

Mechanistic studies on the dehydrogenases of methylotrophic bacteria

1. The influence of substrate binding to reduced trimethylamine dehydrogenase on the intramolecular electron transfer between its prosthetic groups

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The trimethylamine dehydrogenase of bacterium W_3A_1 is reduced with the formation of a triplet state in which two electrons, derived from the substrate, are distributed between the [4Fe–4S] cluster and 6-*S*-cysteinyl-FMN semiquinone. In titration experiments at pH 8.5 about 1.0 mol of dimethylamine or 0.5 mol of trimethylamine per mol of the enzyme is required to titrate the enzyme to an endpoint. At pH values less than 8.0, however, an excess of trimethylamine is required to obtain maximal yield of the $g = 4$ e.p.r. signal, characteristic of the triplet state, or maximal absorbance at 365 nm which indicates formation of the flavin semiquinone. The binding of 0.86 mol of trimethylamine per mol of the enzyme could be detected by a gel chromatographic method. When the enzyme is titrated with dithionite in the presence of tetramethylammonium chloride, an endpoint is reached after the uptake of two electrons which give rise to the triplet state, whereas three electrons are consumed in the absence of tetramethylammonium chloride to reduce the enzyme completely. The enzyme is inhibited noncompetitively by tetramethylammonium chloride and the slopes of double reciprocal plots are a concave upwards function of inhibitor concentration. The data indicate the presence of a binding site for the substrate and other amines on the reduced enzyme which enhances the proportion of enzyme in the triplet state.

Methylotrophic bacteria have the capacity to induce high levels of dehydrogenases specific for the oxidation of methanol and methylated amines (Colby *et al.*, 1979). We have previously reported on properties of one of these enzymes, the trimethylamine dehydrogenase (EC 1.5.99.7) from the methylotrophic bacterium W_3A_1 (Steenkamp *et al.*, 1978*a,b*). This enzyme catalyses the oxidative *N*-demethylation of trimethylamine and uses as an electron acceptor a flavoprotein with properties reminiscent of the mitochondrial electron transfer flavoprotein (Steenkamp & Gallup, 1978). Trimethylamine dehydrogenase is one of the simplest representatives of those complex flavoproteins in which the number of electrons required for reduction of their prosthetic groups exceeds the number of electrons actually transferred from the substrate to an electron acceptor (Palmer & Olson, 1980). The enzyme contains a [4Fe–4S] cluster (Hill *et al.*, 1977) and covalently bound flavin in the unusual form of 6-*S*-cysteinyl-FMN (Steenkamp *et al.*, 1978*b*). While on reduction with dithionite

these prosthetic groups are fully reduced by the uptake of three electrons, only two electrons are taken up from the substrate, and, under certain conditions, are shared between the two prosthetic groups. These groups seem to be located so that, from the neighbouring unpaired spins a triplet state results, which is recognized by a set of characteristic e.p.r. signals (Steenkamp *et al.*, 1978*a*) which are, in fact, the most intense signals originating from such an interaction that have yet been reported for a biological material.

Kinetic studies by the stopped-flow and freeze-quench techniques indicated that the FMN moiety is rapidly reduced to FMNH₂ upon mixing with substrate, whereas the transfer of an electron from FMNH₂ to the [4Fe–4S] cluster proceeded much more slowly (Steenkamp *et al.*, 1978*a,b*). Therefore, it appeared that this enzyme represented one of the few, if not the only, examples where the mode of electron transfer from substrate to flavin and the [4Fe–4S] cluster can be resolved in time. An opportunity to study the function of the individual

redox active components in a complex flavoprotein and to determine their mode of interaction with a natural electron acceptor, therefore, presented itself. An immediately relevant question was whether not only the flavin, but also the [4Fe-4S] centre, are obligatory participants in the transfer of electrons from the substrate to an electron acceptor. This would be the case if the triplet state, i.e., the form containing FMNH[•] and the reduced [4Fe-4S] cluster, could be shown to participate in the overall reaction. While rapid kinetic experiments indicated that the formation of the triplet state is sufficiently fast for it to be an intermediate in catalysis, the correlation between the rates observed in stopped-flow kinetic experiments and those determined by e.p.r. spectroscopy after rapid freezing was unsatisfactory (Steenkamp *et al.*, 1978a). In order to resolve this discrepancy in rates, a more detailed investigation of factors which influence the formation of the triplet species in trimethylamine dehydrogenase was undertaken. The results are reported in two papers. In this first paper evidence is presented that appearance of the triplet state as detected by e.p.r. spectroscopy correlates well with specific absorbance changes in the reduced enzyme. The presence in the reduced enzyme of a substrate binding site which modulates the transfer of electrons from the reduced flavin to the iron-sulphur cluster is demonstrated. Preliminary results from this work have been presented in a symposium on the mechanisms of oxidizing enzymes in La Paz, Mexico, in December 1977.

Materials and methods

Trimethylamine dehydrogenase was purified from bacterium W₃A₁ (Steenkamp & Mallinson, 1976). The enzyme was stored in 20% (v/v) ethylene glycol at -20°C and was transferred into the buffer of choice either by dialysis or by gel chromatography on Sephadex G-25 prior to e.p.r. or stopped-flow experiments. The electron acceptor flavoprotein of trimethylamine dehydrogenase was purified from bacterium W₃A₁ as described (Steenkamp & Gallup, 1978). The dehydrogenase and its electron acceptor were shipped between the two collaborating laboratories in a cold pack at about 0°C in 20% (v/v) ethylene glycol.

Solutions of volatile amines used in anaerobic titrations of the enzyme were made anaerobic under an atmosphere of argon by incubation with 5.6 nM-glucose oxidase, 10 mM-glucose and 10 mM-sodium acetate, pH 5.2, with sufficient catalase to prevent the accumulation of peroxide.

The stoichiometry of binding of trimethylamine to reduced trimethylamine dehydrogenase was estimated by adaptation of the procedure of Hummel & Dreyer (1962). A Sephadex G-25 gel chromatography

column (1.0 cm × 28 cm) was equilibrated with 150 μM-[¹⁴C]trimethylamine at a specific radioactivity of about 30 mCi/mol in 0.1 M-sodium pyrophosphate, pH 7.7. The specific radioactivity of the effluent fractions was determined by comparing the trimethylamine concentration, estimated essentially as described (Large & McDougall, 1975) with radioactivity measurements on aliquots. The air above a solution containing trimethylamine dehydrogenase (22.6 nmol), 20 μmol of glucose and 75 μmol of sodium pyrophosphate/HCl buffer, pH 7.7, in a volume of 0.77 ml was displaced with argon through a serum cap. A mixture of glucose oxidase (1.16 units) and catalase (5.6 units) was added to obtain anaerobicity. The enzyme was reduced by the addition of trimethylammonium chloride to a final concn. of 150 μM, and was subsequently applied under anaerobic conditions to the Sephadex column. Trimethylamine dehydrogenase in the effluent fractions was determined by enzymic assay as described (Colby & Zatman, 1973) and dehydrogenase concentrations were calculated by comparison with the specific activity of the purified enzyme, or by measurement of the absorbance of the triplet state at 365 nm (ϵ 32.1 mm⁻¹·cm⁻¹). The increase in radioactivity of the effluent fractions, coincident with elution of the enzyme, was used to calculate the amount of substrate bound to the reduced form of the enzyme. The experiment was also performed with about 0.3 mM-[¹⁴C]trimethylamine at a specific radioactivity of 30 mCi/mol present in the eluting buffer. In the latter case 90 nmol of the enzyme were passed through the column.

The concentration of trimethylamine dehydrogenase was calculated by using a millimolar absorption coefficient of 29.0 mm⁻¹·cm⁻¹ (Hill *et al.*, 1977) at 443 nm. Absorption spectra were recorded with a Cary 14 spectrophotometer interfaced with a Nova 2/4 computer. Titrations and e.p.r. spectroscopy were carried out essentially as described (Steenkamp *et al.*, 1978a, Hartzell & Beinert, 1976; Orme-Johnson & Beinert, 1969). Anaerobic techniques followed the outlines given by Beinert *et al.* (1978). The signals at $g = 4$ (the so-called half-field signals of the triplet state) were evaluated relative to each other, both by double integration and simple measurement of signal height. There was no significant difference between these two approaches.

Further details of experiments are given in the Figure legends.

Results and discussion

Stoichiometry of reduction of trimethylamine dehydrogenase by trimethylamine

The reduction of trimethylamine dehydrogenase at its pH optimum of 8.5 (Colby & Zatman, 1974)

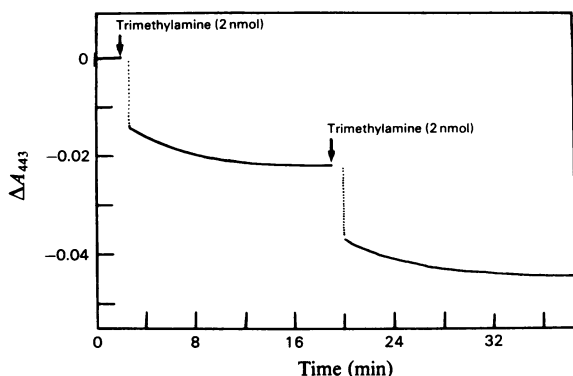


Fig. 1. Absorbance change during the reduction of trimethylamine dehydrogenase by substoichiometric amounts of trimethylamine under anaerobic conditions

A solution of the enzyme (30 nmol of trimethylamine dehydrogenase in 2.4 ml of 0.1 M sodium pyrophosphate/HCl, pH 8.5) was made anaerobic by repeated evacuation and flushing with argon in an anaerobic cuvette (Burleigh *et al.*, 1969). Trimethylamine was added as indicated and subsequent absorbance change was monitored on a Cary 14 spectrophotometer at room temperature.

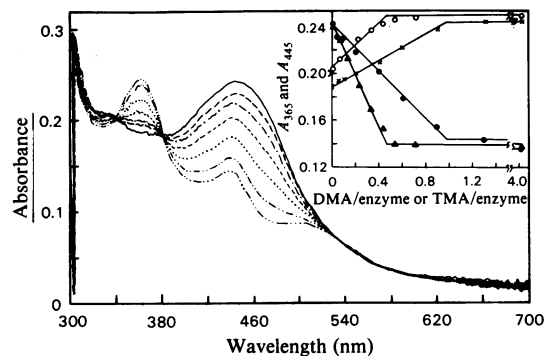


Fig. 2. Titration of trimethylamine dehydrogenase with dimethylamine in 0.1 M-sodium pyrophosphate/HCl, pH 8.5

The solution was made anaerobic as described in the legend of Fig. 1. Spectra were recorded when the decrease in absorbance at 445 nm following the addition of 0 (—), 0.1 (— · —), 0.2 (— · · —), 0.4 (— · · · —), 0.6 (· · · · · —), 0.9 (— · · · · · —), and 4.15 (— · · · · · · —) mol of dimethylamine per mol of enzyme was complete. The inset shows absorbance values at 365 (x) and 445 (O) nm as a function of the ratio of dimethylamine to enzyme. Also shown are the absorbance values at 365 (●) and 445 (▲) nm in an anaerobic titration of the enzyme with trimethylamine under the same conditions of buffer and pH. Abbreviations: DMA, dimethylamine; TMA, trimethylamine.

by substoichiometric amounts of trimethylamine was biphasic (Fig. 1). When sufficient time was allowed for the slow phase of reduction to reach completion, only 0.5 mol of trimethylamine per mol of enzyme was required to titrate the enzyme to an end point (Fig. 2, inset). An explanation for this behaviour became evident when it was found that dimethylamine also slowly reduced the enzyme, 1.0 mol of dimethylamine being required for complete reduction of the enzyme (Fig. 2). Evidence that trimethylamine dehydrogenase released both dimethylamine and formaldehyde upon reduction with trimethylamine was obtained by gel chromatography of the enzyme, reduced with [^{14}C]trimethylamine, on Sephadex G-25 under anaerobic conditions. When the experiment was carried out at pH 7.7 in 0.1 M sodium pyrophosphate/HCl, the radioactivity coincident with the reduced enzyme in the effluent fractions represented only about one-tenth of the expected radioactivity if one of the methyl groups from trimethylamine had remained enzyme-bound, whereas no radioactivity was associated with the enzyme after reduction with substrate at pH 7.0 in 0.1 M-potassium phosphate. The rapid phase in the reduction of trimethylamine dehydrogenase by trimethylamine observed in Fig. 1 was, therefore, due to the utilization of trimethylamine, whereas the slow phase could be ascribed to further reduction by dimethylamine which is released as a product. The rate of reduction of trimethylamine dehydrogenase by dimethylamine decreased with decreasing pH and was imperceptible at pH 7.0.

This observation most probably accounts for a discrepancy in the literature concerning the utilization of dimethylamine as a substrate (Colby & Zatman, 1974; Steenkamp & Mallinson, 1976).

Evidence for complex formation between reduced trimethylamine dehydrogenase and substrate

Whereas spectrophotometric titrations of trimethylamine dehydrogenase with substrates proceeded in an isosbestic manner at pH 8.5, as was the case with dimethylamine (Fig. 2), more complex behaviour was observed at lower pH values. This effect was first noticed when a titration of the enzyme with trimethylamine at pH 7.7 in 0.1 M-sodium pyrophosphate/HCl was monitored both by e.p.r. and absorption spectroscopy (Fig. 3). In this titration only about one-third of the maximal half-field signal at $g=4$ was generated by the addition of a stoichiometric amount of substrate which bleached the 445 nm absorption band of the flavin moiety completely. A substantial excess of substrate was required to maximize the e.p.r. signals characteristic of the triplet state. The enhancement of the half-field signal by the addition of excess substrate was accompanied by relatively minor spectral changes, notably increases in absorbance at 365 and 510 nm. This correlation of spectral changes with the formation of the triplet state is essentially in

agreement with previously reported conclusions which were based on a comparison of stopped-flow kinetic results with e.p.r. and reflectance spectro-

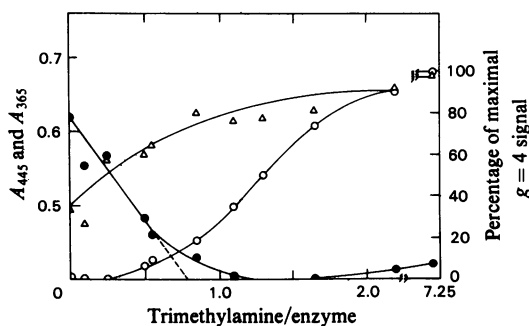


Fig. 3. Titration of trimethylamine dehydrogenase with trimethylamine followed by absorption spectrophotometry and e.p.r. spectroscopy

Individual samples containing $50\ \mu\text{M}$ -trimethylamine dehydrogenase in 0.1M -sodium pyrophosphate/HCl, pH 7.7, were reduced under anaerobic conditions (see the Materials and methods section) with different amounts of trimethylamine. Absorption spectra were recorded 20 min after the addition of substrate at 20°C . The samples were then frozen and e.p.r. spectra were recorded at 13 K. Absorbance at 445 (\bullet) and 365 (Δ) nm and development of the half-field signal at $g = 4.0$ (\circ), characteristic of the triplet state, as a percentage of maximal signal observed with an excess of trimethylamine, are plotted as functions of trimethylamine added per mol of trimethylamine dehydrogenase. With excess trimethylamine, the number of electrons recovered by double integration of the signals centred at $g = 2.0$, characteristic of the triplet state, was $1.4e^-/\text{mol}$ of enzyme.

scopy of samples obtained by the freeze-quench method (Steenkamp *et al.*, 1978a). In the titration experiments described here the reduction of the flavin prosthetic group could, therefore, be observed to precede the subsequent transfer of an electron to the [4Fe-4S] centre.

Close examination of the e.p.r. spectra obtained in the course of the titration shown in Fig. 3 indicated a progressive broadening of the e.p.r. signals centred around $g = 1.93$, suggesting that different species of the enzyme in the triplet state must exist (Fig. 4). The differences between e.p.r. spectra recorded in the early phase of the titration and those obtained at higher trimethylamine concentrations could be rationalized if it was assumed that the substrate binds to the reduced form of the enzyme, and in such a manner that not only the equilibrium in the transfer of an electron from the flavin moiety to the [4Fe-4S] centre, but also the geometry of these prosthetic groups relative to one another is affected (Beinert *et al.*, 1982).

The reduction of the flavin moiety and development of the triplet state of the enzyme was most clearly separated into distinct phases at low pH values. As judged from the height of the e.p.r. signal at $g = 4$, only 26% of the enzyme existed in the triplet state after the addition of a stoichiometric amount of substrate at pH 7.0 in 0.1M -potassium phosphate buffer. In a corresponding spectrophotometric titration (Figs. 5a and 5b) a 10-fold excess of substrate was required to maximize the increase in absorbance at 365 nm, indicative of the triplet state, whereas absorbance spectral changes at 365 nm were complete upon the addition of less than a stoichiometric amount of substrate at pH 8.5 in 0.1M -sodium pyrophosphate/HCl. In a parallel

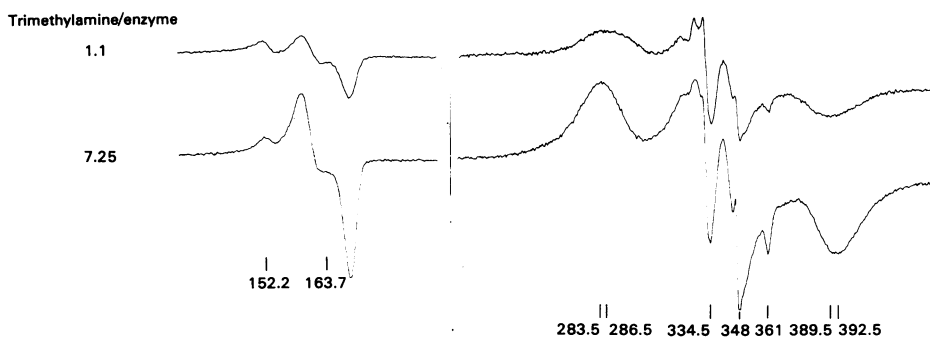


Fig. 4. E.p.r. spectra of samples from Fig. 3 obtained during the anaerobic titration of trimethylamine dehydrogenase with trimethylamine

The samples are those where 1.1 and 7.25 mol of trimethylamine per mol of the enzyme were added. Spectra of the $g \sim 2$ (right) and $g \sim 4$ (left) regions are shown. The numbers along the abscissa indicate mT at 9.2224 GHz. The conditions of e.p.r. spectroscopy were: microwave 2.7 and 9 mW at high and low field, respectively; modulation frequency 100 kHz; amplitude, 0.8 mT; scanning rate 31 and 50 mT/min at high and low field and temperature 13 K. Nine scans were averaged for the high field and three scans for the low field resonances.

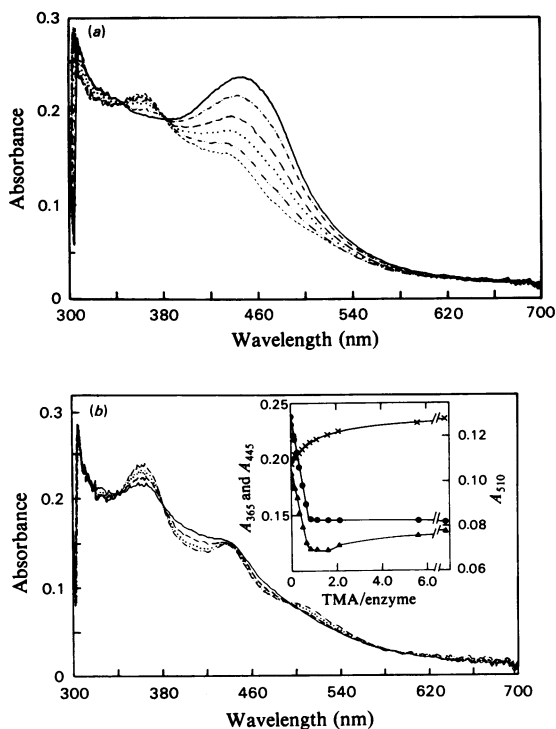


Fig. 5. Anaerobic titration of trimethylamine dehydrogenase with trimethylamine at pH 7.0 in 0.1 M-potassium phosphate

Anaerobicity was achieved as described in the legend to Fig. 1. (a) Titration of the enzyme with a stoichiometric amount of substrate. Spectra were recorded after the addition of 0 (—), 0.254 (— · — ·), 0.335 (— · — ·), 0.51 (· · · ·), 0.66 (— · · · ·), and 1.117 (— · — ·) mol of trimethylamine per mol of the enzyme. (b) Further titration with excess trimethylamine. The spectra were recorded after the addition of 1.117 (— · — ·), 2.13 (— · — ·), 8.22 (· · · ·), and 20.40 (— · — ·) mol of trimethylamine per mol of the enzyme. The inset shows the absorbance values at 365 (x), 445 (O), and 510 (Δ) nm as functions of the ratio of trimethylamine to enzyme. The final level of trimethylamine was 20.4 mol per mol of enzyme. Abbreviation: TMA, trimethylamine.

reduced trimethylamine dehydrogenase with [^{14}C]-trimethylamine in the eluting buffer as described in the Materials and methods section. In two separate experiments in which the eluting buffer contained either 151.4 μM - or 276 μM - [^{14}C]-trimethylamine, the amount of substrate bound to the reduced enzyme was determined to be 0.87 mol of trimethylamine per mol of the dehydrogenase in either case. The binding of substrate to the reduced enzyme did not impair its reoxidation by the electron acceptor flavoprotein, which proceeded at a rate considerably faster than would be necessary for catalytic turnover. When trimethylamine dehydrogenase, reduced with trimethylamine under conditions which should have ensured binding of excess substrate to the reduced enzyme, was rapidly mixed with the electron acceptor flavoprotein using the freeze-quench technique, more than 75% of the $g = 4$ signal disappeared within 6 ms (Steenkamp & Beinert, 1982).

Effect of tetramethylammonium chloride on the formation of the triplet state of trimethylamine dehydrogenase

In view of the foregoing evidence which demonstrated that the binding of trimethylamine to reduced trimethylamine dehydrogenase enhanced the formation of the triplet state, it was of interest to establish whether this property is shared by other amines, such as tetramethylammonium chloride, which protect the enzyme against inhibition by 'suicide inhibitors' of monoamine oxidase (Colby & Zatman, 1974), but do not reduce the enzyme. The steady state inhibition kinetics of tetramethylammonium chloride (Fig. 6) indicated that this could, indeed, be the case since a more complex inhibition pattern was obtained than would be expected of a mere competitive inhibitor. A secondary replot of the linear portions of the slopes in Fig. 6 was concave upwards rather than linear, whereas the non-linearity of double reciprocal plots at high concentrations of tetramethylammonium chloride suggested the contribution of alternative pathways to catalysis (Cleland, 1963). More relevant to this investigation, however, the effect of tetramethylammonium chloride on the intercepts of Fig. 6 indicated binding of the tetramethylammonium ion to a form of trimethylamine dehydrogenase other than the oxidized enzyme. When trimethylamine dehydrogenase was reduced with 0.84 mol of trimethylamine per mol of enzyme at pH 7.7 in 0.1 M-sodium pyrophosphate buffer, the subsequent addition of tetramethylammonium chloride resulted in spectral changes similar to those which characterize the enhancement of the triplet state upon addition of excess trimethylamine as in Fig. 5(b). That these spectral changes do indeed reflect an increase in the proportion of enzyme present in the triplet state was confirmed by e.p.r. spectroscopy

experiment 64% of the maximal e.p.r. signal height at $g = 4$ was obtained when the enzyme was reduced with one mol of substrate per mol of enzyme at pH 8.5, and only 38% of the half-field signal appeared under similar conditions at pH 7.7.

These results suggested the presence of a binding site for the substrate on the reduced form of the enzyme, occupancy of which could modulate the transfer of an electron from the reduced flavin to the [4Fe-4S] cluster. The existence of such a binding site was confirmed by gel chromatography of

which showed that the addition of a 90-fold molar excess of tetramethylammonium chloride to trimethylamine dehydrogenase which had been reduced with a stoichiometric amount of substrate enhanced the $g = 4$ signal height from 38.5 to 90% of the maximum amount obtained in the presence of excess trimethylamine. Moreover, the reduction of the enzyme with dithionite in the presence of a 90-fold excess of tetramethylammonium chloride resulted in the appearance of e.p.r. signals characteristic of the triplet state of the enzyme, rather than the rhombic signal centred at $g = 1.94$, typical of reduced $[4\text{Fe-4S}]$ clusters (Steenkamp *et al.*, 1978a). In agreement with this observation, titration of the enzyme with dithionite in the presence of tetramethylammonium chloride proceeded isobestically as in the titration of the enzyme with substrate in Fig. 2, and terminated after the uptake of one mol of dithionite, rather than 1.5 mol as found in the absence of the amine (Steenkamp *et al.*, 1978a). The final spectrum resembled that of the triplet state. This observation implied that the oxidation-reduction potentials of the flavin and $[4\text{Fe-4S}]$ cluster and/or their geometry relative to one another is markedly influenced by the binding of tetramethylammonium chloride to the enzyme. The species obtained by reduction of trimethylamine dehydrogenase with dithionite in the presence of tetramethylammonium chloride was found to be catalytically competent, since rapid mixing with the oxidized electron acceptor flavoprotein, using the freeze-quench technique, resulted in the disappearance of 75% of the e.p.r. signals characteristic of the triplet state within 6ms. This observation indicated that the triplet state of the enzyme containing bound tetramethylammonium ion is not a 'dead-end' complex (Cleland, 1963), and raised a significant question concerning the participation of the triplet state of the enzyme as an intermediate in catalysis.

If the triplet state of trimethylamine dehydrogenase were an obligatory intermediate in catalysis, it became unclear why the slopes of double reciprocal plots versus tetramethylammonium ion concentration (Fig. 6) should be concave upwards, since high concentrations of the tetramethylammonium ion were found to facilitate the formation of the triplet state and did not interfere with its reoxidation. Moreover, investigation of the steady state kinetics of trimethylamine dehydrogenase at high substrate concentrations indicated that not only the alternative substrate, diethylamine (Steenkamp & Mallinson, 1976), but also trimethylamine, showed partial substrate inhibition. Also this observation was contrary to expectation if the formation of the triplet state were assumed to be obligatory in catalysis. An explanation for these observations was suggested by an analysis of the

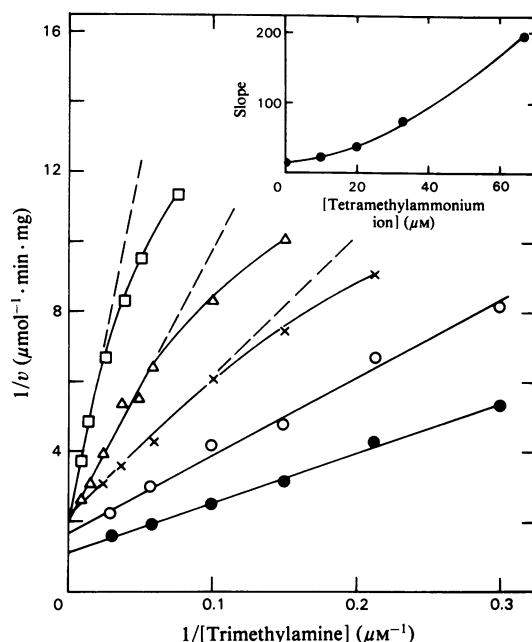


Fig. 6. Inhibition of trimethylamine dehydrogenase by tetramethylammonium chloride

The steady state kinetic assays were conducted at fixed concentrations of $57\ \mu\text{M}$ -dichlorophenolindophenol and $0.67\ \text{mM}$ -phenazine methosulphate. The temperature was 10°C . The tetramethylammonium ion concentrations were 0 (\bullet), 10 (\circ), 20 (\times), 33 (Δ) and 67 (\square) μM . The inset shows a secondary plot of the slopes at high trimethylamine concentrations versus tetramethylammonium ion concentration. Data points shown in the primary plot represent the average of duplicate determinations.

kinetics of formation of the triplet state, the results of which are described in the accompanying paper (Steenkamp & Beinert, 1982).

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