Mechanistic studies on the dehydrogenases of methylotrophic bacteria

2. Kinetic studies on the intramolecular electron transfer in trimethylamine and dimethylamine dehydrogenase

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E.p.r. spectroscopy of the trimethylamine and dimethylamine dehydrogenases of *Hyphomicrobium* X indicates that the substrate-reduced forms of these enzymes exist in the triplet state, which arise through interaction of a reduced [4Fe–4S] cluster and flavosemiquinone, with e.p.r. signals which differ in detail from those of the trimethylamine dehydrogenase of bacterium W₃A₁. Under certain conditions the intramolecular electron transfer between the flavoquinol form of 6-*S*-cysteinyl-FMN and the [4Fe–4S] cluster in all three dehydrogenases was much slower than the preceding reduction of the flavin to the flavoquinol form. Trimethylamine dehydrogenases from both organisms show a time-dependent broadening of the e.p.r. signals centred around $g = 2$ after mixing with trimethylamine. The broadening of the e.p.r. signals could be correlated with an unexpected dependence of the rate of formation of the triplet state on substrate concentration. A model which accounts in a qualitative manner for the substrate dependence of the formation of the triplet state in the trimethylamine dehydrogenase of *Hyphomicrobium* X is proposed. The binding of the substrate to the reduced form of the enzyme seems to result in a conformational change of the enzyme to a form in which the rate of intramolecular electron transfer is decreased. This finding may be correlated with the observation of hyperbolic substrate inhibition for both trimethylamine dehydrogenases. The results indicate the transfer of an electron to the [4Fe–4S] cluster to be an obligatory step in catalysis and suggest that the transfer of electrons from these enzymes to electron acceptors is mediated solely through the [4Fe–4S] cluster.

Trimethylamine dehydrogenase is unique in that electron transfer between its two prosthetic groups, the 6-*S*-cysteinyl-FMN moiety (Steenkamp *et al.*, 1978a) and single [4Fe–4S] cluster (Hill *et al.*, 1977) is a slow step. Thus, complete reduction of the flavin prosthetic group at high substrate concentrations occurs within the dead time of rapid mixing devices, whereas the subsequent transfer of an electron from the flavin to the iron–sulphur cluster can be studied conveniently by stopped-flow spectrophotometry or by application of the freeze-quench technique, followed by e.p.r. spectroscopy. Comparison of the rates of intramolecular electron transfer as observed by these two approaches indicated a considerable discrepancy, since the stopped-flow technique showed an approximately three-fold faster rate than observed by the freeze-quench procedure (Steenkamp *et al.*, 1978a). Three possible explanations for this discrepancy could be invoked.

(i) Using e.p.r. spectroscopy, the progress of the reaction was monitored by observation of the half-field signal ($g = 4$) characteristic of the triplet state, which arises in this enzyme from the close proximity of the flavosemiquinone and reduced iron–sulphur cluster in the substrate reduced form, whereas the increase in absorbance at 365 nm, characteristic of the anionic semiquinone of flavins, was recorded by stopped-flow spectrophotometry as a measure of the same event. Evidently these variables need not necessarily have reflected the same state of the enzyme. (ii) It was reported that pyrophosphate, the buffer used in these experiments, showed pronounced shifts in pH on freezing (Williams-Smith *et al.*, 1977), and although the change in absorbance at 365 nm was correlated well with appearance of the triplet state in equilibrium situations (Steenkamp & Beinert, 1982), a pH effect on the rates could not be ruled out. (iii) Titration of

the enzyme with substrates (Steenkamp & Beinert, 1982) has shown that the transfer of an electron between the flavin and the [4Fe-4S] centre depended, in part, on the occupancy of a binding site for the substrate on the reduced enzyme. This introduced an additional uncertainty, since the rate of intermolecular transfer would, in that case, not necessarily be independent of the enzyme and substrate concentrations, as is usually the case for intramolecular reactions.

It was felt that by a careful examination of these alternatives it may be possible to account for the difference in rates obtained by the two techniques. Moreover, a trimethylamine and dimethylamine dehydrogenase from *Hyphomicrobium* X (Meiberg & Harder, 1979) was found to contain the same prosthetic groups as the trimethylamine dehydrogenase of bacterium W_3A_1 , but differed in specific activity and in the composition of the active site flavin peptide (Steenkamp, 1979). It was, therefore, of interest whether the unique kinetic properties of the enzyme from bacterium W_3A_1 would also be displayed in the *Hyphomicrobium* enzymes, in which case further insight could perhaps be gained from a comparative study.

In this paper the effects of substrate concentration and buffer composition on the rate of intramolecular electron transfer in the trimethylamine dehydrogenase of bacterium W_3A_1 and the tri- and dimethylamine dehydrogenases of *Hyphomicrobium* X are examined.

Materials and methods

Methods used in the preparation of the trimethylamine dehydrogenase and electron acceptor flavoprotein of bacterium W_3A_1 and the conditions of e.p.r. spectroscopy were as described (Steenkamp & Beinert, 1982). Stopped-flow kinetic experiments were performed in a Aminco-Morrow stopped-flow spectrometer interfaced with a Nova 2/4 computer. The stopped-flow computer program allowed for the averaging of data points obtained at corresponding times in successive rapid reaction experiments. The stopped-flow results reported represent the average of at least two, and in most cases three, stopped-flow runs performed under identical conditions. The tri- and dimethylamine dehydrogenases of *Hyphomicrobium* X were prepared as described (Meiberg & Harder, 1979). Other experimental details are described in the legends to Figures and Tables.

Results and discussion

Formation of the triplet state in the tri- and dimethylamine dehydrogenases

Reduction of the trimethylamine and dimethylamine dehydrogenases of *Hyphomicrobium* X

with substrates resulted in the appearance of three banded absorbance spectra indistinguishable from that earlier reported for the enzyme from bacterium W_3A_1 (Steenkamp *et al.*, 1978a). Examination of the three enzymes by e.p.r. spectroscopy, however, indicated significant differences between the three enzymes. Fig. 1 shows e.p.r. spectra in the $g \sim 2$ region obtained with the three dehydrogenases after brief (64–100 ms) and prolonged (2 min) exposure to substrate. The main features seen in these spectra have been described previously (Steenkamp *et al.*, 1978a; Beinert *et al.*, 1982), namely a set of two sharp lines centred at 335 mT at 9.2 GHz and a set

Fig. 1. E.p.r. spectra (first derivative) at $g \sim 2$ of samples of dimethylamine and trimethylamine dehydrogenases that have reacted with $100 \mu\text{M}$ -substrate for various times

The samples are from the experiments shown in Fig. 2. (a) and (b), dimethylamine dehydrogenase at 100 ms and 2 min; (c) and (d), trimethylamine dehydrogenase from *Hyphomicrobium* X at 64 ms and 2 min; (e) and (f), trimethylamine dehydrogenase from bacterium W_3A_1 at 80 ms and 2 min. The conditions of e.p.r. spectroscopy were: microwave power and frequency 2.7 mW and 9.22 GHz, respectively; modulation amplitude and frequency 0.8 mT and 100 kHz, respectively; and temperature 13 K. Four to nine spectra were averaged per sample, as required. Spectra (e) and (f) were recorded at twice the amplification of (a)–(d). Note the outward movement of the broad lines at low and high field in (d) and (f) as compared with (c) and (e), which is not apparent when (a) and (b) are compared.

of broad lines toward low and high field of these. A free-radical signal thought to arise from flavin semiquinone is superimposed on the sharp line at low field, as can be readily seen in Figs. 1(a), 1(e), and 1(f), while the sharp line at high field shows a signal from the reduced Fe-S cluster at ~ 345 and ~ 360 mT which increases with time. This is particularly clear in Fig. 1(d). These signals are approximately spread over a two-fold broader field in the trimethylamine dehydrogenase of bacterium W_3A_1 than in the dimethylamine dehydrogenase of *Hyphomicrobium* X. The corresponding half-field signals at $g \sim 4$ for the samples frozen at 2 min are shown in Fig. 2. The amplitudes are normalized so as to represent the same enzyme concentrations ($102 \mu\text{M}$) for the three enzymes. While the signals are very similar in shape, in contrast to those at $g \sim 2$, they differ widely in intensity. Dimethylamine dehydrogenase, the enzyme that shows no broadening at $g \sim 2$, has the weakest half-field signals, whereas the trimethylamine dehydrogenase of bacterium W_3A_1 , which shows the largest broadening at $g \sim 2$, has unusually intense half-field signals.

Determination of the rate of formation of the triplet state during the reduction of the dimethylamine and trimethylamine dehydrogenases of *Hyphomicrobium* X with their substrates indicated that the transfer of an electron from the flavoquinol species to the iron-sulphur cluster is slow enough to be conveniently followed by e.p.r. spectroscopy of samples collected by the freeze-quench technique. The

buffer used was diethylbarbiturate, which did not appear to undergo significant pH changes upon freezing, as determined by the indicator method (Williams-Smith *et al.*, 1977). The rate of formation of the e.p.r. detectable triplet state was compared with the kinetics of the absorbance change at 365 nm, which may be regarded as indicative of the anionic semiquinone species as it appears in several flavoproteins (Steenkamp *et al.*, 1978a; Palmer & Massey, 1968). In comparing the results of the freeze-quench experiments with the stopped-flow data it should be borne in mind that only eight data points (six in Fig. 3b) were available for construction of a curve depicting the time dependence of the e.p.r. signals, and that the match between this curve and the stopped-flow results was critically dependent on the final value. The results from e.p.r. and absorption spectroscopy obtained for the dimethylamine dehydrogenase from *Hyphomicrobium* X (Fig. 3a) are, therefore, in good agreement. The observed rate of change in absorbance at short times, where substrate depletion was not yet appreciable, was not significantly influenced by variation of the enzyme concentration over an eight-fold range. Although the agreement between the rates observed by the two methods was less satisfactory for the trimethylamine dehydrogenase, both the e.p.r. and stopped-flow spectrophotometric measurements indicated pronounced biphasic kinetics for the formation of the triplet state, which was not apparent for dimethylamine dehydrogenase under the conditions employed in the experiments shown in Figs. 1–3. A further difference between dimethylamine dehydrogenase and the two trimethylamine dehydrogenases was apparent in the e.p.r. spectra collected after various time intervals. Whereas the signal width for the broad outer splitting remained constant with time during the reduction of dimethylamine dehydrogenase, a significant broadening of the signals for the lines characteristic of spin-spin interaction at 285 and 385 mT in Fig. 1(f) was observed for the trimethylamine dehydrogenases. This broadening of the signals was also observed during the substrate-induced enhancement of the triplet state in the trimethylamine dehydrogenase of bacterium W_3A_1 and prompted a more detailed investigation of the substrate dependence of formation of the triplet state in the three enzymes. Nevertheless, the results shown in Fig. 3, especially the reasonable agreement between the rates determined by the stopped-flow and freeze-quench techniques in the case of dimethylamine dehydrogenase, lent further credibility to the utilization of the freeze-quench procedure as a means of following the rate of appearance of paramagnetic species under appropriate conditions. It remained to be established whether the formation of the triplet state and the reduction of the flavin moiety in the

Fig. 2. E.p.r. spectra at $g \sim 4$ of samples of dimethylamine and trimethylamine dehydrogenases that have reacted with $100 \mu\text{M}$ substrate for 2 min

The samples are those used for Figs. 1(b), 1(d) and 1(f) respectively. The conditions of e.p.r. spectroscopy were those of Fig. 1, except that the microwave power was 9 mW and up to 16 spectra were averaged. The amplitudes of the signals were normalized to an enzyme concentration of $102 \mu\text{M}$ as used for the dehydrogenase of *Hyphomicrobium* X.

Fig. 3. Superposition of progress curves of the reaction of dimethylamine and trimethylamine dehydrogenases with their substrates ($100\mu\text{M}$) at 17°C in potassium barbital buffer, pH 7.7, as obtained by spectrophotometry at 365 nm and by e.p.r. spectroscopy

Panels (a), (b), and (c) represent experiments with dimethylamine dehydrogenase and trimethylamine dehydrogenase from *Hyphomicrobium X* and trimethylamine dehydrogenase from bacterium W_3A_1 , respectively. For the trimethylamine dehydrogenases the intensity (obtained by double integration) of the half-field signals at $g = 4$ was compared. Because of the small $g = 4$ signals observed with dimethylamine dehydrogenase the amplitude of the broad lines at $g \sim 2$ was used for evaluation. For details of e.p.r. spectroscopy see the legend to Fig. 1. For the various dehydrogenases the time ranges are shown for which e.p.r. measurements were available. Note the difference of the time axis for (a), (b), and (c). The curves were matched at

Hyphomicrobium enzymes could be kinetically resolved, as was the case for the enzyme from bacterium W_3A_1 .

*Stopped-flow kinetics of the reduction of the trimethylamine dehydrogenases of bacterium W_3A_1 and *Hyphomicrobium X* and the dimethylamine dehydrogenase by their substrates*

From the data presented above and elsewhere (Steenkamp & Beinert, 1982), it is evident that the absorbance change at 365 nm is well correlated with the development of the triplet state, measured by e.p.r. spectroscopy in all three dehydrogenases. Moreover, earlier studies (Steenkamp *et al.*, 1978a) on the trimethylamine dehydrogenase of bacterium W_3A_1 indicated that the development of the $g = 4$ half-field signal is not accompanied by a significant absorbance change at 445 nm where reduction of the flavin moiety can be measured. The two events, reduction of the flavin moiety and formation of the triplet state can, therefore, be observed with minimal interference from one another.

Dimethylamine dehydrogenase. Although not entirely without complicating aspects, the reactions involved in the formation of the triplet state in dimethylamine dehydrogenase were the most readily analysed of those of the three dehydrogenases. When dimethylamine dehydrogenase was reduced with low concentrations of dimethylamine, (up to $\sim 0.4\text{ mM}$) as in the e.p.r. and stopped-flow experiments of Figs. 1–3, the absorbance change at 440 nm , indicative of reduction of the flavin moiety, and the increase in absorbance at 365 nm due to the formation of the triplet state, proceeded at a similar rate (Fig. 4). This meant that the reduction of the flavin moiety could either not be resolved in time from the subsequent transfer of an electron to the $[4\text{Fe-4S}]$ cluster, or else that the absorbance changes at 440 nm and 365 nm were due to the same event. The substrate dependence of the reaction rates at the two wavelengths were, therefore, examined and found to be dissimilar. The absorbance change at 440 nm , due to reduction of the flavin moiety, remained monophasic throughout the range of substrate concentrations and the associated rate constants obeyed a second-order rate law (Fig. 4). The absorbance change at 365 nm , however, became

the starting and final value. For (a) and (c) spectrophotometric curves for different enzyme concentrations correspond to those used for the e.p.r. experiments, i.e. 64.5 , 102 and $61\mu\text{M}$ for (a), (b), and (c), respectively. Symbols: $\circ$ and $\bullet$, high and low enzyme concentration, respectively, and spectrophotometric observation; $\blacktriangle$, observation by e.p.r. at 13 K after freezing.

Fig. 4. Substrate dependence of the reduction of the flavin moiety and of formation of the triplet state in dimethylamine dehydrogenase

The absorbance changes at 440 nm and 365 nm were regarded as measures of the reduction of the flavin and of formation of the triplet state, respectively. Semilogarithmic plots of the absorbance changes were used to calculate first-order rate constants at different substrate concentrations. Dimethylamine dehydrogenase was $7.16 \mu\text{M}$ after mixing. The buffer was 40 mM-potassium barbitol, pH 7.7, and the temperature 17°C . The symbols represent the rate constants determined at 440 and 365 (▲) nm.

Fig. 5. Effect of substrate on the kinetics of formation of the triplet state in dimethylamine dehydrogenase

Conditions were as in Fig. 4. Curves 1 and 2 represent the absorbance changes at 365 nm after mixing dimethylamine dehydrogenase with 0.1 mM- and 2 mM-dimethylamine.

biphasic at higher substrate concentrations (Fig. 5), and when analysed as two simultaneous first order reactions (Fig. 6), rate constants for the faster and more pronounced of the two reactions reached a

Fig. 6. Biphasic kinetics of the formation of the triplet state in dimethylamine dehydrogenase

The absorbance changes from curve 2 in Fig. 5 are analysed as two simultaneous first order reactions.

plateau at about 1.0 mM-dimethylamine (Fig. 4). It is, therefore, evident that reduction of the flavin moiety and transfer of an electron to the iron-sulphur cluster are consecutive reactions, a course of events which seemed intuitively plausible. Moreover, because of the similar magnitudes of the rate constants for reduction of the enzyme to the flavoquinol form and the subsequent intramolecular electron transfer, the kinetics of the rapid phase in formation of the triplet were not adequately described by a first-order rate equation, as evidenced by a downwards curvature of the experimental points at short reaction times (Fig. 6). The results shown in Fig. 4 for the formation of the triplet state are, therefore, presented as apparent first-order rate constants solely to discriminate between the reactions observed at the two wavelengths. The significance of the slower reaction observed at 365 nm will become apparent from an examination of the data obtained for the trimethylamine dehydrogenases, where this reaction is much more prominent.

The trimethylamine dehydrogenases: It had previously been shown (Steenkamp *et al.*, 1978a) that reduction of the flavocoenzyme in the trimethylamine dehydrogenase of bacterium *W₃A₁* is complete within a few ms when the enzyme is mixed with elevated concentrations of substrate (0.5 mM-trimethylamine). The rapid reduction of the flavin moiety appeared to be followed by slow, but minor, absorbance changes which prevented determination of a final absorbance value. The data for the rate of reduction of the flavin moiety at different substrate concentrations were, therefore, analysed by assuming pseudo-first-order kinetics and applying the

Guggenheim procedure. At least 80% of the absorbance change associated with the rapid reduction of the flavin appeared to obey a first-order rate law. Kinetic data for the reduction of flavoenzymes by their substrates are often adequately described by the partial equation:

where E and F are the oxidized and substrate-reduced enzymes, respectively. Provided that the enzyme-substrate complex is in rapid equilibrium with the free enzyme, E, reduction of the flavin

Fig. 7. Substrate dependence of the first order rate constants for the reduction of the flavin moiety in the trimethylamine dehydrogenase of bacterium *W₃A₁*. Enzyme concentrations were 7.0 μ M in 0.1 M-sodium pyrophosphate/HCl, pH 7.7 (Δ) and 7.07 μ M in 40 mM-potassium barbital, pH 7.7 ($\circ$) after mixing. The temperature was 10°C.

moiety is a pseudo-first-order process and double-reciprocal plots of the observed rate constants versus the substrate concentration are linear with intercept and slope equal to $1/k_3$ and k_2/k_1k_3 respectively (Gibson *et al.*, 1964). Such plots for the reduction of the flavin moiety in the trimethylamine dehydrogenase of bacterium *W₃A₁* (Fig. 7) had intercepts which were not sufficiently different from zero to allow the accurate estimation of the rate constant, k_3 . When fitted by simple linear regression the y intercept had 95% confidence intervals of 0.008 ± 1.9 and 0.59 ± 0.66 for rate constants obtained in barbital and pyrophosphate buffers, respectively. This observation suggested that the assumption of rapid equilibrium in the substrate binding step, and consequently also the assumption of pseudo-first-order kinetics, might have been invalid in this case. However, the catalytic-centre activity of trimethylamine dehydrogenase in pyrophosphate buffer at pH 7.7 and 10°C (Table 1) is only 185 min^{-1} , predicting a minimal first-order rate constant of 3.09 s^{-1} for the rate-limiting step in catalysis and, therefore, the reduction of the flavin moiety was clearly not rate-limiting. This conclusion also held true when the measurements were performed in 40 mM-potassium barbital buffer, pH 7.7, the buffer of choice for the e.p.r. experiments, in which case double-reciprocal plots of the observed rate constants against substrate concentration passed through the origin. The second-order rate constant calculated from the data was $68.4 \times 10^4 \text{ M}^{-1} \cdot \text{s}^{-1}$ at 10°C. The close correspondence of the observed rate constants observed in pyrophosphate as opposed to barbital buffer was of some interest since pyrophosphate was found to be an activator of trimethylamine dehydrogenase, as shown below.

Table 1. Comparison of rate constants from stopped-flow data with predicted rate constants from steady state assays. The catalytic centre activities of the enzymes were determined from secondary replots of the apparent maximal velocities obtained at different fixed levels of phenazine methosulphate and variable tri- and dimethylamine concentrations. Observed rate constants for the formation of the triplet state were estimated from semilogarithmic plots of data collected by stopped-flow spectrophotometry at 365 nm. The values for the slow phase observed for the trimethylamine dehydrogenases were obtained by extrapolation to infinite substrate concentration. The value reported for dimethylamine dehydrogenase was obtained from the data shown for the reaction at 2 mM-dimethylamine in Fig. 5 and does not represent an extrapolated value. The buffers were 40 mM-potassium barbital, pH 7.7, for the *Hyphomicrobium* enzymes and 0.1 M-sodium pyrophosphate, pH 7.7, for the enzyme from bacterium *W₃A₁*. Determinations with the *Hyphomicrobium* enzymes were conducted at 17°C and those with the enzyme from bacterium *W₃A₁* at 10°C. N.D., not detected.

Enzyme	Observed rate constant (s^{-1})		Theoretical rate constant from V_{max} , (s^{-1})
	Rapid phase	Slow phase	
Trimethylamine dehydrogenase from bacterium <i>W₃A₁</i>	N.D.	2.22	3.09
Dimethylamine dehydrogenase	~23	1.2	1.64
Trimethylamine dehydrogenase from <i>Hyphomicrobium</i> X	15–23	2.1	2.7

Fig. 8. Substrate dependence of the formation of the triplet state in the trimethylamine dehydrogenase of *Hyphomicrobium X*

The enzyme concentration was $5.9\mu\text{M}$ after mixing and the temperature 17°C . The data represent change in absorbance at 365nm with time after mixing the enzyme with trimethylamine to obtain 0.1 mM- (curve 1) and 1 mM-trimethylamine (curve 2) all in 40 mM-potassium barbital, pH 7.7.

Scheme 1. Mechanism to account for the effect of substrate (trimethylamine) concentration on the rate of intramolecular electron transfer in the trimethylamine dehydrogenases

The binding of trimethylamine to the flavoquinol form of the enzyme, $\text{FH}_2[4\text{Fe}-4\text{S}]^{2+}$ is considered to give rise to a conformational change which alters the rate of transfer of an electron from the flavin to the iron-sulphur cluster.

in which the transfer of an electron to the $[4\text{Fe}-4\text{S}]$ cluster proceeds much more slowly. Such a conformational change is also consistent with the broadening of the e.p.r. signals of the trimethylamine dehydrogenases with time, as in Fig. 1 (Beinert *et al.*, 1982).

When the two phases of the reactions shown in Fig. 8 were treated as two simultaneous first-order processes, the data obtained for the trimethylamine dehydrogenase of *Hyphomicrobium X* yielded linear semilogarithmic plots and only the slow phase had an obvious dependence on substrate concentration (Fig. 9). Unfortunately, the faster phase could only be examined over a limited range of substrate concentrations, since it became negligible at trimethylamine concentrations in excess of $400\mu\text{M}$. A necessary condition for the observation of biphasic kinetics for the transfer of an electron from the reduced flavin to the $[4\text{Fe}-4\text{S}]$ cluster is that the rate of binding of substrate to the reduced form of the enzyme and the consequent conformational change which gives rise to sluggish electron transfer should be such that the species $\text{FH}_2[4\text{Fe}-4\text{S}]^{2+}$ and $\text{FH}_2[4\text{Fe}-4\text{S}]^{2+} \cdot \text{trimethylamine}$ of Scheme 1 are not in rapid equilibrium relative to other interconversions. It can readily be shown by application of the method of Cha (1968), that the assumption of a rapid equilibrium between these species would predict the formation of the triplet state to proceed in a single first order process with rate constant:

$$\begin{aligned}
 k_{\text{obs}} = & \frac{k_3}{1 + \frac{\text{TMA}}{K_1}} + \frac{k_4}{1 + \frac{\text{TMA}}{K_2}} + \frac{k_5}{1 + \frac{K_1}{\text{TMA}}} \\
 & + \frac{k_6}{1 + \frac{K_2}{\text{TMA}}}
 \end{aligned} \quad (1)$$

Reduction of the flavin moiety in the trimethylamine dehydrogenase of *Hyphomicrobium X* was too fast to be followed under conditions which would have ensured pseudo-first-order kinetics and was, therefore, evidently not rate-limiting in catalysis. The second-order rate constant for the reduction of the flavin moiety was about $10^6 \text{M}^{-1} \cdot \text{s}^{-1}$.

The data presented above served to confirm more adequately the sequence of events during the reduction of the three dehydrogenases as involving a rapid conversion of the flavin moiety to the flavoquinol form, followed by a slower transfer of an electron to the $[4\text{Fe}-4\text{S}]$ cluster. In the trimethylamine dehydrogenases, the latter step was biphasic with an unexpected dependence on substrate concentration which could be analysed most satisfactorily for the enzyme from *Hyphomicrobium X* (Fig. 8). At high substrate concentrations the rapid phase in the formation of the triplet state disappeared, and although the total absorbance change remained approximately the same, the entire conversion of the flavoquinol form of either enzyme to the triplet state proceeded by the slower process. This observation could be accounted for if it is assumed that the rate of intramolecular electron transfer is modulated by the binding of excess substrate to the reduced forms of the enzymes. With certain provisions the formation of the triplet states in the trimethylamine dehydrogenases is then adequately described by the reactions shown in Scheme 1. In this Scheme it is assumed that the binding of substrate to the reduced form of the enzyme is associated with a conformational change to a form

Fig. 9. Substrate dependence of the two phases in the formation of the triplet state in the trimethylamine dehydrogenase of *Hyphomicrobium X*

Absorbance changes at 365 nm, as shown in Fig. 8, where analysed as two simultaneous first-order reactions. Data points selected at intervals from traces such as in Fig. 8 were fitted by iteration to a bi-exponential function to obtain the rate constants for the slow and fast phases. Data points represent the variation of the observed first order rate constants, k_{obs} , for the slow (a) and fast (b) phases of the reaction with trimethylamine concentration. Error bars denote the standard errors of the rate constants.

where K_1 and K_2 are dissociation constants of the $\text{FH}_2[4\text{Fe}-4\text{S}]^{2+} \cdot \text{trimethylamine}$ and $\text{FH} \cdot [4\text{Fe}-4\text{S}]^{1+} \cdot \text{trimethylamine}$ complexes, respectively, and the rate constants correspond to those given in Scheme 1.

In the following treatment it is assumed, instead, that rapid interconversion of the species $\text{FH}_2[4\text{Fe}-4\text{S}]^{2+}$, $\text{FH} \cdot [4\text{Fe}-4\text{S}]^{1+}$, and $\text{FH}_2[4\text{Fe}-4\text{S}]^{2+} \cdot \text{trimethylamine}$ gives rise to the fast phase in the

formation of the triplet state. Because of the large difference in rate between the rapid and slow phases of the reactions shown in Fig. 8, the two phases could be treated separately. The rapid phase may be considered as a special case of the general reaction scheme:

Scheme 2.

a solution of which was presented by Lowry & John (1910). A, B, and C may be recognized as the $\text{FH}_2[4\text{Fe}-4\text{S}]^{2+} \cdot \text{trimethylamine}$, $\text{FH}_2[4\text{Fe}-4\text{S}]^{2+}$, and $\text{FH} \cdot [4\text{Fe}-4\text{S}]^{1+}$ species, respectively, of trimethylamine dehydrogenase. By substituting the conditions $A_0 = C_0 = 0$, $B_0 = B$ at time $t = 0$ and defining $k_2 = k_2 S$ in the differential equations given by Capellos & Bielski (1972), the concentration of the various interacting species are obtained as:

$$\text{A} = k_2 S B_0 \left[\frac{k_4}{\lambda_1 \lambda_2} + \frac{k_4 - \lambda_1}{\lambda_1 (\lambda_1 - \lambda_2)} e^{-\lambda_1 t} + \frac{k_4 - \lambda_2}{\lambda_2 (\lambda_2 - \lambda_1)} e^{-\lambda_2 t} \right] \quad (2)$$

$$\text{B} = B_0 \left[\frac{k_1 k_4}{\lambda_1 \lambda_2} + \frac{\lambda_1^2 - \lambda_1 (k_1 + k_4) + k_1 k_4}{\lambda_1 (\lambda_1 - \lambda_2)} e^{-\lambda_1 t} + \frac{\lambda_2^2 - \lambda_2 (k_1 + k_4) + k_1 k_4}{\lambda_2 (\lambda_2 - \lambda_1)} e^{-\lambda_2 t} \right] \quad (3)$$

$$\text{C} = k_3 B_0 \left[\frac{k_1}{\lambda_1 \lambda_2} + \frac{k_1 - \lambda_1}{\lambda_1 (\lambda_1 - \lambda_2)} e^{-\lambda_1 t} + \frac{k_1 - \lambda_2}{\lambda_2 (\lambda_2 - \lambda_1)} e^{-\lambda_2 t} \right] \quad (4)$$

where

$$\lambda_1 = \frac{1}{2} \{ k_1 + k_2 S + k_3 + k_4 - [(k_1 + k_2 S + k_3 + k_4)^2 - 4(k_1 k_3 + k_2 S k_4 + k_1 k_4)]^{1/2} \} \quad (5)$$

and

$$\lambda_2 = \frac{1}{2} \{ k_1 + k_2 S + k_3 + k_4 + [(k_1 + k_2 S + k_3 + k_4)^2 - 4(k_1 k_3 + k_2 S k_4 + k_1 k_4)]^{1/2} \} \quad (6)$$

The change in concentration of the triplet state, $\text{FH} \cdot [4\text{Fe}-4\text{S}]^{1+}$ (denoted by C in Scheme 2) can be observed by stopped-flow spectrophotometry and is, therefore, of particular interest. According to the theoretical considerations presented by Lowry & John (1910) the formation of the triplet state, C, will obey first order kinetics when $k_1 = \lambda_1$, a condition which is met when $k_1 = k_4$. Moreover, the concentration of the triplet state may pass through a maximum when $k_4 < k_1$ and, therefore, does not necessarily increase monotonically. The linearity of

semilogarithmic plots of the rapid phase in the formation of the triplet state in the trimethylamine dehydrogenase of *Hyphomicrobium* X can be accounted for if it is assumed that $k_1 \sim k_4$.

This model also accounts for the disappearance of the rapid phase at high substrate concentrations. It can be shown that the limiting values of the quantities λ_1 and λ_2 at infinite substrate concentrations would be:

$$\lim_{S \rightarrow \infty} \lambda_1 = k_4 \quad \text{and} \quad \lim_{S \rightarrow \infty} \lambda_2 = \lim_{S \rightarrow \infty} k_2 S = \infty \quad (7)$$

At high substrate concentrations the right-hand side of eqn. (4) therefore tends to zero and negligible triplet state will be formed in the rapid phase. The time-dependent broadening of the e.p.r. signals due to spin-spin interaction were, therefore, not observed in our earlier kinetic study (Steenkamp *et al.*, 1978a), which was conducted at high substrate concentrations, since the enzyme in the triplet state must have existed as the $\text{FH}^+[\text{4Fe-4S}]^{1+}$ ·trimethylamine complex at all times.

The slow reaction observed at 365 nm may be ascribed to the more sluggish transfer of an electron from the reduced flavin to the iron-sulphur cluster in the reduced enzyme-substrate complex. Since this reaction is considerably slower than those involved in the rapid phase, the species $\text{FH}_2[\text{4Fe-4S}]^{2+}$, $\text{FH}^+[\text{4Fe-4S}]^{1+}$, and $\text{FH}_2[\text{4Fe-4S}]^{2+}$ ·trimethylamine may be considered as a rapid equilibrium segment relative to the slow phase (Cha, 1968). If the slow phase is assumed to be essentially irreversible under the conditions of the stopped-flow experiments shown in Figs. 8 and 9, the dependence of the observed first order rate constants on the substrate concentration is obtained as:

$$k_{\text{obs.}} = \frac{k_5}{1 + \frac{K_1}{\text{TMA}} \left(1 + \frac{1}{K_2}\right)} \quad (8)$$

and

$$\frac{1}{k_{\text{obs.}}} = \frac{1}{K_5} + \frac{K_1(1+k_2)}{k_5 K_2} \frac{1}{\text{TMA}}$$

$K = k_1/k_2$ and $K = k_4/k_3$ define the equilibria in the rapid equilibrium segment and k_5 is the rate constant for the slow step. The linearity of the plot of $1/k_{\text{obs.}}$ versus $1/[\text{trimethylamine}]$ (Fig. 9) suggests that this relationship is a reasonable approximation for the trimethylamine dehydrogenase of *Hyphomicrobium* X. It can be shown that when the conversion $\text{FH}_2[\text{4Fe-4S}]^{2+}$ ·trimethylamine = $\text{FH}^+[\text{4Fe-4S}]^{1+}$ ·trimethylamine is assumed to be reversible, or if the species $\text{FH}^+[\text{4Fe-4S}]^{1+}$ ·trimethylamine is formed to a significant extent by reversible binding of substrate to $\text{FH}^+[\text{4Fe-4S}]^{1+}$,

Fig. 10. Dependence of the formation of the triplet state in the trimethylamine dehydrogenase of bacterium W_3A_1 on substrate

(a) The enzyme and substrate were mixed to obtain $7.07 \mu\text{M}$ -trimethylamine dehydrogenase and 0.1 mM (curve 1), 0.3 mM (curve 2), and 2 mM-trimethylamine (curve 3), all in 40 mM potassium barbital, pH 7.7. (b) The enzyme and substrate were mixed to obtain $8.28 \mu\text{M}$ -trimethylamine dehydrogenase and 0.1 (curve 1), 0.4 (curve 2), and 1.2 mM-trimethylamine (curve 3), all in 0.1 M-sodium pyrophosphate/HCl, pH 7.7. The inset shows the variation of the observed first order rate constants, $k_{\text{obs.}}$, for the slow phase with substrate (trimethylamine, TMA) concentration. Standard errors of the rate constants were in all cases less than 5% of the observed values.

plots of $1/k_{\text{obs.}}$ versus reciprocal substrate concentration are hyperbolic rather than linear. The rate constants k_6 , k_7 , and k_8 are, therefore, assumed to be negligibly small relative to the remaining constants.

Analysis of the data for formation of the triplet state in the trimethylamine dehydrogenase of bacterium W_3A_1 indicated a more complex situation (Fig. 10). When potassium barbital, pH 7.7, was used as the buffer, semilogarithmic plots for the rapid phase in the formation of the triplet state were non-linear, as might be the case if k_4 and k_1 in Scheme 2 differ significantly. Moreover, semi-

logarithmic plots for the slow phase were also non-linear and showed a distinct lag, which suggested that the conformational change associated with occupancy of the substrate binding site on the reduced enzyme is rate-limiting in the formation of the triplet state by the slower pathway. The assumption of rapid equilibrium between the species $\text{FH}_2[4\text{Fe}-4\text{S}]^{2+}$, $\text{FH}^+[4\text{Fe}-4\text{S}]^{1+}$, and $\text{FH}_2[4\text{Fe}-4\text{S}]^{2+} \cdot \text{trimethylamine}$ is, therefore, invalid for the enzyme from bacterium W_3A_1 .

Whereas the results presented in Figs. 8 and 9 are accounted for in a qualitative manner by the considerations presented above, the data presently available do not permit a detailed quantitative analysis for either of the two trimethylamine dehydrogenases.

Correlation between intramolecular electron transfer and steady state kinetic behaviour

If, indeed, the triplet state is an obligatory intermediate in catalysis its rate of formation should predict a catalytic centre activity in excess of the V_{max} obtained from steady state assays. Moreover, changes in the rate of intramolecular electron transfer should be reflected in a decreased rate of catalysis. These conditions seemed to be met by all three enzymes. Thus, the rate of intramolecular electron transfer for the rapid phase in the formation of the triplet state was much in excess of the calculated rate constant for the rate-limiting step in catalysis for all three enzymes (Table 1).

The trimethylamine dehydrogenases showed partial substrate inhibition at high substrate concentrations (Fig. 11), and, in the case of the trimethylamine dehydrogenase from *Hyphomicrobium* X, the rate constant obtained from the intercept in Fig. 9 predicted a maximal specific activity of $0.86 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ compared with the value of $0.567 \mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ obtained from a determination of V_{app} with phenazine methosulphate variable at a constant level of 20 mM-trimethylamine. The partial nature of the substrate inhibition is most prominently displayed in the case of the trimethylamine dehydrogenase of bacterium W_3A_1 (Fig. 11) and is consistent with a transition from the rapid formation of the triplet state at low substrate concentrations to the much slower intramolecular transfer of an electron in the reduced enzyme-substrate complex $\text{FH}_2[4\text{Fe}-4\text{S}]^{2+} \cdot \text{trimethylamine}$.

In Fig. 11 data are shown for the substrate inhibition of the trimethylamine dehydrogenase of bacterium W_3A_1 both in pyrophosphate and in barbital buffer. Whereas pyrophosphate had little effect on the activity of the *Hyphomicrobium* enzymes, the trimethylamine dehydrogenases of bacterium W_3A_1 was activated about 2.5-fold by pyrophosphate at high substrate concentrations. Comparison of Figs. 10(a) and 10(b) shows that this

Fig. 11. Substrate inhibition of the trimethylamine dehydrogenase of bacterium W_3A_1 (a) and of *Hyphomicrobium* X (b)

(a) Assay mixtures contained, in 3.0 ml, $6 \mu\text{mol}$ of phenazine methosulphate, $0.14 \mu\text{mol}$ of dichlorophenolindophenol, variable amounts of trimethylamine and either 0.3 mmol of sodium pyrophosphate, pH 7.7 (x) or 0.104 mmol of potassium barbital, pH 7.7 (o). The temperature was 10°C . (b) Assay mixtures were as in (a), the buffer being 0.104 mmol of potassium barbital, pH 7.7. The temperature was 17°C .

effect may be traced to a pronounced activation of the rate of formation of the triplet state at high substrate concentrations by pyrophosphate (Fig. 10b). Moreover, semilogarithmic plots were linear in this buffer, which allowed calculation of the rate constants presented in the inset of Fig. 10(b). Plots of the reciprocals of the observed rate constants versus reciprocal substrate concentrations were convex upwards, indicating that the simple relationship of eqn. (8) does not apply. The rate of reduction of the enzyme by substrate, as measured by the absorbance decrease at 445 nm was, however, very similar in barbital and pyrophosphate buffers (Fig. 7).

Substrate inhibition was not observed in the case of the dimethylamine dehydrogenase. Although the reaction rate in steady state assays declined at

substrate concentrations as high as 20 mM-dimethylamine, control experiments in which KCl or NaCl were included in the reaction mixtures indicated that this was probably due to a nonspecific effect of ionic strength on dimethylamine dehydrogenase. In agreement with these observations the slow phase in the formation of the triplet state in dimethylamine dehydrogenase did not become prominent in the range of substrate concentrations used.

The fact that the catalytic activities of all three enzymes were reflected in the variations of the rate of intramolecular electron transfer with substrate concentration and buffer composition indicated that formation of the triplet state was an obligatory step in catalysis even when artificial electron acceptors such as phenazine methosulphate were used in assays. This implies that the electron acceptors do not react significantly with the flavoquinol forms of any of the enzymes. It is, therefore, unlikely that the flavosemiquinone would be accessible to electron acceptors unless the conformation of the enzyme is substantially different in the flavoquinol form as opposed to the triplet state. A more plausible mechanism would involve a reoxidation of the enzyme mediated through the [4Fe-4S] cluster. Evidence in favour of this thesis was sought by examination of the reoxidation of the trimethylamine dehydrogenase of bacterium *W₃A₁* by its flavoprotein electron acceptor (Steenkamp & Gallup, 1978).

When oxidized electron acceptor flavoprotein was mixed with trimethylamine dehydrogenase reduced with a slight excess of substrate, the e.p.r. signals arising from the triplet state in the dehydrogenase disappeared within a few ms (Fig. 12). No evidence for the transient appearance of the reduced iron-sulphur cluster could be obtained, as would have been the case if electrons were transferred to the flavoprotein by reoxidation of the flavosemiquinone of trimethylamine dehydrogenase. This experiment indicated that the reduced iron-sulphur cluster is rapidly reoxidized by the flavoprotein, but could evidently not provide information concerning the rate of reoxidation of the flavosemiquinone in the reduced trimethylamine dehydrogenase. In an attempt to obtain a time resolution of the reoxidation of the two species constituting the triplet state in trimethylamine dehydrogenase, the reaction was also monitored by stopped-flow spectroscopy at more than one wavelength. Since the flavoprotein of bacterium *W₃A₁* is a one-electron acceptor which cannot be reduced past the semiquinone form (Steenkamp & Gallup, 1978), reduced trimethylamine dehydrogenase must transfer two electrons sequentially to the flavoprotein. The reoxidation of trimethylamine dehydrogenase was studied under conditions where the reduction of reoxidized enzyme by excess substrate used in its initial reduction would

Fig. 12. Reoxidation of the trimethylamine dehydrogenase of bacterium *W₃A₁* by its electron acceptor flavoprotein

Trimethylamine dehydrogenase at a concentration of 67 μM was mixed anaerobically with 270 μM -trimethylamine in 0.1 M-sodium pyrophosphate buffer, pH 7.7. This solution was then mixed in equal proportion by the freeze-quench technique with 265 μM -electron acceptor flavoprotein in the same buffer to obtain final concentrations of 33.5 μM -dehydrogenase and 132.5 μM -electron acceptor flavoprotein. The temperature was 13°C. E.p.r. spectra were recorded using a frequency of 9.2 GHz, 2.7 mW microwave power, and 0.5 mT modulation amplitude at 13 K for observation of the signal at $g \sim 4$ (O) ascribed to the triplet state of trimethylamine dehydrogenase. The radical signal at $g \sim 2$ (x) was observed using the same frequency, but 30 μW microwave power and 0.8 mT modulation amplitude at 100 K. The signal at $g \sim 4$ is expressed as a percentage of the signal intensity prior to mixing the enzyme with the flavoprotein, the two-fold dilution being taken into consideration.

not interfere. Such conditions are readily achieved by reducing the enzyme with dimethylamine, a slow substrate (Steenkamp & Beinert, 1982), under argon in the presence of an oxygen-scavenging system composed of glucose oxidase and catalase. When the reaction of the reduced dehydrogenase with the flavoprotein was monitored by stopped-flow spectrophotometry at more than one wavelength, no clear evidence for biphasic behavior could be obtained and the data were of limited utility in resolving the sequential electron transfer steps in time. Whereas studies on the reoxidation of trimethylamine dehydrogenase have not provided definitive evidence

regarding the role of the iron-sulphur cluster in shuttling electrons from the flavin of the dehydrogenase to the flavoprotein, the results also did not contradict such a mechanism which, in view of the obligatory role of the triplet state in catalysis, seemed highly likely.

In summary, the dimethylamine and trimethylamine dehydrogenases appear to function as $2:1e^-$ -transformases (Hemmerich, 1977), the flavin component of which accepts two electrons from the substrate while the $[4Fe-4S]$ cluster provides an output of single electrons to a flavoprotein which functions as a one-electron acceptor (Steenkamp & Gallup, 1978). Although we have not been able to detect the presence of such an electron acceptor in crude extracts of *Hyphomicrobium* X, recent work has shown that the trimethylamine and dimethylamine dehydrogenases from this organism will also reduce the electron acceptor flavoprotein of bacterium W_3A_1 . Variations in the steady state reaction velocity of the enzymes with the composition of the buffer or with substrate concentration was found to be reflected in corresponding variations in the rate of intramolecular electron transfer, rather than in the rate of reduction of the 6-S-cysteinyl-FMN moiety to the flavoquinol form. Reoxidation of the triplet state produced by intramolecular electron transfer in the trimethylamine dehydrogenase of bacterium W_3A_1 was found to proceed at a rate much in excess of the minimal rate for catalytic turnover and can, therefore, not be rate limiting. Seen together, these facts suggest that the transfer of reducing equivalents to electron acceptors is mediated solely via the $[4Fe-4S]$ cluster in these dehydrogenases.

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