Flavinylation in wild-type trimethylamine dehydrogenase and differentially charged mutant enzymes: a study of the protein environment around the N1 of the flavin isoalloxazine

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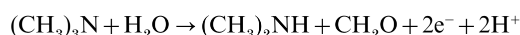
In wild-type trimethylamine dehydrogenase, residue Arg-222 is positioned close to the isoalloxazine N1/C2 positions of the 6S-cysteinyl FMN. The positively charged guanidino group of Arg-222 is thought to stabilize negative charge as it develops at the N1 position of the flavin during flavinylation of the enzyme. Three mutant trimethylamine dehydrogenases were constructed to alter the nature of the charge at residue 222. The amount of active flavinylated enzyme produced in *Escherichia coli* is reduced when Arg-222 is replaced by lysine (mutant R222K). Removal or reversal of the charge at residue 222 (mutants R222V and R222E, respectively) leads to the production of inactive enzymes that are totally devoid of flavin. A comparison of the CD spectra for the wild-type and mutant enzymes revealed no major structural change following mutagenesis. Like the wild-type protein, each mutant enzyme contained stoichiometric amounts of the 4Fe-4S cluster and ADP. Electrospray MS also indicated that the native and recombinant wild-type enzymes were isolated as a mixture of deflavo and holo enzyme, but that each of the mutant enzymes have masses expected for deflavo trimethylamine dehydrogenase. The MS data indicate that the lack of assembly of the mutant proteins with FMN is not due to detectable levels of post-translational modification of significant mass. The experiments reported here indicate that simple mutagenic changes in the FMN-binding site can reduce the proportion of flavinylated enzyme isolated from *Escherichia coli* and that positive charge is required at residue 222 if flavinylation is to proceed.

INTRODUCTION

Trimethylamine dehydrogenase (EC 1.5.99.7) catalyses the oxidative N-demethylation of trimethylamine to yield dimethylamine and formaldehyde [1]



$$(CH_3)_3N + H_2O \rightarrow (CH_3)_2NH + CH_2O + 2e^- + 2H^+$$

The enzyme is responsible for the ability of some methylotrophic bacteria to subsist on trimethylamine as the sole source of carbon. Electrons derived from the oxidation of trimethylamine are transferred to an electron-transferring flavoprotein [2,3] that associates with trimethylamine dehydrogenase as part of the catalytic cycle. The crystal structure of trimethylamine dehydrogenase has been solved at 2.4 Å resolution [4], and recently refined at 1.7 Å (S. A. White and F. S. Mathews, unpublished work) using the amino acid sequence determined from the gene sequence [5]. The enzyme comprises an N-terminal β/α barrel domain that contains the active site and FMN-binding site. A buried loop that binds the 4Fe-4S cluster is located at the C-terminus of the β/α barrel domain. Beyond this loop region, the protein forms two C-terminal domains that are rich in α/β structure and are similar in fold to the dinucleotide-binding domains of glutathione reductase [6–8]. ADP of unknown function is bound in this region of the protein where it occupies a position equivalent to the ADP moiety of FAD in the FAD-binding domain of glutathione reductase and other flavoprotein disulphide oxidoreductases. The structural similarity of the C-terminal domain with dinucleotide-binding domains has led to the proposal that the C-terminal domain of trimethylamine dehydrogenase is a vestigial remnant of an ancestral FAD-binding domain [6,8].

Trimethylamine dehydrogenase and the closely related dimethylamine dehydrogenase of *Hyphomicrobium X* [9,10] are unique in possessing a covalently linked flavin attached by a 6S-cysteinyl FMN bond [11–13]; flavin is usually linked to the 8α-methyl of the isoalloxazine ring in other enzyme systems [14]. In trimethylamine dehydrogenase, activity is retained if the 6S-cysteinyl FMN bond is removed by directed mutagenesis of Cys-30 to Ala-30 [15], but the detailed effects of the mutation on the reductive half-reaction have yet to be determined. A mechanism has been proposed for the flavinylation reaction of trimethylamine dehydrogenase (Figure 1; [15]), and investigated recently by directed mutagenesis and electrospray ionization MS (ESMS) [16,17]. Arginine-222 in the wild-type enzyme is thought to stabilize negative charge as it develops at the N1/O2 carbonyl of the flavin during the flavinylation reaction and is therefore conjectured to facilitate flavinylation of the enzyme. The same residue is thought to serve a similar role during the reductive half-reaction catalysed by the enzyme. Here we show by an analysis of three mutant enzymes with different charges that a positive charge at residue 222 (arginine or lysine) is required for flavinylation. We also demonstrate that simple mutagenic changes in the active site can dramatically affect the relative quantities of flavinylated and deflavo enzyme purified from the host bacterium. The results are discussed in the light of recent work on trimethylamine dehydrogenase and other flavoproteins.

EXPERIMENTAL

Materials

Complex bacteriological medium was prepared as described by Sambrook et al. [18] using materials purchased from Difco Laboratories. *Escherichia coli* strain JM109 [r_k^-, m_k^+, *rec* A1, *sup* E, *end* A1, *hsd* R17, *gyr* A96, *rel* A1, *thi*, Δ(*lac-pro* AB) F′ *tra* D36, *pro* A^+B^+, *lac* I^q, *lac* Z ΔM15] was from Stratagene. Restriction enzymes *Eco*RI, *Hin*dIII and *Kpn*I were purchased from Pharmacia Biotech Inc. Calf intestinal alkaline phosphatase

Abbreviation used: ESMS, electrospray ionization MS.

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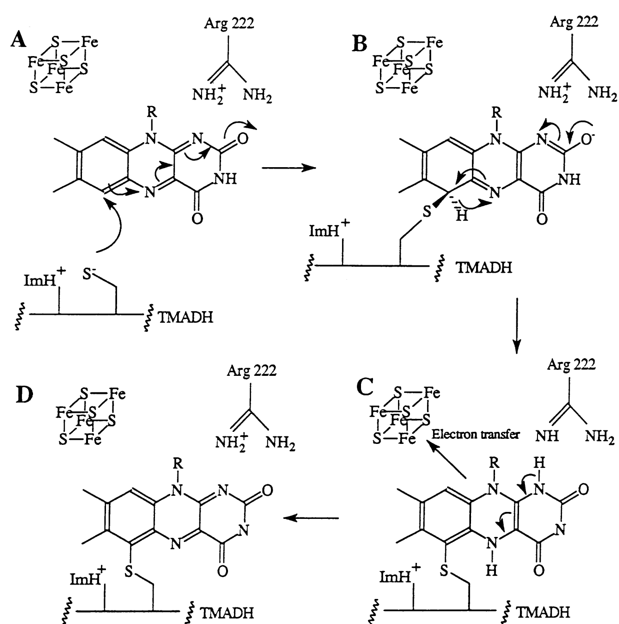

Figure 1 The proposed flavinylation mechanism of trimethylamine dehydrogenase

ImH^+ represents the protonated imidazole side-chain of residue His-29 that is thought to assist in the nucleophilic attack of Cys-30 at the C6 atom of flavin by formation of an imidazolium/thiolate ion-pair. The role of Arg-222 in stabilizing negative charge as it develops at the N1/O2 positions of the flavin is shown. Re-oxidation of the reduced flavin is by internal electron transfer to the 4Fe-4S centre.

was from Boehringer Mannheim. T4 DNA ligase was from Promega; T4 polynucleotide kinase was obtained from Amersham International. Timentin was from Beecham Research Laboratories. Ascorbic acid, dicyclopentadienyliron (ferrocene), riboflavin, sodium hexafluorophosphate, 3-(2-pyridyl)-5,6-bis-(4-phenylsulphonic acid)-1,2,4-triazine (ferrozine) and trimethylamine were from Sigma. Ferricenium hexafluorophosphate was synthesized as described previously [19]. All other chemicals were of analytical quality where possible. Water was glass-distilled and further purified by the Elgastat UHP System, except for HPLC work, where MilliQ-purified water was used (Millipore, Watford, U.K.).

Mutagenesis, plasmid construction and DNA sequencing

Plasmid DNA and bacteriophage replicative-form DNA were prepared by CsCl density-gradient centrifugation [18]. Miniscale plasmid preparations were prepared by modified alkali lysis. Pelleted bacterial cells were resuspended in 10 mM Tris (pH 8.0/1 mM EDTA ('TE Buffer') (150 μl), and lysed with an equal volume of 0.1 % SDS/0.2 M NaOH. Neutralization with 7.5 M ammonium acetate (150 μl) was followed by microcentrifugation and the supernatant was extracted with phenol/chloroform and plasmid material precipitated with isopropanol. Miniscale single-stranded bacteriophage DNA was prepared as described elsewhere [18]. Restriction endonuclease digestion and ligation of DNA were carried out as recommended by the enzyme suppliers.

Site-directed mutagenesis was carried out on the coding strand of the *tmd* gene contained within the M13-based construct M13TM3 [15], using the phosphorothioate method marketed by Amersham International. The degenerate oligonucleotide 5′-ATC CAC GCC GAA T(T/A)(T/A/C) TGT CGC AAT CGC-3′ was used to isolate mutant enzymes in which Arg-222 was exchanged for either a lysine, valine or glutamate residue. Screening for mutant *tmd* genes was based on direct dideoxynucleotide DNA sequencing [20,21] of putative mutant bacteriophage constructs, using the T7 system purchased from Pharmacia. Expression constructs were generated by replacement of the 1.4 kb *Kpn*I fragment found within the wild-type *tmd* gene of plasmid pSV2tmdveg [15] with the analogous fragment released from the mutant bacteriophage by digestion with *Kpn*I. The entire 1.4 kb *Kpn*I fragment containing the desired mutagenic change was resequenced to ensure no spurious mutations had occurred during the mutagenesis reactions.

Protein expression and purification

E. coli strain JM109 transformed with the wild-type [15] and mutant (see below) expression plasmids were cultured in double-strength YT medium (10 g of yeast extract, 10 g of bactotryptone and 5 g of NaCl/l), supplemented with 100 mg/l timentin, 100 mg/l riboflavin and 200 mg/l iron (II) sulphate. Mutant proteins were expressed constitutively under the control of the *Bacillus subtilis veg* promoter [15] in late stationary phase. Cells were harvested and the mutant protein purified as described previously for the wild-type protein [15]. To monitor the purity of enzyme samples, enzymes were submitted to SDS/PAGE in homogeneous 12.5 % Phast gels (Pharmacia Biotechnology) and stained with Coomassie Brilliant Blue R250. Protein concentration was determined by either amino acid analysis (hydrolysis in 6 M HCl for 24 h, Pharmacia Alpha plus II analyser), UV spectroscopy or the Bradford micro-protein assay [22].

Analytical procedures

Each of the enzyme preparations were analysed for the presence of the three co-factors, ADP, FMN and 4Fe-4S centre. For analysis of ADP content, known quantities of protein were precipitated with 0.5 M perchloric acid, and pelleted by microcentrifugation. The UV absorption spectrum of the supernatant was measured and the liberated ADP quantified using an absorption coefficient of 15000 $M^{-1} \cdot cm^{-1}$ at 257 nm and pH 2 [23]. The protein pellet was washed with acetone and resuspended in 6 M guanidine hydrochloride contained in 100 mM potassium phosphate buffer, pH 7.5. The covalently bound flavin was quantified by measuring the quantum yield at the peak of the fluorescence emission (525 nm) following excitation at 442 nm using a Baird Atomic SFR-100 fluorimeter.

The iron content of enzymes was assessed using the procedure of Vanoni et al. [24], adapted for iron analysis of trimethylamine dehydrogenase [15]. Cofactor stoichiometries were calculated by linear, least squares fitting to plots of cofactor concentration versus protein concentration.

ESMS and CD spectroscopy

Analyses of wild-type and mutant trimethylamine dehydrogenases by ESMS were performed using a VG BioQ triple-quadruple instrument (VG Biotech, Altrincham, U.K.), but using only the first analyser. Samples were prepared for ESMS analysis by reverse-phase HPLC of purified enzyme as described by Packman et al. [16]. Instrument settings were also as described in [16]. Replicate measurements were taken from the transformed data. CD spectroscopy was performed using a Jobin Yvon CD6 (Division d'Instruments S.A.), with a 1 mm pathlength. Four scans were averaged, over the range 195 nm to 250 nm. Integration time was one second per nm scanned. No smoothing algorithms were employed. Sample concentration was 0.6 mg/ml.

Measurement of kinetic parameters

Wild-type and mutant enzymes were assayed under steady-state conditions using the artificial electron acceptor ferricenium hexafluorophosphate. Assays were performed in 100 mM potassium phosphate buffer, pH 7.5, at 30 °C, in a final volume of 1 ml, as described previously [16]. Steady-state kinetic parameters were calculated by non-linear least squares fitting of the observed velocities to the modified Michaelis rate equation [25], which takes into account substrate inhibition by trimethylamine (see below). Data fitting was performed using Kaleidograph software (Abelbeck Software, CA, U.S.A.). Units of enzyme activity are expressed as μmol of ferricenium ions reduced per min.

RESULTS AND DISCUSSION

Position Arg-222 and flavinylation

Arg-222 was selected as a target for site-directed mutagenesis because of its inferred importance in the flavinylation mechanism of trimethylamine dehydrogenase (Figure 1). The crystal structure of the enzyme indicates that this residue is ideally placed to stabilize the development of negative charge on the N1/O2 atoms of the flavin during formation of the 6S-cysteinyl FMN (Figure 2). A similar role for arginine has been implicated during the flavinylation reaction of 6-hydroxynicotine oxidase, where the positive charge of Arg-67 in the vicinity of the N1/O2 carbonyl favours the formation of a flavin 'quinone methide' intermediate at the 8α carbon, i.e. the site of imidazole (N3)–(8α)–FAD linkage [26]. A mutant that retains positive charge at this position (Arg-67 to Lys-67) is competent in flavinylation, but flavinylation is abolished in another mutant (Arg-67 to Ala-67) that lacks positive charge at this position [27]. We created three mutants at position 222 in trimethylamine dehydrogenase, designed to retain positive charge (R222K), to remove charge (R222V) and to reverse charge (R222E), with the intention of investigating the effects on flavinylation and also the reductive half-reaction during enzyme catalysis.

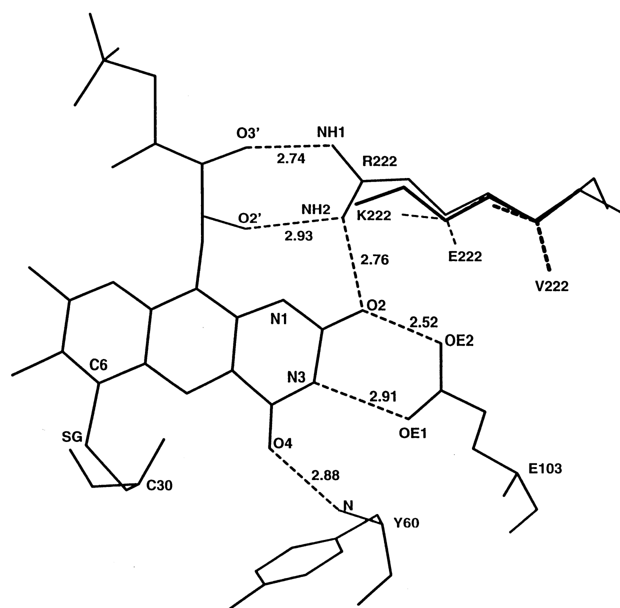

Figure 2 The FMN prosthetic group of trimethylamine dehydrogenase showing interactions of the side-chain of Arg-222 with O2′ and O3′ of the ribityl chain and with O2 of the pyrimidine ring

The replacement side-chains of Lys-222 (heavy solid line), Val-222 (heavy dashed line) and Glu-222 (light dashed line) are superimposed. Also shown are the hydrogen bonds of Glu-103 and Tyr-60 to O2, N3 and O4 and the covalent bond from Cys-30 to C6 of the flavin ring. The hydrogen bond distances are in Å. The amino acid substitutions were modelled using the graphics program TURBO-FRODO [37] on a Silicon Graphics workstation. The side-chain torsion angles of the engineered residues were adjusted manually to match those of Arg-222 and to minimize close contacts with the surrounding atoms. Arg-222 is able to provide a positive charge near the N1 of the flavin ring and stabilize its orientation through hydrogen bonding. Lys-222 can also provide the positive charge, but could hydrogen bond only to the flavin O2 since it would be too far from the ribityl chain. Glu-222 could form a hydrogen bond to O2 of the flavin, but would need to be protonated. Otherwise its negative charge would destabilize a partial negative charge on the flavin ring. Val-222 is much smaller than arginine and appears to make no bad contacts with the protein, but may allow one or two additional water molecules to bind at this site.

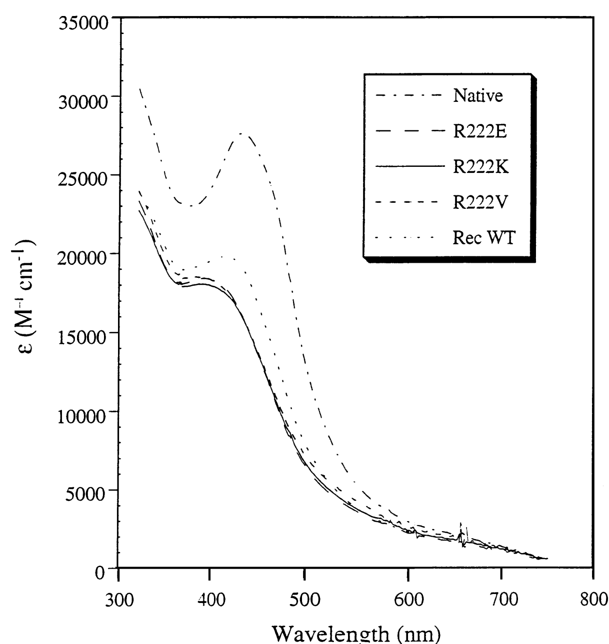

Figure 3 Absorption spectra of native and recombinant forms of trimethylamine dehydrogenase

Molar absorption coefficients refer to a single active site. Spectra were recorded for enzyme samples dissolved in 0.1 M potassium phosphate buffer, pH 7.5. Rec WT, recombinant wild-type.

General properties of the mutant enzymes

Mutant enzymes were constructed and purified to homogeneity as described above. During the purification procedure, no trimethylamine dehydrogenase activity was detected for either the R222V or the R222E mutants and the same was found for pure preparations of these enzymes. In contrast, a small amount of activity was associated with the R222K mutant and the specific activity of the purified R222K mutant (0.04 ± 0.005 units/mg of protein) was 0.6 % of that measured for the native wild-type enzyme (6.5 ± 0.3 units/mg of protein) under standard assay conditions.

Previously, we demonstrated that the recombinant wild-type enzyme is only about 25 % flavinylated; the remaining 75 % of the enzyme is isolated without FMN [15,16]. Also, both the flavinylated and deflavo forms of the recombinant wild-type enzyme were found to be stoichiometrically associated with the 4Fe-4S centre and ADP. In contrast, the native enzyme isolated from *Methylophilus methylotrophus* (W_3A_1) is 96 % flavinylated

Table 1 Cofactor stoichiometries for native and recombinant trimethylamine dehydrogenase

Abbreviations: nd, not detectable; WT, wild type.

Enzyme	ADP (mol/mol of enzyme)	Iron (mol/mol of enzyme)	Fluorescence quantum yield* (arbitrary units/ nmol of protein)	Flavinylation (%)*
Native WT	1.0	3.9	33.5	96
Recombinant WT	1.2	3.5	11.9	35
R222K	1.3	4.3	0.47	1.4
R222V	1.0	4.0	nd	0
R222E	1.0	3.8	nd	0

* Flavinylation levels calculated from fluorescence data assuming native enzyme is 96% flavinylated [16].

[16]. For the Arg-222 mutant enzymes, the possession of little or no activity prompted us to investigate whether this was due to (i) underflavinylation of the mutant enzymes, or (ii) direct catalytic effects during trimethylamine oxidation. The UV–visible absorption spectra of the Arg-222 mutant enzymes were found to be significantly different from the spectra of the recombinant wild-type and wild-type proteins (Figure 3). Decreased absorption at 443 nm suggests that the Arg-222 mutant enzymes are underflavinylated or totally devoid of flavin, which would account for the lack of activity seen for the R222E and R222V mutants and the low level of activity for the R222K mutant, respectively. However, given that deflavo enzyme cannot be active, the R222K mutant enzyme must be assembled with FMN to some extent.

During cofactor evaluation, no FMN was detected spectrophotometrically in the supernatants following perchloric acid treatment of each mutant enzyme. Quantification of ADP and iron in the supernatants demonstrated that ADP and the 4Fe-4S centres are stoichiometrically assembled in the mutant enzymes (Table 1). The observed spectral differences for the mutant enzymes must therefore be attributable to decreased flavin content. Following resolubilization of the precipitated native and recombinant wild-type enzymes in 6 M guanidine hydrochloride, the 6S-cysteinyl FMN can be readily detected spectrophotometrically [15]. For the Arg-222 mutants, however, spectra indicating the presence of 6S-cysteinyl FMN were not observed. Clearly, the mutant enzymes are either devoid of flavin or contain small amounts that cannot be detected using these simple spectrophotometric techniques.

Fluorescence emission following excitation of flavin at 442 nm was found to be a convenient method for identifying small amounts of flavin coupled to protein. Samples of wild-type and mutant forms of trimethylamine dehydrogenase were resolubilized in 6 M guanidine hydrochloride contained in 100 mM potassium phosphate buffer, pH 7.5, following precipitation with perchloric acid. For each enzyme sample, plots of fluorescence quantum yield versus protein concentration were constructed from which the proportion of the enzyme sample coupled to flavin was calculated (assuming the native wild-type enzyme to be 96% flavinylated; [16]). Table 1 indicates that the recombinant wild type is about 35% flavinylated, which is similar to the level determined by absorption spectrophotometry [15], and that the R222K mutant is 1.5% flavinylated. No flavin was detected for the R222E and R222V mutant enzymes. The fluorescence data therefore confirm that the lack of activity seen for these latter two mutants is a consequence of there being no FMN in the active site. The data demonstrate that there is a requirement for positive charge at residue 222 for flavinylation to proceed, and this observation is consistent with the proposed mechanism of flavinylation advanced in our previous work (Figure 1 and [15]). However, the question remains as to why a significant decrease in the amount of flavinylated material is obtained for the R222K mutant enzyme (see below).

A steady-state kinetic analysis of the R222K mutant revealed a K_m for trimethylamine of 129 ± 17 μM, a K_i of 1407 ± 17 μM and a k_{cat} of 13 ± 0.8 s^{-1} (based on concentration of holoenzyme in the holo/deflavo mixture), compared with a K_m of 37 ± 8 μM, a K_i of 523 ± 113 μM and a k_{cat} of 27.5 ± 2.6 s^{-1} for native wild-type protein. Unfortunately, the low flavinylation levels for the R222K mutant prevented an investigation of the reductive half-reaction kinetics using the stopped-flow methods developed for the wild-type enzyme [28]. In the steady state, the conservative exchange of a positively charged arginine residue by a positively charged lysine residue does not adversely affect the kinetic parameters for the oxidative demethylation of trimethylamine. This is intriguing given that all Class I flavin-binding β/α barrel enzymes, including trimethylamine dehydrogenase, possess an arginine residue at the position equivalent to Arg-222 in trimethylamine dehydrogenase, whereas all Class II flavin-binding β/α barrel enzymes contain lysine [8]. The two classes of flavin-binding β/α barrel proteins are defined on the basis of sequence similarity [8]. Although there is high sequence similarity within a given class, the Class I proteins display no significant similarity with Class II proteins. Trimethylamine and dimethylamine dehydrogenases are the only Class I proteins discovered to date that contain covalently linked flavin; the other members of Class I, of which there are currently five, are assembled non-covalently with FMN. Consequently, the selective pressure to retain arginine at this position in the other Class I proteins cannot be attributed to the maintenance of efficient flavinylation chemistry since these enzymes do not contain covalently linked FMN; it is more likely a consequence of enhancing steps in the reductive half-reaction, the effects of which are obscured in the steady state.

The retention of activity for the R222K mutant trimethylamine dehydrogenase contrasts with the findings of Reid and co-workers [29] for the Class II protein, flavocytochrome b_2. Based on crystallographic studies of flavocytochrome b_2 [30], Lys-349 was thought to serve a similar role to Arg-222 in trimethylamine dehydrogenase by stabilizing the anionic form of the flavin hydroquinone in the N1/C2 region of the isoalloxazine ring. Following mutation of Lys-349 to an arginine residue, activity for lactate oxidation was abolished in the mutant flavocytochrome b_2. The lack of activity in this mutant may be a consequence of the engineered arginine side-chain being inappropriately positioned in the N1/O2 vicinity such that it does not contribute to anion stabilization. This would seem likely since the mutation introduces bulk into what is a tightly packed region near the flavin, but in the absence of a crystal structure for the mutant enzyme, the question remains unresolved. By exchanging the arginine residue with a less bulky lysine residue at position 222 of trimethylamine dehydrogenase, the expectation is that steric crowding around the flavin should not be a problem in the R222K mutant enzyme.

Mutant analysis by MS and CD spectroscopy

Homogeneous preparations of all mutants and the wild-type enzymes were analysed by ESMS. Two peaks were seen for the native and recombinant wild-type preparations, corresponding to masses expected for the flavinylated and deflavo forms of the

Table 2 Molecular masses determined by ESMS for native and recombinant trimethylamine dehydrogenases

Numbers of determinations are given in parentheses. Numbers in italics represent expected mass. Abbreviation: ND, not detected.

Enzyme	Molecular mass ± S.D. (Da)			
	Apoenzyme		Flavoenzyme	
Wild-type*	81 500 ± 6 (6)	*81 498*	81 956 ± 5 (7)	*81 951*
Recombinant wild-type*	81 506 ± 2 (4)	*81 498*	81 956 ± 3 (4)	*81 951*
R222K	81 493 ± 3 (3)	*81 470*	ND	*81 923*
R222V†	81 474 ± 20 (2)	*81 441*	ND	*81 894*
R222E	81 477 ± 8 (5)	*81 471*	ND	*81 924*

* Data taken from [16].

† A second peak of mass 81 515 ± 4 Da was detected; this may represent oxidized protein.

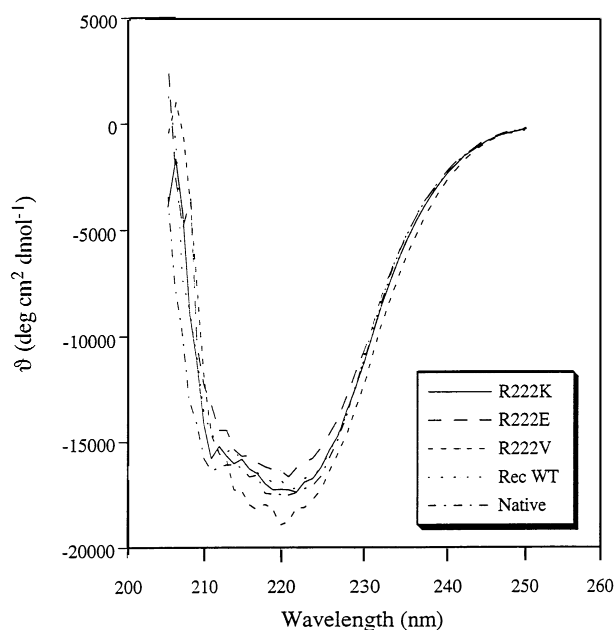

Figure 4 Far-UV CD spectra of native and recombinant trimethylamine dehydrogenases

Spectra were recorded for enzyme samples dissolved in 0.1 M potassium phosphate buffer, pH 7.5. Enzyme was in the oxidized state. Rec WT, recombinant wild-type.

enzyme (Table 2 and [16]). In contrast, each Arg-222 mutant gave a single peak whose mass closely matched that expected for the deflavo form (Table 2). The R222K and R222V mutants each give a mass slightly higher than expected but this may easily reflect simple modifications such as methionine oxidation, or indicate adduction to cations (e.g. Na^+ [+22] or K^+ [+38]). Given the inherent difficulty in assigning accurate masses to proteins of this size (instrument specification approx. 0.01 % ± 8 Da at 80 kDa), it would be inappropriate to assign any significance to the R222K and R222V masses being slightly higher than expected. The R222V mutant, while exhibiting a larger than normal standard deviation, is still quite clearly apoenzyme in form, which is the focus of this analysis. The small amount of flavinylated material detected by fluorescence in the R222K mutant was not detected in ESMS. This is expected because in previous work we demonstrated that the flavinylated form (5 % of total enzyme sample) of a H29Q mutant trimethylamine dehydrogenase is barely detected by ESMS, as the holo enzyme peak is partially obscured by salt adducts of the deflavo enzyme peak [16]. The ESMS data confirm that the Arg-222 mutant enzymes are isolated as *bona fide* deflavo enzymes, and that insertion of flavin is not prevented by any detectable levels of post-translational modification of significant mass when expressed in the heterologous host, *E. coli*.

We were unable to reconstitute the deflavo forms of the Arg-222 mutant enzymes with FMN using procedures published elsewhere [15]. This is in line with reconstitution experiments previously conducted on recombinant wild-type enzyme and the 'as-purified' deflavo form of a C30A mutant in which the 6S-cysteinyl FMN link was removed by directed mutagenesis [15]. The C30A mutant enzyme is isolated with 25 % of the enzyme assembled non-covalently with flavin (holo enzyme) and 75 % in the deflavo form [15]. A reconstitutable form of the C30A enzyme was previously generated *in vitro* by removal of the flavin from C30A holo enzyme, indicating that two forms of the deflavo enzyme exist, i.e. one form that is refractory to reconstitution (the as-purified deflavo enzyme) and another form, prepared *in vitro* from the holo enzyme, which can be reconstituted. In all other respects, e.g. mass, presence of 4Fe-4S centre and ADP, the two forms of the deflavo enzyme are identical [15,16].

Given that the masses obtained for the Arg-222 mutant proteins correspond to those expected for deflavo enzyme, the implication is that the as-purified deflavo forms of the recombinant wild-type enzyme, the C30A mutant and the Arg-222 mutants are locally misfolded in the FMN-binding site. The misfolding must be localized because the deflavo forms are able to bind the 4Fe-4S centre (located only 4.3 Å from the FMN-binding site) and ADP (Table 1). Further evidence that the misfolding is local was obtained from the CD spectra of the native and recombinant wild-type enzymes, and the Arg-222 mutant enzymes. The CD spectra for the recombinant wild-type (25 % flavinylated : 75 % deflavo), the R222K mutant (99 % deflavo) and the R222E and R222V mutants (deflavo) are identical with the spectrum obtained for the fully flavinylated native wild-type enzyme (Figure 4). The spectra indicate that no major structural changes have occurred as a result of mutagenesis.

In the native host *Methylophilus methylotrophus* (W_3A_1), expression levels of trimethylamine dehydrogenase are relatively low (3 % of total cell protein), whereas in *E. coli* the levels are elevated (about 15 % of total cell protein). Biosynthesis of flavin in *E. coli* is constitutive [31] and the host cannot adjust the rate of FMN synthesis to satisfy the demands of a highly expressed protein. This might account for the fact that virtually all the enzyme from the natural host is flavinylated, whereas that purified from *E. coli* is only about 25–35 % flavinylated. In line with this suggestion is the finding that when wild-type trimethylamine dehydrogenase is expressed to very high levels (about 30 % of total cell protein) in *E. coli* using the T7 promoter contained in a vector from the pET series (Novagen), the proportion of flavinylated enzyme is reduced to approx. 12 % (results not shown); under these circumstances flavin synthesis is even less able to match the rate of enzyme synthesis. Some highly expressed recombinant flavoproteins are purified in the holoenzyme form, suggesting that the constitutive synthesis of flavin is able to satisfy protein production. In a few cases, however, the hyperexpression of flavoproteins (e.g. sarcosine oxidase [32], glutathione reductase [33] and lipoamide dehydrogenase [34]) can lead to the production of some deflavo enzyme, but in these cases addition of flavin to cell extracts leads to the full recon-

stitution of these proteins. With trimethylamine dehydrogenase, these simple methods cannot be employed because, in the folded deflavo form, the enzyme is 'locked' and cannot be reconstituted by adding FMN to cell-free extracts or purified deflavo enzyme. In the absence of direct evidence, we speculate that the 'locked' deflavo form is locally misfolded in the active site. This notion of local misfolding is supported, although not unequivocally, by the finding that the 'locked' deflavo form contains the full complement of 4Fe-4S centre and ADP and that a reconstitutable deflavo form of a C30A mutant can be produced from flavo C30A. Structural determination of the recombinant enzymes may provide direct evidence for local misfolding in the active site.

Our R222K (this paper) and H29Q [16] mutant data have now demonstrated that formation of the 'locked' deflavo form of trimethylamine dehydrogenase can be favoured by targeted mutagenesis in the active site. For these mutants, only a small population of the enzyme is able to capture FMN and proceed to flavinylation. The formation of the 'locked' deflavo enzyme means that, unlike for the C30A mutant enzyme [15], it was not possible to generate reconstitutable deflavo forms of the wild-type and mutant enzymes. Consequently, we were unable to assess whether positive charge at position 222 accelerates the formation of the flavinylated enzyme (Figure 1). However, the Arg-222 mutant enzymes have provided insight into the assembly of the enzyme with FMN by showing that single point mutations can favour the formation of the 'locked' deflavo form of the enzyme. This may be the result of an accelerated local misfolding in the active site.

Our data find some parallels with work reported recently for flavocytochrome b_2 [35]. In flavocytochrome b_2, the flavin is non-covalently associated with the protein and the wild-type enzyme is isolated from *E. coli* as the holo enzyme. However, two active-site mutants of the enzyme are isolated as a mixture of holo and deflavo forms [35]. The mutant proteins collapse rapidly to a state that cannot bind flavin, but in all other respects the deflavo forms resemble the wild-type protein. As suggested by Gondry and co-workers [35], a local collapse in structure naturally leads to the question of a possible role for chaperone proteins during flavin incorporation. In this regard, it is interesting to note that mutants of 6-hydroxynicotine oxidase (an enzyme which is covalently linked to FAD) are associated with GroEL, whereas the wild-type enzyme is not [36]. A possible role for chaperone proteins in the flavinylation mechanism of trimethylamine dehydrogenase remains to be explored.

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