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Participation of the Iron–Sulphur Cluster and of the Covalently Bound Coenzyme of Trimethylamine Dehydrogenase in Catalysis

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Bacterial trimethylamine dehydrogenase contains a novel type of covalently bound flavin mononucleotide and a tetrameric iron–sulphur centre. The dehydrogenase takes up 1.5 mol of dithionite/mol of enzyme and is thereby converted into the flavin quinol-reduced (4Fe–4S) form, with the expected bleaching of the visible absorption band of the flavin and the emergence of signals of typical reduced ferredoxin in the electron-paramagnetic-resonance spectrum. On reduction with a slight excess of substrate, however, unusual absorption and electron-paramagnetic-resonance spectra appear quite rapidly. The latter is attributed to extensive interaction between the reduced (4Fe–4S) centre and the flavin semiquinone. The species of enzyme arising during the catalytic cycle were studied by a combination of rapid-freeze e.p.r. and stopped-flow spectrophotometry. The initial reduction of the flavin to the quinol form is far too rapid to be rate-limiting in catalysis, as is the reoxidation of the substrate-reduced enzyme by phenazine methosulphate. Formation of the spin–spin-interacting species from the dihydroflavin is considerably slower, however, and it may be the rate-limiting step in the catalytic cycle, since its rate of formation agrees reasonably well with the catalytic-centre activity determined in steady-state kinetic assays. In addition to the interacting form, a second form of the enzyme was noted during reduction by trimethylamine, differing in absorption spectrum, the structure of which remains to be determined.

Trimethylamine dehydrogenase [trimethylamine–(acceptor) oxidoreductase (demethylating), EC 1.5.99.7], an enzyme from methylotrophic bacteria (Colby & Zatman, 1974), catalyses the oxidative demethylation of trimethylamine to formaldehyde and dimethylamine. The enzyme from the restricted facultative methylotroph, bacterium W3A1 (Colby & Zatman, 1975), has been isolated and shown to have a mol.wt. of 147000 (Steenkamp & Mallinson, 1976) and to contain a single tetrameric iron–sulphur centre (Hill *et al.*, 1977), as well as an unusual yellow chromophore (Steenkamp & Singer, 1976). Recent studies have provided firm evidence that this newly discovered chromophore is a covalently bound flavin derivative, the spectral and fluorescent properties of which are considerably modified by substitution of the flavin in a position other than the 8α-methylene function (D. J. Steenkamp, W. C. Kenney & T. P. Singer, unpublished work).

Our interest in trimethylamine dehydrogenase stems in part from the novel type of covalently bound flavin that it contains, in part from the fact that typical 'suicide' inhibitors of mitochondrial monoamine oxidase (Maycock *et al.*, 1976), such as cyclopropylamines and substituted hydrazines, are also potent inhibitors of this enzyme, although it does not oxidize monoamines (Colby & Zatman, 1974; D. J. Steenkamp & M. Gallup, unpublished work). Another fascinating feature of this enzyme, noted in the present paper, is the intense interaction of the iron–sulphur centre with the flavin on reduction by substrates. As a first step toward the understanding of its reaction mechanism, we have examined the intermediates occurring during catalysis by a combination of absorbance and e.p.r. spectral measurements, and have determined the rates of formation and decay of readily detectable intermediates during the catalytic cycle. The results are described below.

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Materials and Methods

Trimethylamine dehydrogenase was purified from sonicated preparations of bacterium W3A1, as described by Steenkamp & Mallinson (1976), except

that the enzyme was dialysed against 1mM-EDTA before gel exclusion, to minimize interference in e.p.r. experiments by non-specifically bound iron. The enzyme was stored in 20% (v/v) ethylene glycol at −20°C and was transferred to 0.1M-sodium pyrophosphate buffer, pH7.7, by either dialysis or gel exclusion on Sephadex G-25 before e.p.r. or stopped-flow experiments.

For comparison with e.p.r. and stopped-flow analyses, the catalytic centre activity of the enzyme at 18°C was obtained from double-reciprocal plots of steady-state kinetic assays carried out in 0.1M-sodium pyrophosphate buffer, pH7.7, at a fixed saturating concentration of trimethylamine (3.33mM) and variable phenazine methosulphate concentrations.

Stopped-flow kinetic measurements were performed in an Aminco–Morrow stopped-flow spectrophotometer (American Instrument Co., Silver Springs, MD, U.S.A.), interfaced with a Nova 2/4 computer (On Line Instruments, Athens, GA, U.S.A.), and equipped with a Jobin–Yvon prism monochromator which allowed measurements in the spectral range above 390nm. Experiments at wavelengths below 390nm were performed in a Gibson–Durrum stopped-flow spectrophotometer (Durrum Instrument Co., Palo Alto, CA, U.S.A.), equipped with u.v. optics. Trimethylamine dehydrogenase, reduced with a slight excess of substrate, is only slowly reoxidized by atmospheric O_2 over a period of several hours. Consequently, all stopped-flow experiments were carried out under aerobic conditions. Reoxidation of the reduced enzyme by phenazine methosulphate was monitored at 480nm, since at this wavelength reduction of the dye contributed insignificantly to the absorbance change.

Absorbance spectra were recorded with a Cary 14 spectrophotometer. Low-temperature optical reflectance and e.p.r. spectra were measured and anaerobic rapid kinetic experiments by freeze-quenching were carried out and evaluated as described previously (Orme-Johnson *et al.*, 1974*a*,*b*; Beinert *et al.*, 1976). Specific details are given in the Figure legends. The enzyme used in these experiments was shipped between the two collaborating laboratories in solid CO_2 or liquid N_2 in the form of pellets, as obtained in $(NH_4)_2SO_4$ fractionation, and was dialysed before use, as described above. The trimethylamine dehydrogenase–coenzyme peptide was obtained as described by Steenkamp & Singer (1976).

Results

In previous studies it was shown that trimethylamine dehydrogenase contains one (4Fe–4S) core and a yellow organic coenzyme, since shown to be a flavin derivative that is covalently bound to the enzyme (Steenkamp & Singer, 1976; Hill *et al.*, 1977; D. J. Steenkamp, W. C. Kenney & T. P. Singer, unpublished work). Assuming that these are the only reducible groups, the enzyme should theoretically take up 1.5mol of dithionite/mol. This expectation is borne out by the results shown in Fig. 1. During the addition of roughly the first 0.5mol of dithionite/mol of enzyme, the titration proceeds with an increase in absorbance at 365nm (Fig. 1*a*); thereafter, the quasi-isosbestic points at 385nm and 345nm are lost, indicating that more than one group in the enzyme is being reduced (Fig. 1*b*). The titration is complete after the addition of 1.48mol of dithionite/mol of enzyme, in agreement with the uptake of one electron by the iron–sulphur centre and of two electrons by the reducible organic chromophore [cf. Fig. 1*b* (inset) and legend of Fig. 1].

By contrast, anaerobic titration of the enzyme with substrate proceeds isosbestically and generates a pronounced peak at 365nm with shoulders at 430nm and 510nm (Fig. 2). As shown by the e.p.r. results presented below, these marked spectral alterations reflect not merely a perturbation of the chromophores of the enzyme or formation of a charge-transfer complex, as had been reported for many flavoenzymes (Kosower, 1966; Steenkamp *et al.*, 1973), but actual reduction of the enzyme by the substrate. The bleaching is complete after the addition of only a slight molar excess of substrate, indicating that the overall equilibrium is far towards reduction of the enzyme.

The difference between substrate-reduced and dithionite-reduced trimethylamine dehydrogenase is even more dramatic when examined by e.p.r. spectroscopy (Fig. 3). Before reduction the purified enzyme shows only two minor e.p.r. signals, a signal typical of a free radical, at a concentration that represents approx. 5% of the enzyme, and a weak signal at $g = 4.3$, as is found in most biological samples. When the enzyme is reduced anaerobically by an excess of dithionite, a rhombic signal (Fig. 3, bottom curve) with g values of 2.035, 1.925 and 1.85, typical of a reduced iron–sulphur protein, is observed. In the two cases examined, this signal accounted for respectively 1.00 and 1.07 electron equivalents/mol of enzyme, indicating that the enzyme contains a single (4Fe–4S) cluster rather than two (2Fe–2S) clusters, in agreement with results obtained by the core extrusion technique (Hill *et al.*, 1977). When the enzyme is made to react with trimethylamine, however, a very different, complicated set of signals is produced immediately (Fig. 3, top and centre curves), which is seen at 34GHz (top curve, Fig. 3) and 9.2GHz (centre curve, Fig. 3). By integration this set of signals accounts for approx. 1.5 electron equivalents/mol of enzyme. The relationship of the principal lines at these two frequencies is indicated in Fig. 3 by broken lines. In determining what fraction of the iron–sulphur cluster is in the same form after reduction by substrate as after dithionite reduction, com-

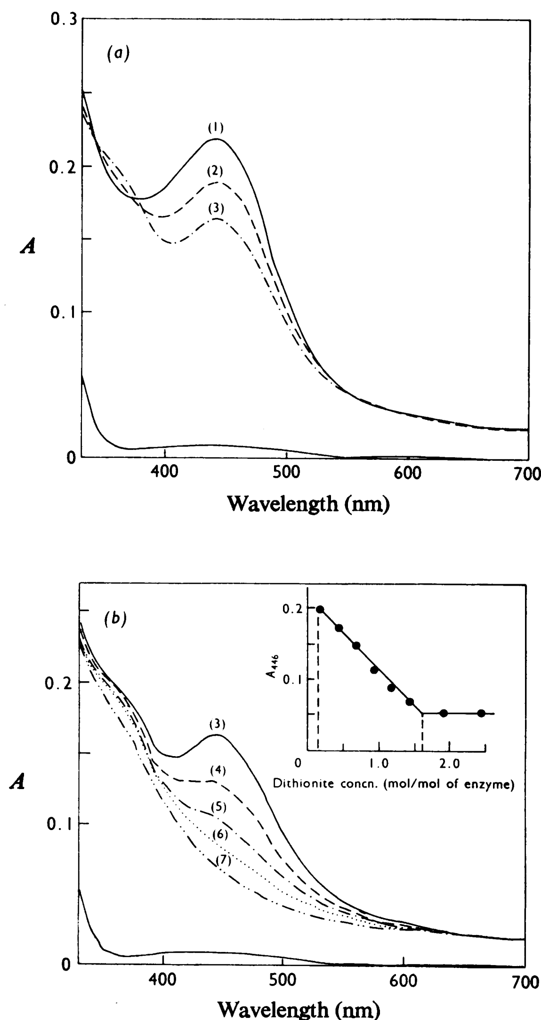


Fig. 1. *Anaerobic dithionite titration of trimethylamine dehydrogenase at pH*7.7 *in* 0.1 M-*sodium pyrophosphate*

(*a*) Initial phase of titration. The curves shown are: (1), oxidized enzyme; (2) and (3), after the addition of 0.42 and 0.66 mol of dithionite/mol of enzyme respectively. (*b*) Later phase of titration. Curves (3), (4), (5), (6) and (7) represent the addition of 0.66, 0.93, 1.17, 1.44 and 1.93 mol of dithionite/mol of enzyme respectively. The dithionite solution was standardized by titration of riboflavin in the same buffer. The inset shows the progress of titration. Note that the first two points, representing the addition of 0 and 0.153 mol of dithionite/mol respectively, gave coincident absorbance spectra [Curve (1), Fig. 1*a*] because of trapped O_2. This quantity of dithionite was therefore subtracted from the total titre of 1.63 mol of dithionite/mol of enzyme giving a net titre of 1.48 mol/per mol of enzyme. The lower-most curve indicates the baseline for the anaerobic cuvette.

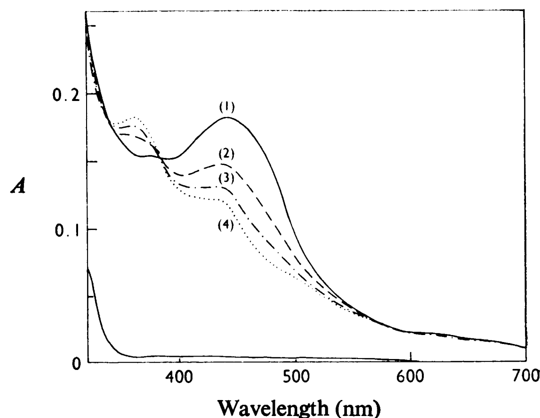


Fig. 2. *Anaerobic titration of trimethylamine dehydrogenase with trimethylamine at pH*7.7 *in* 0.1 M-*sodium pyrophosphate*

The trimethylamine solution was prepared from a titrimetrically standardized stock solution and was made anaerobic under an atmosphere of argon by incubation with 10 pmol of glucose oxidase, 18 μmol of glucose, 18 μmol of sodium acetate, pH 5.2, and sufficient catalase to prevent accumulation of peroxide as judged by independent polarographic measurements, all in 1.8 ml. Curve (1) represents the spectrum of the oxidized enzyme, and curves (2), (3) and (4) show the addition of 0.55, 0.896 and 1.24 mol of trimethylamine/mol of enzyme respectively. The addition of a further 0.7 mol of trimethylamine/mol of enzyme had no significant effect on the spectrum of the substrate-reduced enzyme. The lower-most curve shows the baseline for the anaerobic cuvette.

parison has been based on the intensity of the line at 356 mT, which shows the least interference by overlapping lines after the addition of an excess of trimethylamine (Fig. 3, centre curve). Comparison of the centre and bottom curves, which differ by a factor of 20 in amplification, reveals that only about 5% of the iron–sulphur cluster of the former yields the same signal (i.e. is in the same state) as the dithionite-reduced enzyme. The substrate-reduced form, similarly, shows only weak free-radical signals (e.g. the sharp line in the top spectrum between 1211 and 1227 mT). The broken lines show that the resonances of the reduced cluster are spread out at 34 GHz, as expected for the anisotropic features of a paramagnetic species. Comparison of the 34 GHz and 9.2 GHz spectra, however, also shows that other lines do not spread out to the same extent [cf. the broad ones at 281 and 387 mT (9.2 GHz) and the two more narrow lines in the centre]. The failure of these lines to spread out when the field strength increases is indicative of spin–spin interaction. The fact that spin–spin interaction is indeed taking place is shown by the unusually strong half-field signal around

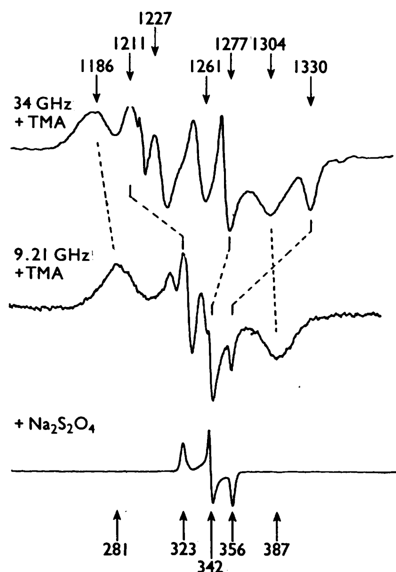


Fig. 3. *E.p.r. spectra of trimethylamine dehydrogenase reduced with an excess of trimethylamine (top and centre curves) or dithionite (bottom curve)*

Enzyme (32 μM) in 0.1 M-pyrophosphate buffer, pH 7.7, was mixed anaerobically with trimethylamine hydrochloride (TMA, final concentration 5 mM) for the centre spectrum, and at approx. 10 times the enzyme concentration for the top spectrum and was frozen approx. 1 min after mixing. The bottom spectrum was obtained after addition of a 10-fold excess of dithionite to enzyme (32 μM). The conditions of e.p.r. spectroscopy were: top spectrum, 34 GHz, ~3 mW, 0.5 mT modulation amplitude, 13 K; centre and bottom spectra, 9.2 GHz, 2.7 mW, 0.8 mT modulation amplitude and 13 K. The amplification of the centre spectrum was 20 times that of the bottom spectrum. The corresponding resonances at 34 and 9.2 GHz are connected by broken lines, to show the large spread of the (sharp) lines of the iron–sulphur centre, as compared with the broad lines, indicating interaction. Two new, sharp lines in the centre in the top and centre spectra, which are not seen in the bottom spectrum (reduced with dithionite), are also due to interaction. The numbers next to the arrows designate magnetic-field strength in mT, the numbers above the traces for the top spectrum and those below the traces for the centre and bottom spectra at 9.21 GHz. When recorded at the same amplification, the peak-to-peak amplitude of the signal at $g = 4$ equals the peak-to-peak vertical distance between the lines at 281 and 387 mT in Fig. 3 (centre).

$g = 4$ (Fig. 4). For the present purposes it may suffice to say that the most likely explanation of these observations is formation in the enzymic reaction of a flavin semiquinone and a reduced iron–sulphur

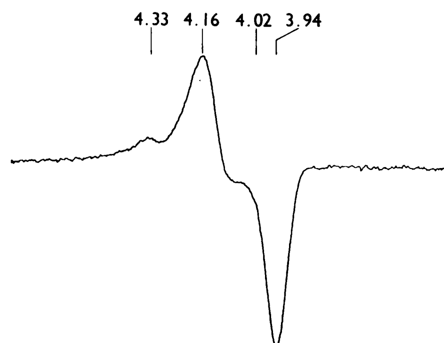


Fig. 4. *Half-field signal of trimethylamine dehydrogenase after reaction with trimethylamine*

Only the signal at $g = 4.3$ is present before addition of the substrate. The sample preparation and conditions of spectroscopy at 9.2 GHz are those of Fig. 3. The numbers represent g values.

cluster, which are sufficiently close to interact, as indicated by the e.p.r. spectra. These observations also imply that trimethylamine furnishes only two electrons to the enzyme, resulting in the formation of a flavin semiquinone and of a reduced iron–sulphur centre. If the flavin were completely reduced, the signal of the iron–sulphur centre would appear with full intensity.

During anaerobic titration with dithionite the principal signals emerging are a free-radical signal (flavin semiquinone) and the signal of the reduced iron–sulphur centre, with only weak signals of the kind observed with trimethylamine as reductant superimposed (results not shown). Though other explanations are possible, this may be interpreted to indicate that the two paramagnetic sites are not in the same favourable position for interaction when dithionite is the reductant as they are in the presence of trimethylamine. This would be compatible with a substrate-induced conformation change in the enzyme.

The time course of the reduction of the enzyme by trimethylamine was investigated by rapid freeze-quenching. Changes detectable by e.p.r. spectroscopy during the reduction were relatively slow. The various lines representing the interaction signals in the centre curve of Fig. 3 appeared at the same rate and, in accord with the data in Fig. 3, only small quantities (approx. 5% of the enzyme concentration) of free-radical and reduced-iron–sulphur signals emerged at any time between 4.5 ms and 1 min. Since the signal at $g = 4$ is least interfered with by other signals, the appearance of this signal is plotted in Fig. 5 against time. This signal appears with a $t_{\frac{1}{2}}$ of approx. 300 ms. Reflectance spectra recorded

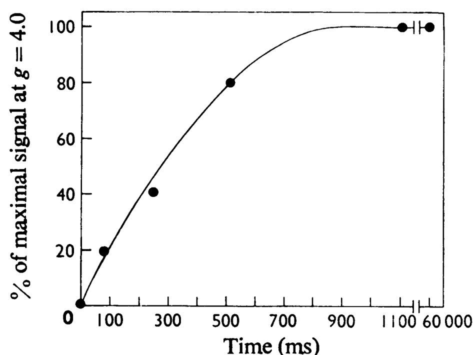


Fig. 5. *Plot of progress of formation of the e.p.r. signal at $g = 4.0$ (see Fig. 4) with time of reaction*
The experiment was that described in the legend to Fig. 6.

on the same samples are shown in Fig. 6. The chromophore at 445nm (flavin) is bleached immediately (the spectrum at 4.5ms was identical with that at 7ms). Absorption at 360 and 520nm develops much more slowly, comparable with the rate of appearance of the e.p.r.-detectable spin–spin interaction. It should be noted that a $t_{\frac{1}{2}}$ of approx. 300ms is compatible with the catalytic-centre activity determined from catalytic assays (at $V_{max.}$), which was found to be 120/min at the same pH and temperature. Since the quinol form of the flavin, produced on reduction with dithionite, does not show absorption peaks at 360 or 520nm, the flavin must be in the radical form. Although from the absorbance changes above it is not possible to decide whether the flavin exists as the free semiquinone or the spin-coupled form after reduction by the substrate, e.p.r. results on the same samples rule out the former possibility, because of the absence of a $g = 2.00$ signal.

This indication that the sharp, e.p.r.-detectable interaction signals are also recognizable by absorbance-spectral changes and that both measurements yield a half-life of approx. 300ms suggested the possibility of following the formation of this intermediate by stopped-flow spectrophotometry. Initial measurements involved the bleaching of the 445nm absorbance peak of the flavin. On mixing the dehydrogenase with 1mM-trimethylamine at 18°C in the stopped-flow apparatus, the spectral change observed at this wavelength was complete within the dead time of the instrument, indicating extremely fast reduction of some component of the enzyme. At a somewhat lower (0.5mM) trimethylamine concentration, the half-life for this reaction was estimated to be about 1.5–2ms.

Because of the difficulty in measuring accurately small spectral changes occurring near the dead-time

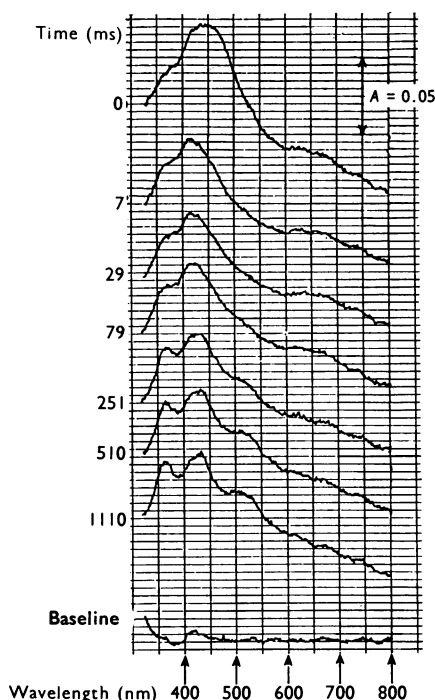


Fig. 6. *Low-temperature (100K) reflectance spectra of trimethylamine dehydrogenase reacting with trimethylamine*
The enzyme (60μM) was dissolved in 0.1M-sodium pyrophosphate buffer, pH7.7, and mixed at 15°C with an equal volume of 2mM-trimethylamine hydrochloride in the same buffer in the rapid freeze–quench apparatus. The samples shown were frozen after the reaction times indicated.

of the instrument, this fast reaction was not investigated further. We attempted to characterize instead the absorption spectrum of the rapidly formed intermediate by back-extrapolation from subsequent slow spectral changes.

Before determining the spectrum in this manner, Iwas necessary to study the time course of the slow reactions that follow this initial, rapid bleaching of the enzyme. Two relatively slow reactions have been detected (Fig. 7), the rates of which appeared to be independent of trimethylamine concentration in the range 0.5–5mM. Thus, a reaction with $t_{\frac{1}{2}}$ approx. 80ms at 18°C could be observed at 365nm (curve A) and also at longer wavelengths in the region 490–600nm (curve B). Fortunately, this reaction showed relatively little spectral overlap with a slower reaction, with $t_{\frac{1}{2}}$ approx. 200ms, that could hardly be detected at 600nm, but that was the predominant reaction observed at times longer than 50ms in the spectral region 390–430nm (curves C and D). Consequently,

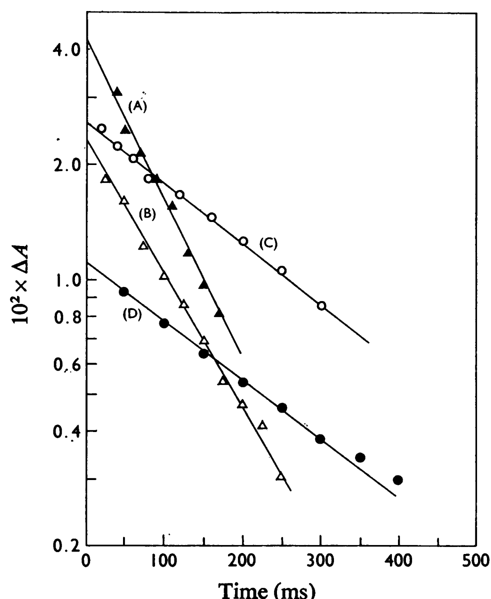


Fig. 7. *Semilogarithmic plots obtained from stopped-flow experiments at different wavelengths*

Trimethylamine dehydrogenase was mixed with 0.5mM-trimethylamine (final concentration) at 18°C in 0.1M-sodium pyrophosphate buffer, pH7.7. For purposes of comparison, the results from different experiments were all normalized to an enzyme concentration of 9μM. The actual enzyme concentrations after mixing were 4.5–5μM. The reaction was observed at: (A), 365nm (▲); (B), 520nm (△); (C), 410nm (○); (D), 430nm (●). The ΔA value on the ordinate denotes the magnitude of the difference in absorbance between the end of the reaction and at time t, but not its sign: in curves (A) and (B), the ΔA value represents an increase in colour, but in curves (C) and (D) a decrease.

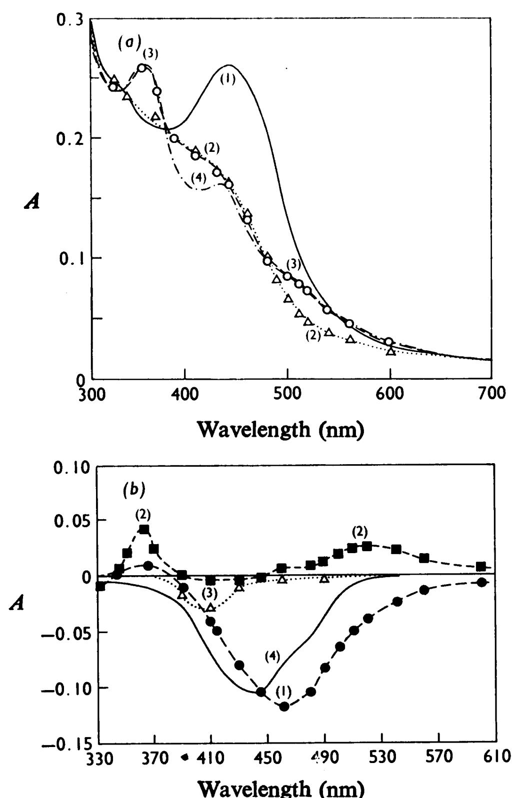


Fig. 8. *Effect of trimethylamine on the absorbance spectral properties of trimethylamine dehydrogenase*

(*a*) Spectral intermediates obtained during the reduction of trimethylamine dehydrogenase by trimethylamine. Reaction conditions were as described in the legend to Fig. 7. The curves represent: (1), the oxidized enzyme at a concentration of 9μM (——); (2), after a rapid bleaching with $t_{\frac{1}{2}}$ approx. 1.5–2.0ms (△···△); (3), after a slower reaction with $t_{\frac{1}{2}}$ approx. 80ms (○—○); (4), the final spectrum after several minutes (–·–·–). (*b*) Difference spectra of the results shown in Fig. 8(*a*). The curves represent: (1), reaction intermediate formed by fast reaction ($t_{\frac{1}{2}}$ approx. 1.5–2.0ms) minus oxidized enzyme; (2), reaction intermediate formed by slower reaction ($t_{\frac{1}{2}}$ approx. 80ms) minus intermediate formed by fast reaction ($t_{\frac{1}{2}}$ approx. 1.5–2.0ms); (3), final spectrum minus reaction intermediate that appeared with $t_{\frac{1}{2}}$ approx. 80ms; (4), difference spectrum between dithionite-reduced and oxidized trimethylamine dehydrogenase–coenzyme peptide at a concentration of 9μM.

the spectrum of the intermediate that appears with $t_{\frac{1}{2}}$ approx. 1.5–2.0ms at 18°C could readily be ascertained as described (Cattalini *et al.*, 1968; Hiyama & Ke, 1971). The results, however, do not allow a decision as to whether the reactions with $t_{\frac{1}{2}}$ approx. 80ms and 200ms are simultaneous or consecutive, since they are not sufficiently well separated in time, and even a small spectral overlap might have obscured any initial lag in the reaction with $t_{\frac{1}{2}}$ approx. 200ms, as may be expected if these reactions were consecutive. Therefore, the fact that semilogarithmic plots of the reaction with $t_{\frac{1}{2}}$ approx. 200ms appeared linear from about 50ms up to 400ms when observed at 390, 410 and 430nm may be fortuitous. As an approximation it was decided that extrapolation of the slow spectral changes observed in the reaction with $t_{\frac{1}{2}}$ approx. 200ms to zero time would give a reasonable, although only qualitative, estimate of the total spectral change involved in this reaction.

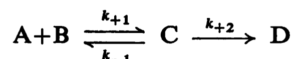
The spectra of the intermediates obtained by these procedures are shown in Fig. 8. Fig. 8(*b*) shows that the difference spectrum between the oxidized enzyme

and the product of the reaction with $t_{\frac{1}{2}}$ approx. 1.5–2.0ms (curve 1) shows a close similarity to the difference spectrum obtained by dithionite titration of an equimolar amount of the flavin peptide (Steenkamp & Singer, 1976) isolated from the dehydrogenase (curve 4). Although the difference spectrum for dithionite titration of this flavin peptide is blue-shifted by about 25nm relative to the difference spectrum for the rapid bleaching of the enzyme by substrate, the influence of the protein environment on the spectrum of the flavin may well account for the discrepancy. Therefore the earliest detectable event on mixing enzyme with substrate appears to involve a reduction of the coenzyme moiety, so that the development of the spin-coupled signal observed by e.p.r. spectroscopy must result from a transfer of an electron from the reduced coenzyme to the (4Fe–4S) core.

Correlation of the reactions with $t_{\frac{1}{2}}$ approx. 80ms and 200ms respectively with the rate of development of the unusual interaction spectrum observed in e.p.r. spectroscopy also proved to be problematic. Although superficial comparison of the rates involved suggests that the reaction with $t_{\frac{1}{2}}$ approx. 200ms, observed by the stopped-flow method, may be identified with the appearance of the interaction signal $g = 4$ (Fig. 5), the results obtained from reflectance spectral measurements on the samples that were used for e.p.r. measurements (Fig. 6) do not support this supposition. The timed series of reflectance spectra (Fig. 6) agree with the spectral changes observed by stopped-flow kinetic measurements (Fig. 8) as regards the conclusion that the bleaching of the absorbance band at 445nm of the enzyme is rapid and is followed by the slower development of absorbance bands at about 520nm and 365nm in both sets of measurements. It is evident, however, that the rate of increase of the band at 520nm observed by stopped-flow spectrophotometry is substantially faster than the increase in the intensity of the band at 520nm observed in the reflectance spectra or the rate of appearance of the spin–spin-interaction signal in the e.p.r. experiments. There is no satisfactory explanation for this discrepancy at this time (see the Discussion section). Considering that the reflectance spectra were measured on the same samples as were used for e.p.r. studies, despite the qualitative nature of the former method, it might be argued that it may be more readily correlated with the changes in the e.p.r spectra, and that consequently the increased absorbance at 365 and 520nm reflects more clearly the formation of the semiquinone species, which, according to e.p.r. results, is in the interacting state, than does the slower decrease in absorbance at 410nm noted in the stopped-flow experiments, although both reactions are fast enough to be catalytically significant.

This assignment is also in accord with the spectral changes to be expected during the formation of such an interacting flavin radical–(4Fe–4S) species, in view of the published spectra of flavosemiquinone–iron chelates (Müller *et al.*, 1968).

The reoxidation of the enzyme was examined by stopped-flow spectrophotometry at 480nm at variable phenazine methosulphate concentrations. A double-reciprocal plot (Fig. 9) of the observed rate constant against phenazine methosulphate concentration showed a non-zero intercept, indicating a partial reaction sequence of the type



$$\mathrm{A+B} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \mathrm{C} \xrightarrow{k_{+2}} \mathrm{D}$$

The rate constant k_{+2} at infinite phenazine methosulphate concentration of about $16\,s^{-1}$ predicts a catalytic-centre activity of 1000mol/min per mol of enzyme, about 10-fold faster than the rate calculated for infinite phenazine methosulphate concentration from steady-state kinetic measurements. Clearly, therefore, reoxidation of the enzyme by phenazine methosulphate is not rate-limiting in catalytic assays.

The rate of reoxidation of the enzyme by phenazine

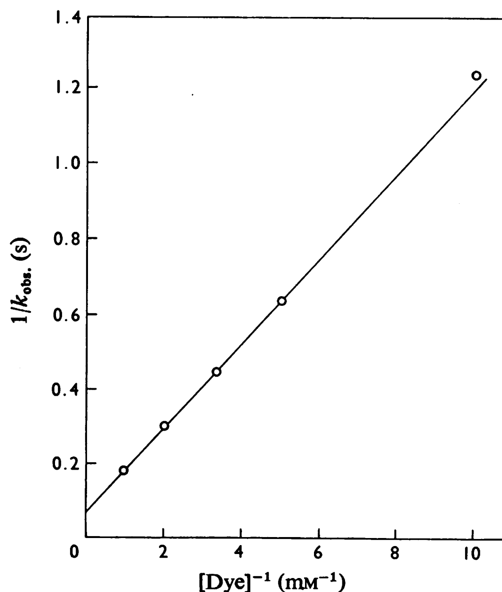


Fig. 9. *Double-reciprocal plot of the observed rate constant for reoxidation of the enzyme by phenazine methosulphate*

The reaction was measured in a stopped-flow apparatus at 480nm and 18°C, with in one syringe enzyme (9.5μM) that had been reduced by an approximately 3-fold excess of trimethylamine, and various concentrations of phenazine methosulphate in the other, to yield the final dye concentrations shown on the abscissa. The buffer was 0.1 M-sodium pyrophosphate, pH7.7, in both syringes. The enzyme concentration after mixing was 4.75μM.

methosulphate was also studied by the rapid-freeze e.p.r. technique. On mixing enzyme (30μM), previously reduced with 1 mM-trimethylamine, with 1 mM-phenazine methosulphate (all final concentrations) at pH 7.7 and 18 °C, all e.p.r. signals given by the interaction of flavin semiquinone and the reduced iron–sulphur cluster disappeared by 8 ms, as did the weak signal corresponding to the non-interacting iron–sulphur cluster (cf. Fig. 3 and its discussion). As early as 4.5 ms after mixing, only approx. 10% of these signals were detectable. Because of the presence of a strong free-radical signal at $g = 2.0$ from phenazine methosulphate, the signal corresponding to the free (non-interacting) semiquinone could not be followed in these experiments.

Discussion

Even before the studies described in the present paper, trimethylamine dehydrogenase attracted considerable interest, partly because it contains an entirely new type of flavin coenzyme (Steenkamp & Singer, 1976; D. J. Steenkamp, W. C. Kenney & T. P. Singer, unpublished work), and partly because it is severely inhibited by substances previously regarded as specific inhibitors of mitochondrial monoamine oxidase (Colby & Zatman, 1974). The results presented above call attention to two more unusual properties of this enzyme: a very intense interaction between its flavin semiquinone and its iron–sulphur cluster on reduction with the substrate, and the fact that the rate of formation of this species from the reduced flavoprotein seems to be the rate-limiting step in catalysis.

Evidence for the spin–spin interaction is presented in Fig. 3 and is verified by the uncommonly strong half-field signal at $g = 4$ (Fig. 4). It appears that the interaction observed in this enzyme is the most extensive among known complex flavoproteins (R. Sands, personal communication).

Evidence that formation of the interacting species may be rate-limiting in the catalytic cycle comes from comparison of rapid-freeze e.p.r. and spectrophotometric results with catalytic-centre activity determined in steady-state assays. From the extremely rapid bleaching of the band at 445 nm on addition of the substrate, it seems clear that reduction of the flavin chromophore precedes any interaction of the substrate with the iron–sulphur centre of the enzyme, and occurs at a rate that is several orders of magnitude faster than the catalytic-centre activity of this enzyme. Subsequent events, leading to the formation of the interacting species, are substantially slower and in the range predicted from steady-state kinetic data. At least two spectrally recognized intermediates form from the initial flavin quinol enzyme (Figs. 7 and 8), distinguishable by their visible spectra and rates of formation. Although the structure of neither species has been fully established, and hence it is not known which one corresponds to the interacting species shown in the upper two e.p.r. spectra in Fig. 3, it seems clear from Fig. 5 that the rate of formation of the interacting species is the slowest step in catalysis and the one that most clearly corresponds to the rate predicted from steady-state kinetics.

As noted above, the rate of formation of the interacting species from the dihydroflavin appears to be considerably faster when measured by stopped-flow ($t_{\frac{1}{2}}$ approx. 80 ms, Fig. 7) than by e.p.r. or reflectance spectra ($t_{\frac{1}{2}}$ approx. 300 ms, Figs. 5 and 6). Although it remains for future work to provide an explanation of this discrepancy, it is noteworthy that the enzyme concentrations in the e.p.r. experiments were over 6-fold higher than in the stopped-flow studies (the high enzyme concentration required for well-resolved e.p.r. spectra could not be duplicated in the stopped-flow experiments because of the limited quantity of enzyme available). Although the apparent first-order character of the absorption changes involved in the transition from the dihydroflavin to the radical form (Fig. 7) would suggest that the enzyme concentration should not affect the rate, it is conceivable that the resulting change in the enzyme/substrate ratio may influence the rate.

Regardless of this uncertainty, it is of great interest that the transfer of electrons from reduced flavin to the iron–sulphur centre, which is most probably an intramolecular event, is so much slower than either the initial reduction or reoxidation by phenazine methosulphate, especially since the strong spin–spin interaction between the resultant flavin radical and the reduced iron–sulphur centre suggests a very close proximity between these two species. No indication of the occurrence of this interacting form was noticed during titration with dithionite, showing that in the conformation obtained in chemical reduction of the enzyme, the flavin and iron–sulphur species are not juxtaposed. These observations suggest that the transfer of electrons to the iron–sulphur centre is accompanied by slow conformational changes in the enzyme, which bring the flavin and iron–sulphur loci in close contact with each other, or by the slow making and breaking of covalent bonds between enzyme-bound intermediates originating from the substrate and the flavin component. It is noteworthy that intramolecular electron transfer is generally not rate-limiting in complex flavoproteins (Edmondson *et al.*, 1973; Olson *et al.*, 1974; Beinert *et al.*, 1976) and that, hence, the mechanism of action of this enzyme may be somewhat unique.

During the reoxidation of the enzyme by phenazine methosulphate, the sequence of events seems to be in reverse order to that observed during reduction. The strong e.p.r.-detectable interaction signals disappear immediately on mixing the previously reduced enzyme with the dye, suggesting very fast reoxidation

of one of the interacting components. The slower spectral change observed by the stopped-flow method at 480nm, during reoxidation of the enzyme by phenazine methosulphate, accounts for most of the spectral difference between oxidized and substrate-reduced enzyme and most probably reflects a slower reoxidation of the flavin component.

One may rationalize the fact that the disappearance of the spin–spin-interaction e.p.r. signal is much faster than the return of A_{480} by considering that the absorbance change corresponding to the oxidation of tetrameric iron–sulphur clusters is only about $2\,\text{mM}^{-1}\cdot\text{cm}^{-1}$ at 480nm (Gillum *et al.*, 1977), where the measurements were carried out (Fig. 9), whereas reoxidation of the flavin moiety of the interacting species should be accompanied by an A_{480} change of approx. $10\,\text{mM}^{-1}\cdot\text{cm}^{-1}$ (Fig. 8). The fact that oxidation of the reduced (4Fe–4S) component represents only a small fraction of the observed change at this wavelength, coupled with the possibility that it may be fast enough to be within the dead-time of the apparatus, might account for the fact that the rapid phase was missed in the stopped-flow experiments summarized in Fig. 9.

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