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Microcoulometric analysis of trimethylamine dehydrogenase

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Trimethylamine dehydrogenase, which contains one covalently bound 6-*S*-cysteinyl-FMN and one Fe_4S_4 cluster per subunit of molecular mass 83000 Da, was purified to homogeneity from the methylotrophic bacterium W_3A_1. Microcoulometry at pH 7 in 50 mM-Mops buffer containing 0.1 mM-EDTA and 0.1 M-KCl revealed that the native enzyme required the addition of 3 reducing equivalents per subunit for complete reduction. In contrast, under identical conditions the phenylhydrazine-inhibited enzyme required the addition of 0.9 reducing equivalent per subunit with a midpoint potential of +110 mV. Least-squares analysis of the microcoulometric data obtained for the native enzyme, assuming uptake of 1 electron by Fe_4S_4 and 2 electrons by FMN, indicated midpoint potentials of +44 mV and +36 mV for the FMN/$FMN^{\cdot-}$ and $FMN^{\cdot-}/FMNH_2$ couples respectively and +102 mV for reduction of the Fe_4S_4 cluster.

INTRODUCTION

Trimethylamine dehydrogenase catalyses the initial step in carbon metabolism in methylotrophic bacteria grown on trimethylamine. The enzyme isolated from the bacterium W_3A_1 is a homodimer of subunits, of molecular mass 83000 Da, each of which contains one covalently bound flavin, 6-*S*-cysteinyl-FMN, and one Fe_4S_4 cluster (Steenkamp *et al.*, 1978; Hill *et al.*, 1977). Substrate reduction occurs at the FMN, whereas electron egress, to an electron-transferring flavoprotein, occurs via the Fe_4S_4 cluster (Steenkamp & Beinert, 1982*b*). X-ray-diffraction analysis at a resolution of 0.24 nm (2.4 Å) has shown that both of these cofactors are located within a 'large' domain that comprises the major portion of each subunit's tertiary structure (Lim *et al.*, 1986).

Stoichiometric titrations of the enzyme have shown that during reduction with substrate trimethylamine dehydrogenase accepts 2 reducing equivalents. These electrons are redistributed to form flavin free radical and reduced Fe_4S_4 with the formation of an unusual spin-coupled system that gives rise to both anomalous visible-absorption and e.p.r. spectra (Steenkamp & Beinert, 1982*a*,*b*). In contrast, dithionite titrations result in the addition of 3 electrons per subunit and appearance of an e.p.r. signal typical of a reduced Fe_4S_4 cluster and an increase in absorbance at 365 nm, characteristic of formation of an anionic flavin semiquinone (Steenkamp *et al.*, 1978).

The enzyme can be inactivated by incubation with various monoamine oxidase inhibitors, such as phenylhydrazine, which results in phenylation of the 4-position of the FMN cofactor, rendering it redox-inactive. Thus inactivated enzyme only accepts 1 electron, reducing the Fe_4S_4 cluster.

We have utilized microcoulometry to determine the oxidation–reduction midpoint potentials of the FMN and Fe_4S_4 prosthetic groups and compared these values with those previously obtained by spectroelectrochemistry (Stankovich & Steenkamp, 1987).

MATERIALS AND METHODS

Trimethylamine dehydrogenase was isolated from the bacterium W_3A_1 and assayed as previously described (Steenkamp & Mallinson, 1976), with the modification that during enzyme purification the final gel-filtration step with Sephadex G-200 was replaced by preparative h.p.l.c. with a TSK 4000 gel-exclusion column, the enzyme being eluted with 50 mM-Mops buffer, pH 7.0, containing 0.1 mM-EDTA. The enzyme exhibited a specific activity of 1.1 units/mg and an A_{280}/A_{448} ratio of 7.3. Enzyme concentration, given in terms of individual subunits, was quantified by using an ϵ_{443} value of 29 $mM^{-1} \cdot cm^{-1}$ (Hill *et al.*, 1977).

Enzyme containing inactivated flavin was produced by treating the native enzyme (18.5 μM-FMN), in 50 mM-pyrophosphate buffer, pH 8.2, containing 0.1 mM-EDTA, with excess phenylhydrazine (9.7 mM) and phenazine methosulphate (520 μM) at 25 °C until all enzyme activity was abolished (Colby & Zatman, 1974). Control samples of native enzyme incubated under identical conditions in the absence of phenylhydrazine showed no loss of activity. The modified enzyme was extensively dialysed against 50 mM-Mops buffer, pH 7, containing 0.1 mM-EDTA, and concentrated by ultrafiltration, and the concentration of enzyme subunits was quantified by using an ϵ_{443} value of 14.3 $mM^{-1} \cdot cm^{-1}$ (Kasprzak *et al.*, 1983).

Microcoulometry was performed as described by Spence *et al.* (1982) in the presence of the following mediators (each present at 167 μM): dichlorophenolindophenol ($E'_0 = +216$ mV), 1,4-naphthoquinone-2-sulphonate ($E'_0 = +110$ mV), Toluidine Blue ($E'_0 = +34$ mV), pyocyanine ($E'_0 = -60$ mV), indigodisulphonate ($E'_0 = -125$ mV), anthraquinone-1,5-disulphonate ($E'_0 = -225$ mV), Safranine T ($E'_0 = -289$ mV), benzyl viologen ($E'_0 = -311$ mV) and methyl viologen ($E'_0 = -440$ mV).

Oxidation–reduction midpoint potentials, expressed relative to the standard hydrogen electrode, were ob-

Abbreviations used: Fe_4S_4, non-haem iron sulphur.

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tained from the microcoulometric data by comparison with theoretical $n = 1$ or $n = 2$ Nernst equations added together to represent total electron uptake by the redox centres present in the enzyme by the use of non-linear least-squares analysis. Previous estimates of the error involved in determining midpoint potentials by microcoulometric analysis have indicated the values to be accurate to approx. ± 10 mV (Spence *et al.*, 1982).

RESULTS AND DISCUSSION

The results of microcoulometric titrations of native trimethylamine dehydrogenase and the phenylhydrazine-inactivated enzyme are shown in Fig. 1. Titrations, performed in a reductive mode, showed that neither form of the enzyme accepted reducing equivalents at potentials more positive than +200 mV. However, as the applied potential was decreased, reducing equivalents were taken up by the two forms of the enzyme over the potential range +150 mV to 0 mV for the phenylhydrazine-inactivated enzyme and +150 mV to −100 mV for the native enzyme. Determination of electron uptake at significantly more-negative potentials, within the range −200 to −500 mV, showed that the native enzyme only accepted a maximum of 3 reducing equivalents per subunit (results not shown). Thus the native enzyme required the addition, in an apparent single wave, of 3 reducing equivalents per subunit for total reduction with a midpoint potential of approx. +50 mV. In contrast, the phenylhydrazine-inactivated enzyme required the addition of 0.9 reducing equivalent per subunit in an $n = 1$ Nernst process with a midpoint potential of +110 mV. Combination of the data obtained for both the native and the phenylhydrazine-inactivated enzyme indicated that complete reduction of each subunit in native trimethylamine dehydrogenase could be accounted for by addition of 2 reducing equivalents to FMN and 1 reducing equivalent to the Fe_4S_4 cluster, suggesting the enzyme contained a full complement of cofactors and that other functional groups did not participate in electron-transfer reactions. In contrast, for the phenylhydrazine-inactivated enzyme, which contains redox-inactive phenylated FMN, only approximately 1 reducing equivalent was added, to the Fe_4S_4 cluster. The lower-than-expected level of electron uptake for the phenylhydrazine-inactivated enzyme may have been due to some autoreduction of the enzyme during the preparation of the sample. However, these stoichiometries were consistent with previous studies involving dithionite titrations (Steenkamp *et al.*, 1978) that indicated that the native enzyme accepted 3 electrons whereas the phenylhydrazone-inactivated enzyme would be expected to take up only 1 electron. In addition, the redox stoichiometries obtained for both the native and the phenylhydrazine-inactivated enzyme indicated that neither preparation of the enzyme was contaminated by extraneous redox-active proteins.

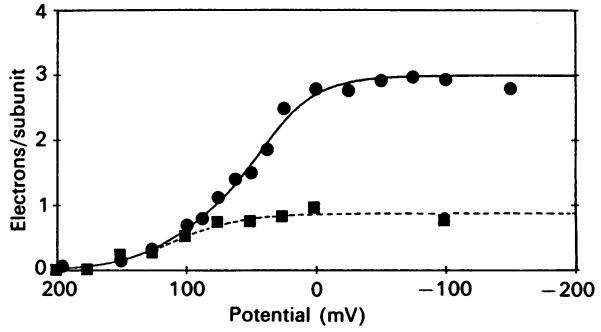

Fig. 1. Microcoulometric titrations of trimethylamine dehydrogenase

Titrations of native enzyme (●) and phenylhydrazine-inactivated enzyme (■) were performed in 50 mM-Mops buffer, pH 7.0, containing 0.1 mM-EDTA and 0.1 M-KCl in the presence of dye mediators described in the Materials and methods section. Each data point represents the electron uptake following the addition of 0.96–2.0 nmol of enzyme subunits to the titration vessel and is considered to be accurate to $\pm 2 \times 10^{-10}$ C (0.13 electron/FMN) and ± 10 mV. The continuous and broken lines were fitted by using the equations given in the text and the midpoint potentials given in Table 1. For the phenylhydrazine-inactivated enzyme, the theoretical maximum electron uptake has been decreased to 0.9 electron per subunit to reflect the experimentally observed electron uptake at full reduction.

Table 1. Oxidation–reduction midpoint potentials obtained for trimethylamine dehydrogenase

Redox couple	E'_0 (mV) Native enzyme	E'_0 (mV) Phenylhydrazine-inactivated enzyme
$Fe_4S_{4ox./red.}$	+102	+110
$FMN/FMN^{\cdot -}$	+44	–
$FMN^{\cdot -}/FMNH_2$	+36	–

Analysis of the experimental data points was carried out by using computer-simulated electron-uptake curves generated by using the equations:

$$n = 1/[1 + 10^{(E-E\{FMN1\})/59} + 10^{(E\{FMN2\}-E)/59}] + 2/[1 + 10^{(E-E\{FMN2\})/59} + 10^{(2E-E\{FMN1\}-E\{FMN2\})/59}] + 1/[1 + 10^{(E-E\{Fe/S\})/59}]$$

for the native enzyme and:

$$n = 1/[1 + 10^{(E-E\{Fe/S\})/59}]$$

for the phenylhydrazine-inactivated enzyme, where n represents the number of electrons taken up per subunit, E is the applied potential, $E\{FMN1\}$ and $E\{FMN2\}$ correspond to the potentials of the $FMN/FMN^{\cdot -}$ and $FMN^{\cdot -}/FMNH_2$ couples and $E\{Fe/S\}$ corresponds to the midpoint potential of the Fe_4S_4 cluster.

The midpoint potentials obtained by non-linear least-squares analysis of the titration curves are given in Table 1. The electron-uptake behaviour of the native enzyme was best-fitted by using potentials of +44 mV and +36 mV for the $FMN/FMN^{\cdot -}$ and $FMN^{\cdot -}/FMNH_2$ couples respectively and +102 mV for the Fe_4S_4 cluster. This latter potential was found to be in good agreement with the value of +110 mV found directly for the Fe_4S_4 cluster in the phenylhydrazine-inactivated enzyme. (We have analysed the midpoint potential of the Fe_4S_4 cluster in the phenylhydrazine-inactivated enzyme separately to ensure that any possible modification of this centre would not influence the results obtained for the native enzyme.) The optimum values obtained for the two flavin

couples by using non-linear least-squares analysis suggested that during reduction significant amounts of $FMN^{\cdot-}$ semiquinone, of the order of 38% of the total flavin content, would be generated, in agreement with the results of previous dithionite titrations (Steenkamp *et al.*, 1978) that indicated formation of an anionic free-radical signal. Altering the values of the $FMN/FMN^{\cdot-}$ and $FMN^{\cdot-}/FMNH_2$ redox couples such that the amount of $FMN^{\cdot-}$ semiquinone would be either increased or decreased, although the overall $n = 2$ redox potential $FMN/FMNH_2$ remained unchanged (+40 mV), resulted in electron-uptake curves that were either of decreased or of increased slope respectively, and which did not fit the data points as well as the curve generated by using the values given in Table 1.

Previous spectroelectrochemical titrations of trimethylamine dehydrogenase (Stankovich & Steenkamp, 1987) have yielded values significantly different from those determined by microcoulometry. Titrations of the phenylhydrazine-inactivated enzyme, performed at pH 8.5, yielded two potentials of 0 mV and −66 mV for the Fe_4S_4 clusters, suggesting non-equivalence of these redox centres. This behaviour was retained for the native enzyme, the two potentials being shifted to +70 mV and −43 mV, which was interpreted in terms of the oxidation state of the flavin influencing the midpoint potentials of the Fe_4S_4 clusters. In the native enzyme the FMN exhibited midpoint potentials of −8 mV and −29 mV. In contrast, our results indicate that the two Fe_4S_4 clusters in the phenylhydrazine-inactivated enzyme are isopotential, suggesting very similar environments in the two subunits. In addition, the potentials for the Fe_4S_4 clusters were very similar in the native enzyme, suggesting that the redox state of the flavin has little influence on the midpoint potential of the Fe_4S_4 cluster. However, direct comparison of the potentials obtained for the flavin centre by microcoulometric and spectroelectrochemical titrations is difficult because of the different conditions involved and the unknown temperature- and pH-dependencies of the $FMN/FMN^{\cdot-}$ and $FMN^{\cdot-}/FMNH_2$ redox couples, although temperature effects would be expected to be minimal compared with the magnitude of the possible pH effects. Pollock *et al.* (1988) have reported preliminary results of the effects of pH on the midpoint potentials of trimethylamine dehydrogenase measured by using potentiometric titrations. At pH 7 the Fe_4S_4 and $FMN/FMNH_2$ redox couples were determined to have potentials of +89 mV and +61 mV respectively, whereas at pH 9.1 these values were shifted to +65 mV and −20 mV, indicating that pH changes had a more significant effect on the measured oxidation–reduction midpoint potential of the flavin centre than on that of the Fe_4S_4 cluster.

The electron-transfer sequence of the trimethylamine dehydrogenase reaction has been suggested to involve the transfer of reducing equivalents from trimethylamine to the oxidized FMN, followed by intramolecular electron transfer from the reduced flavin to oxidized Fe_4S_4, and finally the transfer of reducing equivalents from reduced Fe_4S_4 to the physiological electron acceptor, an FAD-containing flavoprotein. The separation in potential between the Fe_4S_4 cluster midpoint potential and the potentials of the $FMN/FMN^{\cdot-}$ and $FMN^{\cdot-}/FMNH_2$ couples indicate that both the fully reduced and partially reduced forms of the flavin are thermodynamically capable of transferring a single electron to the oxidized Fe_4S_4 cluster.

Comparison of the potentials obtained for the FMN prosthetic group with those of free riboflavin ($Rfl_{ox.}/Rfl_{sq} = -231$ mV, $Rfl_{sq}/Rfl_{hq} = -167$ mV) and FMN ($FMN/FMNH^{\cdot} = -238$ mV and $FMNH^{\cdot}/FMNH_2 = -172$ mV) and other flavoproteins (Muller, 1987) indicated that the potentials for the two flavin couples in trimethylamine dehydrogenase are significantly more positive than those for other FMN-containing flavoproteins, such as the flavodoxins, suggesting an increased electron affinity, and the separation of the two redox couples indicates greater stabilization of the flavin semiquinone.

X-ray-diffraction data (Lim *et al.*, 1986) has indicated that the Fe_4S_4 cluster in trimethylamine dehydrogenase is in some respects structurally similar to that of the ferredoxin isolated from *Pseudomonas aerogenes* in having a similar number of amino acids residues separating the cysteine residues that comprise part of the cluster within the amino acid sequence. However, the midpoint potential for this ferredoxin is significantly lower (−400 mV; Mortenson & Nakos, 1973) than that found for the Fe_4S_4 cluster of trimethylamine dehydrogenase, suggesting significant differences in the overall protein environments of the two Fe_4S_4 clusters.

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