Identity of the subunits and the stoichiometry of prosthetic groups in trimethylamine dehydrogenase and dimethylamine dehydrogenase

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Trimethylamine dehydrogenases from bacterium *W₃A₁* and *Hyphomicrobium* X and the dimethylamine dehydrogenase from *Hyphomicrobium* X were found to contain only one kind of subunit. The millimolar absorption coefficient of a single [4Fe-4S] cluster in trimethylamine dehydrogenase from bacterium *W₃A₁* was estimated to be $14.8\text{ mm}^{-1}\cdot\text{cm}^{-1}$ at 443 nm. From this value a 1:1 stoichiometry of the prosthetic groups, 6-S-cysteinyl-FMN and the [4Fe-4S] cluster, was established. Millimolar absorption coefficients of the three enzymes were in the range $49.4\text{--}58.7\text{ mm}^{-1}\cdot\text{cm}^{-1}$ at approx. 440 nm. This range of values is consistent with the presence of two [4Fe-4S] clusters and two flavin residues, for which the millimolar absorption coefficient had earlier been found to be $12.3\text{ mm}^{-1}\cdot\text{cm}^{-1}$ at 437 nm. The *N*-terminal amino acid was alanine in each of the three enzymes. Sequence analysis of the first 15 residues from the *N*-terminus of dimethylamine dehydrogenase indicated a single unique sequence. Two identical subunits, each containing covalently bound 6-S-cysteinyl-FMN and a [4Fe-4S] cluster, in each of the enzymes are therefore indicated.

Trimethylamine dehydrogenase (EC 1.5.99.7) catalyses the oxidative *N*-demethylation of trimethylamine in certain methylotrophic bacteria (Colby & Zatman, 1974; Steenkamp & Mallinson, 1976; Meiberg & Harder, 1979). The enzyme was shown to contain the unusual covalently bound flavin, 6-S-cysteinyl-FMN (Steenkamp *et al.*, 1978c) and a single [4Fe-4S] cluster (Hill *et al.*, 1977). Its molecular weight was estimated to be 147 000 and 161 000 by sedimentation equilibrium (Steenkamp & Mallinson, 1976) and gel chromatography (Colby & Zatman, 1974), respectively. Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis indicated the presence of two non-identical subunits with molecular weights of 70 000–80 000 (Colby & Zatman, 1971; Steenkamp & Mallinson, 1976).

Trimethylamine dehydrogenase was therefore visualized as a dimer comprised of two dissimilar subunits with the flavin and [4Fe-4S] cluster either both located in one of the subunits or located in separate subunits. This visualization was based in part on an estimated millimolar absorption coefficient of $29\text{ mm}^{-1}\cdot\text{cm}^{-1}$ at 443 nm, as estimated by dry-weight determination of solutions of known

absorbance. The latter value was consistent with the presence of a single [4Fe-4S] cluster in the enzyme as determined by the cluster-extrusion technique (Hill *et al.*, 1977), with the stoichiometry of dithionite or substrate required for the reduction of the enzyme (Steenkamp *et al.*, 1978d), with the quantification of the rhombic e.p.r. signals of the [4Fe-4S]¹⁺ cluster in the dithionite-reduced enzyme (Steenkamp *et al.*, 1978a), and with complete inactivation of the enzyme by the incorporation of 1.08 mol of phenylhydrazine/mol of the enzyme (Nagy *et al.*, 1979).

With time, however, a few observations were made that did not agree with the conception of trimethylamine dehydrogenase presented above. When the two protein bands observed after sodium dodecyl sulphate / polyacrylamide - gel electrophoresis were irradiated to generate the fluorescent photoproduct of 6-S-cysteinyl-FMN (Steenkamp *et al.*, 1978b), both bands became fluorescent. If, indeed, the two protein bands were representative of two dissimilar subunits of trimethylamine dehydrogenase, this observation suggested the presence of covalently bound flavin in each subunit. Moreover, X-ray-crystallographic data (Lim *et al.*, 1982) indicated the presence of two, rather than one,

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iron-sulphur clusters in the enzyme. In view of these observations, it became important to examine more closely the stoichiometry of the prosthetic groups and the subunit composition of the enzyme.

In the present paper evidence is given that the trimethylamine dehydrogenase of bacterium *W₃A₁* and also the trimethylamine dehydrogenase and dimethylamine dehydrogenase of *Hyphomicrobium* X (Meiberg & Harder, 1979; Steenkamp, 1979) are dimeric enzymes comprised of two identical subunits, each containing 6-*S*-cysteinyl-FMN and a [4Fe-4S] cluster.

Methods

Bacterium *W₃A₁* and *Hyphomicrobium* X were grown in batch culture (Colby & Zatman, 1974; Meiberg & Harder, 1979). The trimethylamine dehydrogenase of bacterium *W₃A₁* and the trimethylamine dehydrogenase and dimethylamine dehydrogenase of *Hyphomicrobium* X were purified as described previously (Steenkamp & Mallinson, 1976; Meiberg & Harder, 1979). Further purification was effected by chromatography on hydroxyapatite columns with a linear gradient of 10-200 mM-potassium phosphate buffer, pH 7.2. Although no impurities were apparent on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fig. 1, gels 1a-3a), the A_{440}/A_{280} ratio of the enzymes is increased by about 8% on chromatography on hydroxyapatite, showing the removal of contaminants that were not detected by the sodium dodecyl sulphate / polyacrylamide - gel electrophoresis.

Dithionite titrations of the enzymes were performed in an anaerobic cuvette with dithionite that had been standardized by titration of riboflavin (ϵ_{445} taken as $12 \text{ mm}^{-1} \cdot \text{cm}^{-1}$). Trimethylamine dehydrogenase of bacterium *W₃A₁* was inactivated with phenylhydrazine by aerobic incubation of the enzyme in 0.1 M-sodium pyrophosphate/HCl buffer, pH 7.7, with 2 mM-phenylhydrazine for several hours at 30°C. A control sample of the enzyme, incubated under similar conditions but without phenylhydrazine, retained activity for the duration of the incubation. The molar absorption coefficient of the [4Fe-4S] clusters in the enzyme was estimated by dithionite titration of the inactive enzyme after removal of excess phenylhydrazine by chromatography on Sephadex G-25 with 0.1 M-sodium pyrophosphate/HCl buffer, pH 7.7, as the eluent.

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed as described by Weber & Osborn (1969). Polyacrylamide-gel electrophoresis under denaturing conditions was performed in the presence of 8 M-urea. Carboxymethylation was performed as follows. The enzymes in 6.5 M-guanidinium chloride containing 0.17 M-2-

mercaptoethanol were immersed in a boiling-water bath for 3 min and cooled in ice. The thiol concentration was determined by using Ellman's (1959) reagent. Sodium iodoacetate was added to obtain a 40 mM excess over the thiol concentration. The pH was adjusted to 7-8 and the mixture was incubated at 37°C until thiols could no longer be detected. Excess iodoacetate was destroyed by addition of 2-mercaptoethanol to a final concentration of 0.25 M. The samples were subsequently dialysed for 4 h against 8 M-urea that had been purified by passage through a mixed-bed resin consisting of Bio-Rad AG-50W-X8 (H^+ form) and Bio-Rad AG-1-X2 (OH^- form). Electrophoresis of the samples was performed in the discontinuous gel electrophoresis system no. 6 described by Maurer (1971) with the modification that 8 M-urea was included in all components except the electrode buffer solution. About 0.2 ml of 8 M-urea in electrode buffer was layered on top of the spacer gel, and the sample, after its density had been increased by addition of glycerol, was layered underneath.

Amino acid analyses were performed on a Durrum D-500 amino acid analyser, and handling losses were compensated for by the use of norleucine as an internal standard. Protein samples were hydrolysed for 24 h at 110°C in a partial vacuum under argon in 4 M-methanesulphonic acid containing 0.2% tryptamine (Simpson *et al.*, 1976). Cysteine was determined as cysteic acid after performic acid oxidation (Hirs, 1967) and hydrolysis of the samples in 6 M-HCl for 24 h at 110°C in a partial vacuum under argon. *N*-Terminal amino acid sequence analysis of dimethylamine dehydrogenase was performed on a Beckman model 890C sequencer. Alanine was detected by a dansylation (5 - dimethylaminonaphthalene - 1 - sulphonylation) method (Gray, 1972) as the *N*-terminal amino acid in each of the three enzymes. Dansyl-alanine released by acid hydrolysis of dansylated protein was quantified on a reverse-phase high-pressure liquid-chromatography column (Ultrasphere-ODS; Altex, Berkeley, CA, U.S.A.) with a Beckman model 332 liquid chromatograph equipped with a C-R1A (Shimadzu, Kyoto, Japan) integrator. The mobile phase consisted of methanol/water (21:29, v/v) supplemented with 0.6% acetic acid and 0.008% triethylamine (Tapuhi *et al.*, 1981). The flow rate was 0.7 ml/min and the temperature 30°C. Dansyl-amino acids in the effluent were detected fluorimetrically and quantified by comparison of the peak area with that of dansyl-alanine standards. The latter were obtained by dansylation of known amounts of alanine, the products then being treated with 6 M-HCl under conditions employed for the hydrolysis of the dansylated protein samples (for 16 h at 110°C). The validity of the method was further ascertained by addition of various amounts of

alanine as an internal standard to the protein samples before dansylation and hydrolysis.

The molar absorption coefficient of the enzyme, ϵ_λ , was calculated from the relationship:

$$\epsilon_\lambda = \frac{A_\lambda(M_r - M_c)}{\sum_i (a_i R_i)} \quad (1)$$

where A_λ is the absorbance of the sample in a cuvette of 1 cm pathlength, a_i is the amount of amino acid, obtained from amino acid analysis, R_i is the residual molecular weight of the amino acid, and M_r and M_c denote the molecular weight of the enzyme and prosthetic groups respectively. The sum in eqn. (1) should be extended to all amino acids present in the enzyme; in order to estimate M_c the stoichiometry of the prosthetic groups with respect to the enzyme must be known or assumed. The correction introduced by using $M_r - M_c$ instead of M_r is, however, small.

Results and discussion

When trimethylamine dehydrogenase from either bacterium W_3A_1 or *Hyphomicrobium* X and dimethylamine dehydrogenase were examined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis by the method initially described by Weber & Osborn (1969), two protein bands with molecular weights in the range 70 000–80 000 were observed either by the fluorescence of the fluorescent photoproduct of 6-S-cysteinyl-FMN or after staining for protein. This observation appears to have been due to incomplete denaturation of the protein, since after brief heat treatment in the presence of sodium dodecyl sulphate, as recommended later (Weber *et al.*, 1972), only a single protein band, coincident with the fluorescent photoproduct, was detected in each of the three enzymes (Fig. 1). After carboxymethylation, each of the proteins also migrated as a single band during disc polyacrylamide-gel electrophoresis in the presence of 8 M-urea (Fig. 1). The subunit molecular weights were estimated to be 83 000 for trimethylamine dehydrogenase from either organism and 78 500 for dimethylamine dehydrogenase (Fig. 2). It is therefore evident that each of the enzymes is comprised of two very similar, if not identical, subunits.

Identity of the subunits would require the presence of two flavin moieties and two [4Fe-4S] clusters per mol of enzyme. The millimolar absorption coefficient of a 6-S-cysteinyl-FMN dodecapeptide at 437 nm has been estimated to be $12.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$, whereas the millimolar absorption coefficient of [4Fe-4S] clusters may be expected to vary in the range $12\text{--}20 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ at about 440 nm (Malkin, 1973). It is evident that the literature value for the millimolar absorption coefficient of these enzymes,

Fig. 1. *Electrophoresis of the subunits of trimethylamine dehydrogenase and dimethylamine dehydrogenase*
Results of sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in 5% polyacrylamide gels of bacterium W_3A_1 trimethylamine dehydrogenase (1) *Hyphomicrobium* X trimethylamine dehydrogenase (2) and *Hyphomicrobium* X dimethylamine dehydrogenase (3), before (a) and after (b) chromatography on hydroxyapatite, are shown. Gels 4, 5 and 6 represent the results of disc gel electrophoresis in 7.5% polyacrylamide gels in the presence of 8 M-urea of carboxymethylated trimethylamine dehydrogenases from bacterium W_3A_1 and *Hyphomicrobium* X and of dimethylamine dehydrogenase respectively.

Fig. 2. Determination of the molecular weights of trimethylamine dehydrogenase and dimethylamine dehydrogenase by using sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

Marker proteins were phosphorylase *b* (Seery *et al.*, 1967), bovine serum albumin (Brown, 1975), ovalbumin (Warner, 1954) and carbonic anhydrase (Davis, 1971). Arrows pointing upwards and downwards indicate R_F values obtained for trimethylamine dehydrogenase from either organism and for dimethylamine dehydrogenase respectively.

Table 1. Amino acid compositions of trimethylamine dehydrogenase from bacterium W_3A_1 and of trimethylamine dehydrogenase and dimethylamine dehydrogenase from *Hyphomicrobium X*

Amino acid	Amino acid composition (mol of residue/mol of enzyme)		
	Bacterium W_3A_1 trimethylamine dehydrogenase	<i>Hypho-</i> <i>microbium X</i> trimethylamine dehydrogenase	<i>Hypho-</i> <i>microbium X</i> dimethylamine dehydrogenase
Asp	146.8	139.9	154.5
Thr	80.2	74.5	83.5
Ser	77.0	62.2	58.5
Glu	163.3	165.5	151.2
Pro	93.1	86.2	79.1
Gly	138.5	149.7	134.3
Ala	130.5	143.5	121.4
Val	78.1	88.9	76.1
Met	31.4	20.6	22.6
Ile	77.0	69.2	62.9
Leu	98.3	102.1	90.8
Tyr	62.7	65.1	59.9
Phe	39.3	43.4	34.5
His	44.6	49.9	45.2
Lys	81.4	83.4	65.6
Trp	35.9	26.3	28.4
Arg	91.8	102.0	110.9
Cys	17.1	21.2	19.5

which had earlier been estimated to be $29 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ at about 440 nm (Hill *et al.*, 1977), would exclude the presence of two flavin moieties and two [4Fe-4S] clusters in the enzyme. It therefore seemed essential to re-assess the accuracy of this value. It was considered that determination of the molar absorption coefficients of the three dehydrogenases, which appear to be very similar in overall composition (Steenkamp, 1979), would increase confidence in the validity of the results.

Initially our approach was, in part, similar to that of Hong & Rabinowitz (1970). In their determination of the molar absorption coefficient of clostridial ferredoxin, these workers quantified the amount of protein by dry-weight determinations, C-terminal end-group analysis and amino acid analysis. The trimethylamine dehydrogenases and dimethylamine dehydrogenase, however, were not soluble in distilled water and were precipitated on prolonged dialysis. The molar absorption coefficient of each of the three dehydrogenases was therefore estimated by drying samples of solutions, of known absorbance, that had been dialysed extensively against $1 \text{ mM-NH}_4\text{HCO}_3$, for 72 h at 110°C in a vacuum oven, to constant weight. Values determined in this manner were about 16% lower than the corresponding values derived from amino acid analysis of enzyme solutions of known absorbance. The discrepancy between the values based on

dry-weight determinations and amino acid analysis is most probably due to the presence of counterions; the presence of an HCO_3^- ion to neutralize each lysine and arginine residue in the trimethylamine dehydrogenase of *Hyphomicrobium X* (Table 1), for instance, would effectively increase its molecular weight by 11300. Estimation of the protein content from dry-weight determinations was therefore considered to be unreliable. Each of these methods, moreover, suffers from the deficiency that the molecular weight of a protein must be accurately known in order to determine the molar absorption. If the molecular weights of the trimethylamine dehydrogenases and dimethylamine dehydrogenase are assumed to be 166000 and 157000 respectively, as estimated from sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, the millimolar absorption coefficients of the enzymes, based on amino acid analysis, are $53\text{--}54 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ at 443 nm for the trimethylamine dehydrogenases and $49.4 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ for dimethylamine dehydrogenase. If a molecular weight of 147000, determined for the trimethylamine dehydrogenase of bacterium W_3A_1 by sedimentation equilibrium, is used in the calculations, a millimolar absorption coefficient of only $47.1 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ at 443 nm is obtained for this enzyme.

Fig. 3. Dithionite titration of phenylhydrazine-inactivated trimethylamine dehydrogenase

The enzyme was contained in a volume of 2.41 ml of 0.1 M-sodium pyrophosphate/HCl buffer, pH 7.7. The curves represent spectra of the oxidized inactive enzyme (—) and after the addition of 4.4 (—), 8.9 (.....), 13.3 (---), 17.76 (----) and 31.1 nmol of dithionite (·-·-·). The total volume increment was 14 μ l and was considered negligible.

It was therefore decided to determine the molar absorption coefficient by independent procedures, such as dithionite titration or *N*-terminal end-group analysis, which would not be influenced by uncertainties in the determination of the molecular weight. The results of dithionite titration of each of the three enzymes indicated the molar absorption coefficient to be a multiple of $9.3\text{--}9.8\text{ mm}^{-1}\cdot\text{cm}^{-1}$ at about 440 nm. In terms of these values an enzyme titrated by the uptake of five electron equivalents would have a molar absorption coefficient in the range $46.5\text{--}49.0\text{ mm}^{-1}\cdot\text{cm}^{-1}$; for a six-electron acceptor the corresponding values are $55.8\text{--}58.8\text{ mm}^{-1}\cdot\text{cm}^{-1}$. The former situation would pertain, for instance, to an enzyme containing one flavin moiety and three iron-sulphur clusters, the latter in an enzyme with two flavin moieties and two iron-sulphur clusters. In order to distinguish between these possibilities, it was necessary to determine the ratio in which the flavin coenzyme and [4Fe-4S] cluster occur in the enzymes. The assumption that these entities are present in equimolar amounts in the dehydrogenases was crucial in the interpretation of earlier studies of their reaction mechanism (Steenkamp *et al.*, 1978*a,d*; Steenkamp & Beinert, 1982*a,b*). It was therefore also important to establish the validity of this assumption. Inactivation of the trimethylamine dehydrogenase of bacterium W_3A_1 by treatment with the monoamine

oxidase inhibitor phenylhydrazine results in extensive bleaching of the flavin chromophore owing to the addition of the phenyl moiety at the 4(*a*)-position of the flavin to form a stable adduct (Nagy *et al.*, 1979). The millimolar absorption coefficient of the [4Fe-4S] cluster in the enzyme was estimated to be $14.8\text{ mm}^{-1}\cdot\text{cm}^{-1}$ at 443 nm by titration of the inactive enzyme with dithionite (Fig. 3). Since the millimolar absorption coefficient of a 6-*S*-cysteinyl-FMN dodecapeptide had earlier been determined to be $12.3\text{ mm}^{-1}\cdot\text{cm}^{-1}$, the ratio in which the flavo-coenzyme and [4Fe-4S] cluster occur in the enzyme can be established by selective destruction of either of them. Relevant findings were presented by Hill *et al.* (1977) (see Fig. 3). Treatment of the unfolded enzyme in hexamethylphosphoramide with mersalyl resulted in a pronounced decrease in visible absorbance of the enzyme. The ratio of the remaining absorbance, due to the covalently bound flavin coenzyme, to that of the enzyme before destruction of the [4Fe-4S] cluster was 0.46. If it is assumed that the flavin coenzyme and [4Fe-4S] cluster are present in equimolar amounts, a corresponding expected ratio of 0.454 may be calculated, based on the molar absorption coefficient values for the flavin coenzyme and [4Fe-4S] cluster. Equimolar quantities of flavin and [4Fe-4S] cluster are therefore indicated. Acceptable values for the molar absorption coefficient of trimethylamine dehydrogenase therefore have to be a multiple of about $27.1\text{ mm}^{-1}\cdot\text{cm}^{-1}$ at 443 nm.

If the dehydrogenases are assumed to be dimeric, with each subunit containing a flavin moiety and a [4Fe-4S] cluster, the millimolar absorption coefficient should be about $54.2\text{ mm}^{-1}\cdot\text{cm}^{-1}$. This value is in satisfactory agreement with the value that was obtained based on amino acid analysis (Table 2), provided that the molecular weights from sodium dodecyl sulphate / polyacrylamide - gel electrophoresis, rather than the values reported earlier (Steenkamp & Mallinson, 1976; Steenkamp, 1979), are assumed. It is known that sedimentation-equilibrium studies conducted at pH values substantially different from the isoelectric point of a protein can lead to underestimation of its molecular weight (Schachman, 1958; Johnson *et al.*, 1954). Howard & Solomonson (1982) have reported on factors contributing to the underestimation of the molecular weight of nitrate reductase as determined by sedimentation equilibrium. The main source of error resided in an underestimation of the partial specific volume by computation from the amino acid composition, a method that was also used in the determination of the molecular weight of trimethylamine dehydrogenase (Steenkamp & Mallinson, 1976).

Further evidence for the identity of the subunits in the enzymes was obtained by qualitative analysis for *N*-terminal amino acid after dansylation. Dansyl-

Table 2. *Some properties of trimethylamine dehydrogenase and dimethylamine dehydrogenase*

The molecular weights were obtained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Molar absorption coefficients were obtained (i) by dithionite titration of the dehydrogenases with dithionite standardized against riboflavin ($\epsilon_{445} = 12.0 \text{ mm}^{-1} \cdot \text{cm}^{-1}$), or (ii) by summation of the amino acid contents of samples of dehydrogenase solutions of known absorbance, to which a correction for the contribution of two FMN moieties and two [4Fe-4S] clusters was applied. The number of *N*-terminal alanine residues, Fe and S contents, [4Fe-4S] clusters extruded and phenyl residues bound during inactivation of trimethylamine dehydrogenase with phenylhydrazine were calculated assuming millimolar absorption coefficients of $54.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ at 443 nm and 440 nm for trimethylamine dehydrogenase and dimethylamine dehydrogenase respectively. This value reflects an average of millimolar absorption coefficients for the three enzymes as determined by the two methods.

Source	Bacterium W_3A_1 trimethylamine dehydrogenase	<i>Hyphomicrobium</i> X trimethylamine dehydrogenase	<i>Hyphomicrobium</i> X dimethylamine dehydrogenase	Reference
Molecular weight	166 000	166 000	157 000	Present paper
Molar absorption ($\text{mm}^{-1} \cdot \text{cm}^{-1}$)				
From dithionite titration	55.8	56.6	58.7	Present paper
From amino acid analysis	52.3	53.9	49.4	Present paper
<i>N</i> -Terminal alanine residues (mol/mol of enzyme)	2.0	2.06	1.62	Present paper
Fe content (mol of Fe/mol of enzyme)	7.9	8.5	8.5	Hill <i>et al.</i> (1977); Steenkamp (1979)
S content (mol of S-mol of enzyme)	7.5	7.3	7.3	Hill <i>et al.</i> (1977); Steenkamp (1979)
[4Fe-4S] clusters extruded				Hill <i>et al.</i> (1977)
With <i>p</i> -methoxybenzenethiol	2.07			
With <i>o</i> -xylene- $\alpha\alpha'$ -dithiol	1.84			
Phenylhydrazine inactivation (mol of phenyl groups bound/ mol of enzyme)	2.03			Nagy <i>et al.</i> (1979)

alanine was identified by t.l.c. after dansylation and hydrolysis of each of the enzymes. Quantification by a high-pressure liquid-chromatography method as outlined in the Methods section indicated the presence of two *N*-terminal alanine residues if a molar absorption coefficient of $54.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ is assumed (Table 2). Although the value of $\epsilon_{443} = 54.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ has been rather arbitrarily chosen, it does represent the average of the molar absorption coefficients reported in Table 2 as determined for the three enzymes by two different methods, and corresponds closely to a summation of the molar absorption coefficients of two 6-*S*-cysteinyl-FMN residues and two [4Fe-4S] clusters as determined in independent experiments. Further refinement of the data would only be possible once more accurate values for the molecular weights of the enzymes are available. That the presence of two *N*-terminal alanine residues in each of the enzymes indeed indicated identity of the subunits was further conclusively demonstrated in the case of dimethylamine dehydrogenase by *N*-terminal sequence analysis. A single unique sequence

was found. Moreover, a single flavin peptide of unique sequence had earlier been isolated from the trimethylamine dehydrogenase of bacterium W_3A_1 (Kenney *et al.*, 1978).

Conclusion

The properties of trimethylamine dehydrogenase and dimethylamine dehydrogenase reported here are entirely in accord with a preliminary X-ray-crystallographic study on the trimethylamine dehydrogenase of bacterium W_3A_1 (Lim *et al.*, 1982) and necessitates a revision of some of the values reported in earlier studies. Such a revision is presented in Table 2, where a molar absorption coefficient of $54.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$, presenting an average of the molar absorption coefficients of the three enzymes as estimated by dithionite titration and by amino acid analysis, was used to re-evaluate some of the data from earlier publications.

The data presented leave little doubt that the trimethylamine dehydrogenases and dimethylamine dehydrogenase are comprised of two identical

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
Ala-Arg-Asp-Pro-Arg-Phe-Asp-Ile-Leu-Phe-Thr-Pro-Leu-Lys-Leu-

subunits, each containing a covalently bound flavin moiety and a [4Fe-4S] cluster.

The question also arises whether this perception of the structure of these enzymes necessitates a revision of earlier kinetic data (Steenkamp *et al.*, 1978a,d; Steenkamp & Beinert, 1982a,b). Since the millimolar absorption coefficient of $29 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ used throughout the kinetic studies corresponds closely to the most reliable estimate of the corresponding parameter for a subunit, this does not appear to be so. There is little definitive evidence that the two subunits present in the enzymes react at dissimilar rates during the reductive half-reaction. In the case of the trimethylamine dehydrogenase of bacterium W_3A_1 the non-linear dependence on substrate of the slow intramolecular transfer of an electron from the flavin to the iron-sulphur cluster (Fig. 10 in Steenkamp & Beinert, 1982b), which could not previously be accounted for, may reflect a certain degree of co-operativity between the subunits.

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