreptococcus elsdenii electron transfer flavoprotein (20), the new flavoprotein had negligible diaphorase activity.

Trimethylamine dehydrogenase in association with its electron acceptor flavoprotein constitute a triad of oxidation-reduction active groups which conform well to the biological $(2 \times 1 e^-)$-transformase unit recently propounded by Hemmerich (21), and, in view of the relative stability of the participating components, should be a valuable model system for further investigation of the transformase concept.
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