The effect of tetrahydrofolate on the reduction of electron transfer flavoprotein by sarcosine and dimethylglycine dehydrogenases

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Pig liver electron transfer flavoprotein (ETF) is rapidly reduced by sarcosine and dimethylglycine dehydrogenases to the anionic semiquinone form, the subsequent formation of the flavoquinol form being a much slower process. In the presence of tetrahydrofolate the yield of anionic semiquinone at the end of the rapid phase of reduction of ETF is only about 10% less than without tetrahydrofolate, as judged by e.p.r. spectroscopy. Tetrahydrofolate does not alter the rate of reduction of ETF by either sarcosine or dimethylglycine dehydrogenase. Nevertheless, it was clearly demonstrated that tetrahydrofolate is a substrate for both sarcosine and dimethylglycine dehydrogenases and is converted to $N^{5,10}$ -methylenetetrahydrofolate.

Sarcosine and dimethylglycine dehydrogenases (EC 1.5.99.1 and 1.5.99.2) are flavoproteins which catalyse the oxidative *N*-demethylation of sarcosine and dimethylglycine (MacKenzie & Abeles, 1956; MacKenzie & Frisell, 1958). Although oxidative *N*-demethylations are of general occurrence in biochemistry, the immediate fate of the methyl groups which are oxidized to the oxidation level of formaldehyde is not unequivocally understood because of facile addition reactions of formaldehyde with nucleophiles. It is not always apparent whether adduct formation of formaldehyde with biologically important nucleophiles such as N-5 of H_4 PteGlu or thiol groups, as in glutathione, is enzyme-catalysed or not. The sarcosine and dimethylglycine dehydrogenases provide interesting cases in point. The rapid incorporation of radioactive carbon from the methyl group of sarcosine, but not from free formaldehyde, into serine was reported by MacKenzie (1955) and Lewis *et al.* (1978), who postulated that 'active' rather than free formaldehyde is formed as a product in the oxidation of sarcosine to glycine. The nature of 'active formaldehyde', presumably an adduct, was unclear. While liver mitochondria isolated from folate-deficient rats showed a de-

creased incorporation of radioactivity from the *N*-methyl group of sarcosine into serine, sarcosine oxidase activity was unaffected (Dac & Wriston, 1958), indicating that H_4 PteGlu did not participate directly in the oxidation of sarcosine. More recently, however, the identification of dimethylglycine and sarcosine dehydrogenases as folate-binding proteins in rat liver mitochondria by Wittwer & Wagner (1980, 1981*a,b*) led to the proposal that these enzymes use H_4 PteGlu as a co-substrate in the direct synthesis of 5,10- CH_2-H_4 PteGlu from sarcosine and dimethylglycine. However, since formaldehyde condenses non-enzymically with H_4 PteGlu to form 5,10- CH_2-H_4 PteGlu, positive identification of 5,10- CH_2-H_4 PteGlu as the immediate reaction product in the oxidative *N*-demethylation of dimethylglycine was not possible (Wittwer & Wagner, 1981*b*).

If indeed H_4 PteGlu is a co-substrate in the oxidative demethylation of sarcosine or dimethylglycine it may be expected that H_4 PteGlu should stimulate the turnover rate of the sarcosine and dimethylglycine dehydrogenases markedly, by analogy with the rate-enhancing effect of H_4 PteGlu on the interconversion of formaldehyde and glycine to serine by serine hydroxymethyltransferase (Chen & Schirch, 1973). Since H_4 PteGlu reacts rapidly with the dyes commonly used as electron acceptors in assays of flavoprotein dehydrogenases *in vitro*, the effect of H_4 PteGlu on the reaction kinetics of these enzymes cannot be ascertained in such assays. In this study the effect of H_4 PteGlu on the rate of

Abbreviations used: ETF, electron transfer flavoprotein; H_4 PteGlu, tetrahydrofolate; 5,10- CH_2-H_4 PteGlu, $N^{5,10}$ -methylenetetrahydrofolate; 10-CHO- H_4 PteGlu, 10-formyltetrahydrofolate; SDS, sodium dodecyl sulphate.

reduction of the physiological oxidant, the electron transfer flavoprotein (ETF), by purified preparations of dimethylglycine and sarcosine dehydrogenases from pig liver mitochondria is examined. Evidence confirming the earlier proposals of Wittwer & Wagner (1980, 1981b) that 5,10-CH₂-H₄PteGlu is a direct reaction product of the oxidative *N*-demethylation of sarcosine and dimethylglycine is presented.

Materials and methods

Chemicals

Folinic acid, folic acid, sodium borohydride, 5,10-CH₂-H₄PteGlu dehydrogenase (EC 1.5.1.5), formaldehyde dehydrogenase (Ando *et al.*, 1979) and ω -aminohexyl-agarose were obtained from Sigma and were used without further purification. Molecular weight markers used in polyacrylamide-gel electrophoresis were obtained from BDH. H₄PteGlu and 5,10-CH₂-H₄PteGlu were synthesized by modification of established procedures (Scrimgeour & Vitols, 1966; Blair & Saunders, 1970). Folic acid (20 mg) was reduced using 60 mg of NaBH₄ in a final volume of 1.5 ml. After 45 min the borohydride was destroyed by acidification with glacial acetic acid or 6M-HCl and the pH was adjusted to approx. 7.5 with KOH. Untreated folate and dihydrofolate were removed anaerobically under argon by selective batch adsorption to DEAE-cellulose acetate or chloride under conditions where tetrahydrofolate is not adsorbed. In the preparation of H₄PteGlu for kinetic experiments it was essential to use chloride as the anion, because sarcosine and dimethylglycine dehydrogenases are competitively inhibited by acetate (MacKenzie, 1955). 5,10-CH₂-H₄PteGlu was synthesized by treating H₄PteGlu with a two-fold excess of formaldehyde. The concentrations of H₄PteGlu and of 5,10-CH₂-H₄PteGlu were determined from their absorption coefficients of 29 mm⁻¹·cm⁻¹ at 297 nm and 33 mm⁻¹·cm⁻¹ at 292 nm, respectively (Kallen, 1971). The concentration of 5,10-CH₂-H₄PteGlu determined by means of the NADP-linked conversion to 10-HCO-H₄PteGlu (Ramasastry & Blakley, 1964) in the presence of 2 mM- β -mercaptoethanol was generally approx. 90% of the value calculated from the absorbance at 292 nm.

Purification of enzymes and of ETF

The purification and characterization of sarcosine and dimethylglycine dehydrogenases and of ETF by modifications of existing procedures (Hoskins & MacKenzie, 1961; Frisell & MacKenzie, 1962, 1970; Wittwer & Wagner, 1981a) will be described in detail in a separate communication and are only briefly summarized here. Pig liver mitochondria were prepared essentially as described

(Schnaitman & Greenawalt, 1968) and subjected to one freeze-thaw cycle followed by osmotic lysis and sonication (Hoskins & MacKenzie, 1961) to release soluble enzymes. Further fractionation involved (NH₄)₂SO₄ precipitation and chromatography on DEAE-cellulose. Dimethylglycine and sarcosine dehydrogenases were further purified by chromatography on Sephadex G-150, hydroxylapatite, and a second DEAE-cellulose column. Alternatively, the enzymes obtained from the gel chromatography step were subjected to affinity chromatography as described (Wittwer & Wagner, 1981b).

ETF was eluted from DEAE-cellulose chromatography of the (NH₄)₂SO₄ precipitate at low ionic strength and was further purified by chromatography on hydroxylapatite and CM-Sephadex.

Sarcosine and dimethylglycine dehydrogenases were assayed as described (Wittwer & Wagner, 1980). Protein was determined by the Lowry (Lowry *et al.*, 1951) and biuret (Gornall *et al.*, 1949) methods. Polyacrylamide-gel electrophoresis was performed in the system of Davis (1964) and with SDS and β -mercaptoethanol as described (Weber & Osborn, 1969). Sarcosine dehydrogenase was detected on gels run under non-denaturing conditions by using an activity stain (Wittwer & Wagner, 1981b).

Further experimental details are given in the Figure legends.

Results and discussion

Properties of purified dimethylglycine and sarcosine dehydrogenases and of ETF

Sarcosine and dimethylglycine dehydrogenases, purified by affinity chromatography, migrated as single bands with subunit *M_r* values of 91 000 and 93 000, respectively, on SDS/polyacrylamide-gel electrophoresis. Yellow fluorescence co-migrated with the protein in each case, indicating the presence of covalently bound flavin. The number of FAD residues in the two enzymes was estimated by assuming an absorption coefficient of 11.3 mm⁻¹·cm⁻¹ at 459 nm and 457 nm for dimethylglycine dehydrogenase and sarcosine dehydrogenase, respectively, and that the Lowry procedure with bovine serum albumin as a standard accurately reflects the protein content of the enzymes. Based on these assumptions, dimethylglycine dehydrogenase and sarcosine dehydrogenase contained 0.93 and 1.19 mol of FAD, respectively, per mol of subunit. The specific activity of purified sarcosine dehydrogenase was 263 nmol·min⁻¹·mg⁻¹ and that of dimethylglycine dehydrogenase was 157 nmol·min⁻¹·mg⁻¹. These activities are about 500-fold less than reported by Wittwer & Wagner (1980), but our specific activities agree more closely with that reported by other workers (Frisell & MacKenzie, 1962; Hoskins & Bjur, 1964) at corresponding stages of purification.

The reason for the discrepancy is uncertain, particularly since the data presented in Table 1 clearly indicate that a high proportion of either sarcosine or dimethylglycine dehydrogenases in our preparations were catalytically competent. A decision regarding the validity of the unusually high specific activities reported by Wittwer & Wagner (1980) will, therefore, have to await independent confirmation. Recovery of the enzymes from affinity chromatography was, however, low and variable, due to irreversible adsorption to the affinity matrix.

Since absolute purity of the enzymes was not essential for the work reported here, affinity chromatography was replaced by chromatography on hydroxyapatite and DEAE-cellulose, which gave a recovery of about 60% overall of the activity applied to these two steps. Sarcosine dehydrogenase so obtained was about 60% pure as judged by the ratio of FAD to protein, and polyacrylamide-gel electrophoresis in the system of Davis (1964) showed the presence of a diffuse band of protein not coincident with the activity stain. The purity of the dimethylglycine dehydrogenase preparation was about 80% as judged by the ratio of enzyme bound flavin to protein. These preparations were used to investigate the effect of tetrahydrofolate on the rate of reduction of ETF by dimethylglycine or sarcosine.

ETF obtained as described in the Materials and methods section had a ratio of absorbance at 270 nm to that at 437 nm of 6.3, comparable with the best preparations reported in the literature (Hall & Kamin, 1975; Crane & Beinert, 1956). The molar absorption coefficient of ETF at 437 nm was determined by direct comparison of the absorption spectra of ETF and the FAD released from ETF by treatment with 4.5 M-guanidine hydrochloride (Thorpe *et al.*, 1979). Two such determinations gave absorption coefficients of $13.6 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ and $13.5 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ relative to that of FAD for which $\epsilon_{445} = 11.75 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ (L. D. Arscott & C. H. Williams, Jr., unpublished results).

Formation of an anionic semiquinone of ETF

Reduction of ETF by either sarcosine or dimethylglycine in the presence of their dehydrogenases rapidly gave rise to a spectrum resembling that of an anionic semiquinone (Fig. 1). When the absorbance at 370 nm was used to calculate an absorption coefficient, a value of $16.2 \text{ mm}^{-1} \cdot \text{cm}^{-1}$ was found, in close agreement with that reported for the anionic semiquinones of other flavoproteins (Massey *et al.*, 1966). The spectrum of the anionic semiquinone gradually changed over a period of several hours to that resembling the flavoquinol form. The system had therefore not yet reached thermodynamic equilibrium when maximal formation of the flavosemiquinone was observed.

Fig. 1. Reduction of pig liver ETF by sarcosine dehydrogenase

Reaction mixtures contained 10.65 nmol of ETF, 30.6 μmol of potassium barbital, pH 8.0, 0.8 μmol of sarcosine and 20 μmol of glucose in 0.89 ml of 10% (v/v) ethylene glycol. The air in the cuvette was displaced with argon through a silicone rubber stopper and the solution was rendered anaerobic by the addition of glucose oxidase (0.42 unit) and catalase (5.8 units). The reaction was started by adding 5 μl of a sarcosine dehydrogenase solution containing 6.2 μM enzyme-bound flavin. The spectrum of reduced ETF (broken line) was recorded after completion of the rapid phase of reduction. The solid line represents oxidized ETF. The temperature was 25°C.

Nonetheless, in view of the large negative free energy change of about -25 kJ (Kallen & Jencks, 1966) for the condensation of formaldehyde with H_4PteGlu , its presence could conceivably alter the proportion of free radical formed in favour of the flavoquinol form during the rapid phase of reduction of ETF by sarcosine or dimethylglycine dehydrogenase. Direct comparison of the rates of reduction of ETF in the presence and absence of H_4PteGlu would only be valid if the respective proportions of flavosemiquinone formed are similar. Consequently, the yield of flavosemiquinone in the reduction of ETF by sarcosine dehydrogenase in the presence and absence of H_4PteGlu was quantified. Difference spectra

between oxidized ETF and the species present at the end of the rapid phase of reduction of ETF by sarcosine dehydrogenase in the presence and absence of $H_4PteGlu$ (Fig. 2), indicated that a somewhat larger proportion of the flavoquinol form of ETF is formed in the presence of $H_4PteGlu$. This conclusion was further substantiated by examination of the same samples by e.p.r. spectroscopy. The e.p.r. signals ($g=2$) had a width of about 15G, characteristic of anionic flavosemiquinone species (Beinert, 1972). Integration of the signals indicated that the presence of $H_4PteGlu$ decreased the amount of anionic semiquinone formed by only about 10%, which was unlikely to influence the results of kinetic experiments significantly.

Earlier studies (Hall & Lambeth, 1980) have demonstrated that the flavosemiquinone form of ETF is a catalytically important intermediate in its reduction by general acyl-CoA dehydrogenase. These authors also concluded that further reduction of ETF to the flavoquinol form is not due to disproportionation of the radical, but to a slower reduction of ETF semiquinone by general acyl-CoA dehydrogenase. In a more recent study of the reduction of ETF by the general acyl-CoA dehydrogenase (Beckmann *et al.*, 1981) the ETF semiquinone accumulated in an isosbestic manner during the course of the reaction, indicating a much slower reduction of ETF semiquinone to the flavoquinol form than reported by Hall & Lambeth (1980). This discrepancy suggests that the buffer conditions may be important in determining the behaviour of the

system. The results reported in this paper reinforce the conclusion that the reduction of ETF semiquinone to the flavoquinol form is too slow to be catalytically important in the transfer of electrons from dehydrogenases to the ubiquinone pool. Reduction of the anionic semiquinone of ETF in the presence of either dimethylglycine or sarcosine dehydrogenases proceeds only very slowly and the accumulation of the anionic semiquinone is, therefore, more conspicuous than in the earlier study of Hall & Lambeth (1980).

Effect of $H_4PteGlu$ on the reduction of ETF by dimethylglycine and sarcosine dehydrogenases

The presence of $H_4PteGlu$ had no effect on the rate of reduction of ETF by either dimethylglycine or sarcosine dehydrogenase (Fig. 3). This surprising observation suggested either that the *N*-demethylations catalysed by sarcosine and dimethylglycine dehydrogenases are exceptional cases in which $H_4PteGlu$ can function as a co-substrate without influencing the catalytic turnover rate, or that the proposal of Wittwer & Wagner (1980, 1981b), based almost entirely on binding studies, that these enzymes synthesize 5,10- $CH_2-H_4PteGlu$ directly, was erroneous. It, therefore, became essential to obtain independent evidence that the sarcosine and dimethylglycine dehydrogenases do actually utilize $H_4PteGlu$ for the enzymic synthesis of 5,10- $CH_2-H_4PteGlu$.

Although the non-enzymic condensation of formaldehyde with $H_4PteGlu$ (eqn. 1 in Scheme 1) is very favourable (Kallen & Jencks, 1966), the reaction rate is slow at physiological concentrations of the reactants (Jaenicke, 1971; Krebs *et al.*, 1976) especially in the presence of thiols (Kallen & Jencks, 1966). The formation of 5,10- $CH_2-H_4PteGlu$, rather than of free formaldehyde, in a reaction can, therefore, be established by observing the rate of reduction of $NADP^+$ catalysed by 5,10- $CH_2-H_4PteGlu$ dehydrogenase (eqn. 2 of Scheme 1). Under appropriate conditions, such as the presence of a thiol as an activator of the enzyme (Ramasastry & Blakley, 1962) and at pH values >7 , the non-enzymic condensation of formaldehyde with $H_4PteGlu$ is severely rate limiting in the overall reactions of Scheme 1, as shown in Fig. 4. The immediate formation of either formaldehyde or 5,10- $CH_2-H_4PteGlu$ upon reduction of sarcosine or dimethylglycine dehydrogenases by their respective substrates could, therefore, be detected by linking these reductive half-reactions to a reaction catalysed by an appropriate $NAD(P)$ -dependent dehydrogenase. The reaction traces consisted of a rapid decrease in absorbance measured at 340nm (completed within the mixing time) due to bleaching of sarcosine or dimethylglycine dehydrogenases by substrate, followed by a more gradual increase in

Fig. 2. Comparison of the difference spectrum for reduction of ETF by sarcosine dehydrogenase in the presence and absence of $H_4PteGlu$

The solid line represents the difference spectrum between oxidized and reduced ETF for the curves shown in Fig. 1. The broken line represents data obtained under identical conditions, but in the presence of 46.2 nmol of ($\pm$)- $H_4PteGlu$.

Fig. 3. Effect of $H_4PteGlu$ on the rate of reduction of ETF by sarcosine and dimethylglycine dehydrogenases (a) Reaction mixtures contained 6.84 nmol of ETF, 10 μ mol of glucose and 29.2 μ mol of potassium barbital, pH 8.0, in 0.81 ml of 10% (v/v) ethylene glycol. The air in the cuvette was displaced with argon through a silicone rubber stopper. The solution was rendered anaerobic by the addition of glucose oxidase (0.42 unit) and catalase (5.8 units) and 5 μ l of a solution containing 6.2 μ M sarcosine dehydrogenase enzyme-bound flavin was added and the reaction was started with various amounts of sarcosine. (b) To the anaerobic reaction mixtures prepared as described in (a) was added 10 μ l of a solution containing 3.6 μ M dimethylglycine dehydrogenase enzyme-bound flavin and the reaction was started with various amounts of dimethylglycine. The temperature was 25°C. Triangles and circles in (a) and (b) represent reaction rates in the absence and presence, respectively, of 38 μ M-($\pm$)- $H_4PteGlu$.

absorbance due to the formation of NAD(P)H by the NAD(P)-dependent dehydrogenase. Thus, the release of formaldehyde could be detected by observing the increase in absorbance at 340 nm due to the reduction of NAD^+ ($\Delta\epsilon = 6.22 \text{ mm}^{-1} \cdot \text{cm}^{-1}$)

by formaldehyde dehydrogenase (Fig. 5), whereas the release of 5,10- $CH_2-H_4PteGlu$ could be quantified similarly (Ramasastry & Blakley, 1964) by measuring the NADPH formed in the oxidation of 5,10- $CH_2-H_4PteGlu$ to 10-CHO- $H_4PteGlu$ by 5,10- $CH_2-H_4PteGlu$ dehydrogenase and cyclohydrolase ($\Delta\epsilon = 7.1 \text{ mm}^{-1} \cdot \text{cm}^{-1}$).

In either case the initial absorbance at 340 nm immediately after addition of substrate was estimated from the absorbance change which accompanied complete reduction of sarcosine and dimethylglycine dehydrogenases by their substrates in spectrophotometric titrations (Figs. 6 and 7) and was subtracted from the final absorbance to obtain the absorbance change for the production of NAD(P)H. This procedure could, unfortunately, not be applied legitimately to the release of products from the sarcosine dehydrogenase preparation iso-

Fig. 4. Rate of NADPH formation by 5,10- $CH_2-H_4PteGlu$ dehydrogenase in the presence of 5,10- $CH_2-H_4PteGlu$ compared with $H_4PteGlu$ plus formaldehyde. Reaction mixtures comprised 50 μ mol of potassium barbital buffer, pH 7.5, 2 μ mol of $NADP^+$, 1 μ mol of cysteine, and 0.04 units of 5,10- $CH_2-H_4PteGlu$ dehydrogenase in a volume of 1.04 ml. After equilibrium at 30°C in the temperature-controlled cuvette compartment of a Cary 219 spectrophotometer and extensive bubbling with argon, reactions were started by the addition of 6.6, 13.2, and 19.8 nmol of ($\pm$)-5,10- $CH_2-H_4PteGlu$ to obtain curves (2), (3) and (4), respectively. Curve (1) was obtained upon the addition of 80 nmol of formaldehyde to an assay mixture which contained, in addition, 92.5 nmol of ($\pm$)- $H_4PteGlu$. Mixing was affected by bubbling with argon. The traces represent absorbance changes at 340 nm.

Scheme 1. Reactions involved in the conversion of formaldehyde and H_4PteGlu to 10-CHO- H_4PteGlu . Both enzymes are present in commercial preparations of 5,10- $\text{CH}_2\text{-H}_4\text{PteGlu}$ dehydrogenase.

Fig. 5. Enzymic analysis of the products formed in the reduction of sarcosine or dimethylglycine dehydrogenases with their substrates

Sarcosine and dimethylglycine dehydrogenases which had been purified by affinity chromatography were equilibrated with 40 mM-potassium barbital, pH 7.7, by passage through a Sephadex G-25 column equilibrated with this buffer. Reaction mixtures containing the enzymes were made anaerobic by the inclusion of glucose oxidase (1 unit) and catalase (2.3 μg). After replacing the air in a 1 ml cuvette fitted with a silicone rubber stopper with argon, 4 μmol of glucose was added. Assay mixtures contained, in addition: (a) 10.15 nmol of sarcosine dehydrogenase, 0.4 μmol of NAD^+ , 80 μg of formaldehyde dehydrogenase and 36 μmol of potassium barbital, pH 7.7, in 0.955 ml; (b) as (a) but with 0.125 μmol of ($\pm$)- H_4PteGlu also present in a final volume of 1.0 ml; (c) 11.26 nmol of sarcosine dehydrogenase, 2 μmol of NADP^+ , 0.04 units of 5,10- $\text{CH}_2\text{-H}_4\text{PteGlu}$ dehydrogenase, 1 μmol of cysteine, 0.125 μmol of ($\pm$)- H_4PteGlu and 36 μmol of potassium barbital, pH 7.7, in 1.0 ml; (d) 6.8 nmol of dimethylglycine dehydrogenase, formaldehyde dehydrogenase (60 μg), 0.4 μmol of NAD and 30 μmol of potassium barbital in 0.8 ml; (e) as (d) but with 0.1 μmol of ($\pm$)- H_4PteGlu present in a final volume of 0.84 ml; (f) as (c) but with 8.2 nmol of dimethylglycine dehydrogenase instead of sarcosine dehydrogenase. Other additions to the mixtures are as shown. Spectral changes were recorded on a Cary 219 spectrophotometer at 340 nm and the offset on the instrument was used to reset the baseline following the addition of ($\pm$)-5,10- $\text{CH}_2\text{-H}_4\text{PteGlu}$ in (c) and (f) and prior to the addition of formaldehyde in (a) and (d). The experiment was conducted at room temperature. Abbreviation used: Me_2Gly , dimethylglycine.

Fig. 6. Anaerobic titration of sarcosine dehydrogenase with sarcosine

Anaerobiosis was achieved as described in Fig. 5. The solution contained 9.4 nmol of sarcosine dehydrogenase enzyme-bound flavin in a final volume of 0.925 ml of 40 mM-potassium barbital, pH 7.7. Spectra shown were recorded after the addition of 1.78 (—), 3.56 (·····), 5.34 (— · — · —), and 9.49 (— — —) nmol of sarcosine. The inset shows the decrease in absorbance at 452 nm as a function of sarcosine added. Sarcosine dehydrogenase used in this experiment was obtained by chromatography on hydroxyapatite and DEAE-cellulose and corresponds to preparation B of Table 1.

Fig. 7. Anaerobic titration of dimethylglycine dehydrogenase with dimethylglycine

Anaerobiosis was achieved as described in the legend of Fig. 5. The solution contained 8.1 nmol of dimethylglycine dehydrogenase enzyme-bound flavin in a final volume of 0.925 ml of 40 mM-potassium barbital, pH 7.7. Spectra were recorded after the addition of 2.26 (—), 4.52 (— · — · —), 6.78 (·····), and 12.43 nmol of dimethylglycine (— — —). The inset shows the decrease in absorbance at 459 nm as a function of dimethylglycine added. Dimethylglycine dehydrogenase used in this experiment was obtained by affinity chromatography and corresponds to preparation A in Table 1. Abbreviation used: Me₂Gly, dimethylglycine.

lated by affinity chromatography. Only about 50% of the enzyme in this preparation was rapidly reduced by substrate, the remainder requiring up to 30 min to reach equilibrium after each successive addition of substrate during spectrophotometric titrations. Consequently, the absorbance change at 340 nm, used to calculate the amount of product released, was estimated by logarithmic extrapolation of the progress curves for the production of NAD(P)H to the time of addition of sarcosine. Enzyme concentrations were calculated assuming an absorption coefficient of $11.3 \text{ mM}^{-1} \cdot \text{cm}^{-1}$ for free FAD at 450 nm. The amount of substrate required for complete reduction of either dehydrogenase was estimated from spectrophotometric titrations (Figs. 6 and 7) and should correspond to the maximum amount of product which can be expected in this kind of experiment. It is evident from the data presented that formaldehyde is a product of the reduction of the sarcosine and dimethylglycine dehydrogenases in the absence of H₄PteGlu (Fig. 5 and Table 1). In the presence of H₄PteGlu, however, 5,10-CH₂-H₄PteGlu becomes the major product (Figs. 5c and 5f and Table 1).

In Table 1 data are presented for the dimethylglycine and sarcosine dehydrogenases purified by either affinity chromatography or by chromatography on DEAE-cellulose and hydroxylapatite, which correspond to the preparations used in experiments illustrated by Fig. 3. Reduction of sarcosine dehydrogenase by substrate gave rise to a larger proportion of anionic semiquinone in the enzyme isolated by affinity chromatography than by hydroxyapatite and DEAE-cellulose chromatography, and required correspondingly less substrate (0.59 as opposed to 0.80 mol of substrate/mol of enzyme-bound flavin). A further difference between the two sarcosine dehydrogenase preparations was reflected in their absorbance maxima (452 nm for preparation B as opposed to 457 nm for preparation A in Table 1). It is uncertain whether these differences were due to an altered proportion of isoenzymes of sarcosine dehydrogenase (Wittwer & Wagner, 1981b) or due to the difference in purification technique. Moreover, since enzyme isolations were performed during different seasons, fluctuations in the isoenzyme patterns due to dietary and seasonal effects may also have to be considered as contributing factors. Although the reasons underly-

Table 1. *Effect of H₄PteGlu on the release of products by dimethylglycine or sarcosine dehydrogenases*

The amounts of products released upon the reduction of either dimethylglycine or sarcosine dehydrogenases by their respective substrates was determined as described in the legend to Fig. 6. Substrate consumed represents mol of substrate, estimated from spectrophotometric titrations, which was required for the reduction of either enzyme per mol of enzyme bound flavin. Products released represents mol of product detected per mol of enzyme-bound flavin. The letter A designates preparations obtained by affinity chromatography with folic acid as the immobilized absorbent and the letter B designates preparations obtained by chromatography on hydroxyapatite and DEAE-cellulose. N.T., not tested (insufficient enzyme was available for spectrophotometric titration); N.D., not detected.

Enzyme preparations	(±)-H ₄ PteGlu (μM)	Substrate consumed	Products released	
			CH ₂ O	5,10-CH ₂ -H ₄ PteGlu
Dimethylglycine dehydrogenase				
A	0.0	0.81	0.83	—
	125	0.81	~0.14	0.55
B	110	N.T.	N.D.	0.61
Sarcosine dehydrogenase				
A	0.0	0.59	0.49	—
	125	0.59	N.D.	0.36
B	0.0	0.80	0.74	—
	73	0.80	~0.12	0.69

ing the different yields of semiquinone in different preparations of sarcosine dehydrogenase are at present being actively investigated, these variations are only of relevance inasmuch as they affect the yield of product which can theoretically be expected in a single turnover of the enzyme.

The results reported in Table 1 clearly indicate that both sarcosine and dimethylglycine dehydrogenases synthesize 5,10-CH₂-H₄PteGlu. The utilization of H₄PteGlu as a substrate, however, has no effect on the rate of reduction of ETF by these enzymes. This observation suggests that the release of products is not rate limiting in the reaction mechanism of the enzymes, and moreover, that the binding of H₄PteGlu to either enzyme has no effect on the rate limiting step. The synthesis of 5,10-CH₂-H₄PteGlu may depend, therefore, solely on the availability of H₄PteGlu, which would determine the incorporation of formaldehyde from the mitochondrial one-carbon cycle (MacKenzie & Frisell, 1958) into biosynthetic pathways.

The results reported here raise a question of wider relevance. The enhancement of catalytic turnover of an enzyme by a compound is clearly an inadequate criterion by which to judge its possible participation in an enzymic reaction. In essence, whether a compound is utilized as a substrate can only be ascertained by product analysis.

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