Interaction of Tetrahydrofolate and Other Folate Derivatives with Bacterial Sarcosine Oxidase†

Kalla Kvalnes-Krick and Marilyn Schuman Jorns*

Department of Biological Chemistry, Hahnemann University School of Medicine, Philadelphia, Pennsylvania 19102

Received February 5, 1987; Revised Manuscript Received July 13, 1987

ABSTRACT: Sarcosine oxidase from *Corynebacterium* sp. P-1 binds 2 mol of tetrahydrofolate/mol of enzyme (K_D = 8.8 μM). The same stoichiometry is observed with tetrahydropteroyltetraglutamate (K_D = 15.4 μM). Binding is also observed with pteroyltetraglutamate and with 5-formyltetrahydrofolate. In the case of the pteroylmonoglutamates, binding appears to be sensitive to changes in the pteridine ring since no binding is observed with 5-methyltetrahydrofolate or with folate. Sarcosine oxidase can be specifically adsorbed onto an affinity matrix prepared by coupling 5-formyltetrahydrofolate to AH-Sepharose. Tetrahydrofolate does not affect the rate of sarcosine oxidation but does block the formation of formaldehyde as a final product. In the presence of tetrahydrofolate, sarcosine oxidation is accompanied by the formation of 5,10-methylenetetrahydrofolate at a rate that exceeds the rate at which formaldehyde (or a precursor) can be released into solution and which is also considerably faster than the nonenzymic reaction of free formaldehyde with tetrahydrofolate. It is suggested that tetrahydrofolate may serve primarily to trap formaldehyde as it is formed at the active site during sarcosine oxidation. The existence of a catalytically significant binding site for tetrahydrofolate appears to be a general property of sarcosine oxidizing enzymes since similar results have previously been obtained with mammalian sarcosine dehydrogenase, an enzyme that is structurally and mechanistically very different from bacterial sarcosine oxidase.

Sarcosine oxidase catalyzes the oxidative demethylation of sarcosine to yield glycine, formaldehyde, and hydrogen peroxide (eq 1). Sarcosine oxidase is produced as an inducible

$$CH_3NHCH_2COOH + O_2 + H_2O \rightarrow H_2CO + NH_2CH_2COOH + H_2O_2 \quad (1)$$

enzyme when *Corynebacterium* sp. P-1 is grown with sarcosine as source of carbon and nitrogen (Kvalnes-Krick & Jorns, 1986). The enzyme contains 1 mol of covalently bound flavin [8α-(N^3-histidyl)-FAD][1] plus 1 mol of noncovalently bound flavin (FAD) per mol of enzyme (M_r 168 000). The noncovalent flavin appears to function as a dehydrogenase flavin, accepting electrons from sarcosine. The electrons are transferred to the covalent flavin, which, acting as an oxidase flavin, reduces oxygen to hydrogen peroxide. Sarcosine oxidase is composed of four nonidentical subunits (M_r 100 000, 42 000, 20 000, and 6000). The covalent flavin is attached to the subunit with a molecular weight of 42 000. The presence of four dissimilar subunits is rather unusual, but recent studies show that the subunit composition is not a proteolytic artifact but rather a genuine property of the enzyme as it exists in vivo (Kvalnes-Krick & Jorns, 1986). Another sarcosine oxidase with similar properties has been isolated from *Corynebacterium* sp. U-96 (Kvalnes-Krick & Jorns, 1986; Jorns, 1985; Hayashi et al., 1980, 1982; Suzuki, 1981).

The presence of both covalent and noncovalent flavin distinguishes sarcosine oxidase from *Corynebacteria* sp. P-1 and sp. U-96 from all other known flavoproteins. The complex quaternary structure observed for the corynebacterial enzymes is also unique as compared with other sarcosine oxidizing enzymes. For example, sarcosine dehydrogenase from rat liver is a monomeric protein (M_r 94 000) (Sato et al., 1981) and contains only covalently bound flavin [1 mol of 8α-(N^3-histidyl)-FAD/mol of enzyme] (Cook et al., 1984; Sato et al., 1981; Patek & Frisell, 1972). The mammalian enzyme has been shown to bind tetrahydrofolate (H_4folate) and in the presence of H_4folate forms 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4folate), instead of formaldehyde, as a product of sarcosine oxidation (Wittwer & Wagner, 1981; Steenkamp & Husain, 1982; Porter et al., 1985). The studies described in this paper were conducted to determine whether the presence of a catalytically significant binding site for H_4folate might be a general property of sarcosine oxidizing enzymes that could be detected with sarcosine oxidase from *Corynebacterium* sp. P-1, an enzyme that is structurally and mechanistically very different from mammalian sarcosine dehydrogenase.

Experimental Procedures

Materials. AH-Sepharose-4B was obtained from Pharmacia. DEAE-cellulose (DE-52) was from Whatman. Bio-Gel P-10 and 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride (EDAC) were purchased from Bio-Rad. 5-Formyltetrahydrofolate (5-HCO-H_4folate) was obtained from Lederle Labs or from Sigma. Folate, 5-methyltetrahydrofolate (5-CH_3-H_4folate), 5,10-CH_2-H_4folate dehydrogenase, formaldehyde dehydrogenase, NAD^+, and $NADP^+$ were also obtained from Sigma. Sodium borohydride was purchased from Fisher. NADH and NADH peroxidase were from Boehringer Mannheim Biochemicals. NADPH was purchased from Pabst Labs. Pteroyltetraglutamate ($PteGlu_4$)

† This work was supported in part by Grant GM 31704 from the National Institutes of Health.

* Author to whom correspondence should be addressed.

[1] Abbreviations: FAD, flavin adenine dinucleotide; H_4folate, tetrahydrofolate; 5,10-CH_2-H_4folate, 5,10-methylenetetrahydrofolate; $PteGlu_4$, pteroyltetraglutamate; $H_4PteGlu_4$, tetrahydropteroyltetraglutamate; 5-HCO-H_4folate, 5-formyltetrahydrofolate; 5-CH_3-H_4folate, 5-methyltetrahydrofolate; DEAE, diethylaminoethyl; NAD, nicotinamide adenine dinucleotide; NADP, NAD phosphate; NADH, reduced NAD; NADPH, reduced NADP.

0006-2960/87/0426-7391$01.50/0

was obtained from Dr. B. Schircks Laboratories.

Isolation and Assay of Sarcosine Oxidase. Sarcosine oxidase was purified from *Corynebacterium* sp. P-1 as previously described (Kvalnes-Krick & Jorns, 1986). Protein concentration was determined from the absorbance of the purified enzyme at 280 nm by using the extinction coefficient ($E^{1\%}$ = 13.1) reported by Suzuki (1981). Enzyme flavin concentration was determined by using the extinction coefficient (ϵ_{450} = 12.7 × 10^3 M^{-1} cm^{-1}) reported by Kvalnes-Krick and Jorns (1986). Sarcosine oxidase activity was measured at 25 °C by using a NADH peroxidase coupled assay. Reactions were initiated by adding 7.6 pmol of sarcosine oxidase to 360 μL of 0.33 M potassium phosphate buffer, pH 7.0, containing 0.12 M sarcosine, 0.14 mM NADH, and NADH peroxidase (0.022 units). For experiments with H_4folate the assay mixture also included β-mercaptoethanol (10 mM), which did not affect activity. NADH oxidation was monitored by following the decrease in absorbance at 340 nm (ϵ_{340} = 6.2 × 10^3 M^{-1} cm^{-1}). Reaction rates were corrected for a very small blank reaction (A_{340} = 0.006/min). A unit of activity was defined as the production of 1 μmol of H_2O_2/min.

Assay of Formaldehyde Dehydrogenase Activity in Crude Extracts of Corynebacterium sp. P-1. Cells were lysed as previously described (Kvalnes-Krick & Jorns, 1986). The supernatant obtained after centrifugation was assayed for formaldehyde dehydrogenase activity at 37 °C. Assays were initiated by adding crude extract (100 μL) to 500 μL of 50 mM potassium phosphate buffer, pH 7.5, containing 1.0 mM formaldehyde plus 0.6 mM NAD^+ or 0.6 mM $NADP^+$. For some experiments, as indicated, the reaction buffer also contained 1.0 mM glutathione, 1.0 mM coenzyme A, or 1.0 mM dithioerythritol. Reactions were monitored at 340 nm.

Preparation of Folate Derivatives. H_4folate was prepared by reduction of folate with sodium borohydride as described by Steenkamp and Husain (1982). The concentration of H_4folate was calculated from its absorption at 297 nm (ϵ_{297} = 2.9 × 10^4 M^{-1} cm^{-1}) (Kallen, 1971) or after conversion to 5,10-methylenetetrahydrofolate (ϵ_{355} = 2.5 × 10^4 M^{-1} cm^{-1}) according to the method of Scrimgeour (1980). $PteGlu_4$ was converted to tetrahydropteroyltetraglutamate ($H_4PteGlu_4$) by reaction with borohydride, similar to the procedure used with folate except that DEAE chromatography was omitted because the conversion was quantitative and pure product precipitated during the acidification step. The concentration of $H_4PteGlu_4$ was determined by the same methods used for H_4folate. 5,10-CH_2-H_4folate was synthesized by reaction of H_4folate (0.49 mM) with a 2-fold excess of formaldehyde in 50 mM potassium phosphate buffer, pH 7.5, for 15 min at 0 °C under anaerobic conditions. The concentration of 5,10-CH_2-H_4folate was calculated from its absorption at 292 nm (ϵ_{292} = 3.3 × 10^4 M^{-1} cm^{-1}) (Kallen, 1971) or by the amount of $NADP^+$ converted to NADPH in the presence of 5,10-CH_2-H_4folate dehydrogenase (Ramasastri & Blakely, 1964).

Affinity Chromatography. 5-HCO-H_4folate was coupled to AH-Sepharose 4B according to the procedure described by Wittwer and Wagner (1981). Sarcosine oxidase (19.5 nmol) was applied to a 5-HCO-H_4folate–Sepharose column (0.4 × 1.6 cm) equilibrated with 0.02 M potassium phosphate, pH 7.2. After 2 h at 3 °C, the column was washed with 0.02 M potassium phosphate, pH 7.2 (12.2 mL) and then with 0.02 M potassium phosphate, pH 7.2, containing 1.0 M KCl (10.0 mL). Sarcosine oxidase eluted when the latter buffer was augmented with 5-HCO-H_4folate (0.02 M).

Determination of Binding Constants and Binding Stoichiometry. Binding parameters were determined by gel filtration according to the method of Hummel and Dreyer (1962). All operations were conducted under yellow light. Sarcosine oxidase (6.5–7.0 nmol) was incubated under anaerobic conditions for 30 min at 3 °C in 250 μL of 50 mM potassium phosphate buffer, pH 7.5, containing 30 mM β-mercaptoethanol plus a given amount of a particular folate derivative. (For some experiments, as indicated, β-mercaptoethanol was omitted.) The sample was then anaerobically transferred to a Bio-Gel P-10 column (0.7 × 34 cm) equilibrated just before use with the same anaerobic buffer. The buffer reservoir was continually flushed with argon during chromatography. Column fractions (350 μL) were collected, and absorbance values were immediately measured at a wavelength corresponding to the absorption maximum of the particular folate derivative. The data were analyzed according to the method of Ackers (1973) to determine the number of moles of enzyme-bound folate derivative.

Results

Fate of Formaldehyde Produced during Sarcosine Oxidation. No odor of formaldehyde is detectable when *Corynebacterium* sp. P-1 is grown with sarcosine as source of carbon and nitrogen except when the culture goes into stationary phase. This suggested that the formaldehyde produced from sarcosine oxidation in growing cultures did not accumulate but was further metabolized. To determine whether the formaldehyde might undergo oxidation to formic acid or to the corresponding thioester, crude extracts of *Corynebacterium* sp. P-1 were tested for formaldehyde dehydrogenase activity. No activity was detected with NAD^+ or $NADP^+$ as electron acceptor in the presence or absence of various thiols (glutathione, coenzyme A, dithioerythritol).

As an alternate possibility, the further metabolism of formaldehyde might require entry into the one-carbon pool via incorporation into H_4folate. Preliminary evidence to evaluate this hypothesis was sought in studies to determine whether sarcosine oxidase might possess a binding site for H_4folate. To this end, the enzyme was applied to a 5-HCO-$H_4PteGlu$ affinity column prepared by coupling 5-HCO-H_4-folate to AH-Sepharose. 5-HCO-H_4folate was selected for these studies because the compound is stable, unlike H_4folate. Sarcosine oxidase bound to the column as a narrow, intense yellow band and was not eluted even when the column was washed with buffer containing 1.0 M KCl. Elution did occur when 20 mM 5-HCO-H_4folate was added to the buffer. The results indicate that sarcosine oxidase does contain a H_4folate binding site and suggested that the enzyme might be important in the subsequent metabolism of formaldehyde. It should be noted that affinity chromatography of pure sarcosine oxidase did not affect its specific activity, as judged by analysis of the eluted enzyme. However, only one-third of the applied enzyme was recovered. This low yield is due to irreversible absorption on the column, as evidenced by the residual yellow color. A similar problem with this affinity matrix has been reported for other H_4folate-binding proteins (Steenkamp & Husain, 1982).

Stoichiometry and Binding Constants for Complexes Formed with Sarcosine Oxidase and Folate Derivatives. Binding parameters were measured by gel filtration according to the method of Hummel and Dreyer (1962). Since H_4folate is sensitive to light and air oxidation, experiments were conducted under yellow light and anaerobic conditions using buffers containing 30 mM β-mercaptoethanol. Prior to chromatography sarcosine oxidase was mixed with the desired concentration of H_4folate and incubated for 30 min. The data obtained with H_4folate yield a straight line when plotted ac-

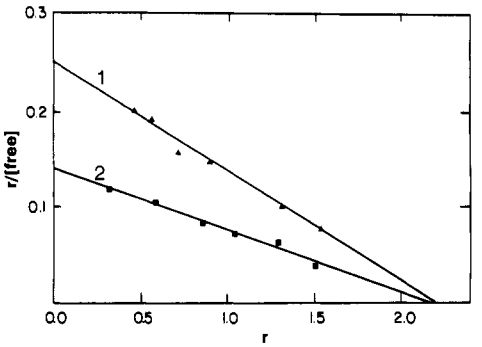

FIGURE 1: Binding of tetrahydrofolate and tetrahydropteroyltetraglutamate to sarcosine oxidase. Sarcosine oxidase was subjected to anaerobic chromatography on Bio-Gel P-10, as detailed under Experimental Procedures, in the presence of various amounts of (6*R*,*S*)-H_4folate (6.0–40.0 μM) or (6*R*,*S*)-$H_4PteGlu_4$ (5.4–79.4 μM). The results obtained with H_4folate and $H_4PteGlu_4$ are plotted according to the method of Scatchard (1949) in lines 1 and 2, respectively. [Free] refers to the concentration of free (6*S*)-H_4folate or (6*S*)-$H_4PteGlu_4$, and *r* is the number of moles of bound ligand per mole of enzyme.

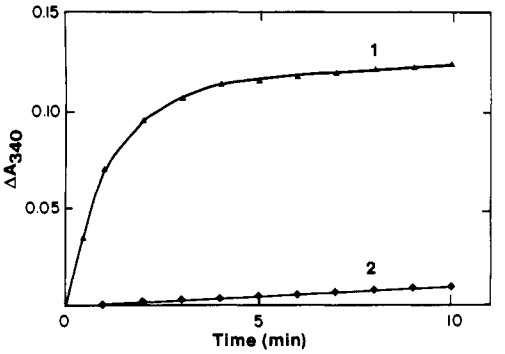

FIGURE 2: Effect of tetrahydrofolate on formaldehyde formation during sarcosine oxidation. A solution containing sarcosine oxidase (21.4 μM with respect to flavin) and formaldehyde dehydrogenase (80 μg) plus 0.4 mM NAD^+ in 1.0 mL of 50 mM potassium phosphate buffer, pH 7.5, was made anaerobic in a specially constructed cuvette as previously described (Jorns & Hersh, 1975). Sarcosine (4.0 mM) was added from a sidearm, and absorbance changes were monitored at 340 nm. The results are shown in curve 1 where A_{340} at $t = 0$ corresponds to the A_{340} observed after the small, initial, rapid decrease in absorption due to flavin reduction. The results shown in curve 2 were obtained when the experiment was repeated in the presence of 0.12 mM (6*R*,*S*)-H_4folate. Both reactions were conducted at 25 °C.

cording to the method of Scatchard (1949) (Figure 1). From this plot it is estimated that sarcosine oxidase binds 2.19 mol of H_4folate/mol of enzyme. The enzyme appears to exhibit a similar affinity for both ligands. A value of 8.8 μM is calculated for the dissociation constant. In this calculation it is assumed that the enzyme only binds the natural 6*S* isomer in the (6*R*,*S*)-H_4folate used in these studies. It should be noted that β-mercaptoethanol, used to prevent oxidation of tetrahydrofolate, also reduces the flavins in sarcosine oxidase. The reaction is relatively slow, but complete reduction does occur during the 30-min incubation before chromatography. This means that the complex formed in these experiments involved fully reduced rather than oxidized enzyme.

To determine whether different binding properties might be observed for oxidized versus reduced enzyme, additional experiments were conducted with 5-HCO-H_4folate since this derivative is stable in the absence of β-mercaptoethanol. In studies conducted under anaerobic conditions, similar binding of (6*R*,*S*)-5-HCO-H_4folate (13.6 μM) was observed in the presence or absence of β-mercaptoethanol. The results indicate that binding is unaffected by the redox state of the enzyme. At this ligand concentration, 0.44 mol of 5-HCO-H_4folate is bound per mol of enzyme. Under comparable conditions, 0.95 mol of H_4folate is bound. This suggests that the 5-formyl derivative is a somewhat weaker ligand. No binding was detected with oxidized or reduced enzyme and (6*R*,*S*)-5-CH_3-H_4folate (8.4–22.5 μM) or with oxidized enzyme and folate (23.5 μM).

Intracellularly, folate derivatives are present mainly as polyglutamate derivatives. In studies with various folate-dependent enzymes, it has been found that the pteroylpolyglutamate derivatives are more tightly bound than the corresponding monoglutamates [see Matthews and Baugh (1980) and references cited therein]. Since folate tetraglutamate derivatives predominate in corynebacteria (Foo et al., 1980), we measured the binding of $H_4PteGlu_4$ to reduced sarcosine oxidase. The binding stoichiometry (2.15 mol of $H_4PteGlu_4$/mol of enzyme), determined from a Scatchard plot (Figure 1), is very similar to that observed for H_4folate, but the dissociation constant for the tetraglutamate derivative (K_D = 15.4 μM) is about 2-fold larger. The corresponding oxidized tetraglutamate derivative, $PteGlu_4$, also functions as a ligand for sarcosine oxidase. This differs from results obtained with the monoglutamate analogues where binding was not detectable with folate. The extent of binding observed in a study conducted with 8.7 μM $PteGlu_4$ and oxidized enzyme (0.35 mol of $PteGlu_4$/mol of enzyme) is similar to that observed at the same concentration of (6*R*,*S*)-$H_4PteGlu_4$ and reduced enzyme.

Effect of Tetrahydrofolate on Catalysis by Sarcosine Oxidase. We have developed a rapid spectrophotometric assay for sarcosine oxidase. Enzyme activity is measured by monitoring the disappearance of NADH at 340 nm in a NADH peroxidase coupled assay. Reaction rates (measured at saturating sarcosine and ambient oxygen concentrations) were unaffected upon addition of (6*R*,*S*)-H_4folate (100 μM), similar to results reported for mammalian sarcosine dehydrogenase (Porter et al., 1985; Steenkamp & Husain, 1982).

Other studies were conducted to determine whether H_4folate might affect formaldehyde production. When excess sarcosine is added to an anaerobic solution of sarcosine oxidase, both of the flavins in the enzyme are rapidly reduced (Kvalnes-Krick & Jorns, 1986). Since the enzyme cannot turn over, the amount of substrate oxidized should be stoichiometric with enzyme flavin. At 340 nm, the reduction of the enzyme is accompanied by a small, rapid decrease in absorbance. When sarcosine is added to the enzyme in the presence of NAD^+ and formaldehyde dehydrogenase, the initial rapid decrease in absorbance at 340 nm is followed by a slow increase in absorbance due to NADH formation (Figure 2). The amount of formaldehyde formed under these conditions is stoichiometric with enzyme flavin (0.94 mol/mol of flavin). An identical kinetic trace for NADH formation was obtained by the direct addition of a similar amount of formaldehyde to reaction mixtures containing only NAD^+ and formaldehyde dehydrogenase (data not shown). This shows that the observed rate of NADH production in the sarcosine-initiated reaction is not limited by the rate of release of formaldehyde from sarcosine oxidase. No formaldehyde was detected when the experiment was repeated in the presence of a small excess of H_4folate (3 mol of (6*S*)-H_4folate/mol of flavin) (Figure 2). The results show that although H_4folate does not affect the rate of sarcosine oxidation, it does cause a change in the reaction products.

Further studies were conducted to determine whether sarcosine oxidation in the presence of H_4folate yields 5,10-CH_2-H_4folate in place of formaldehyde. In these experiments sarcosine was added under anaerobic conditions to reaction

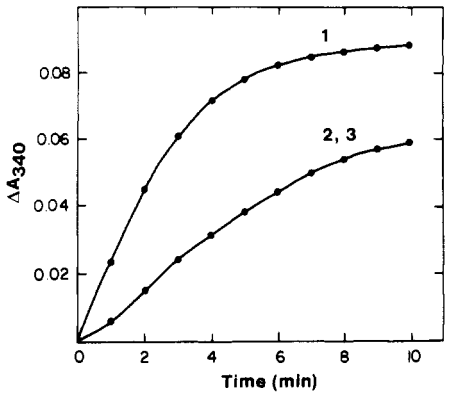

FIGURE 3: Formation of 5,10-methylenetetrahydrofolate during sarcosine oxidation in the presence of tetrahydrofolate. A cuvette containing sarcosine oxidase (20.7 μM with respect to flavin), 5,10-CH_2H_4folate dehydrogenase (0.04 units), and 2.0 mM NAD^+ plus 1.0 mM cysteine in 1.0 mL of 50 mM potassium phosphate buffer, pH 7.5, was made anaerobic. (6*R*,*S*)-H_4folate (0.10 mM) was then added to the anaerobic buffer. The reaction shown in curve 1 was initiated at 25 °C by addition of sarcosine (4.0 mM) from a sidearm. The A_{340} at $t = 0$ is defined as described in the legend to Figure 3. Curves 2 and 3 superimpose. These reactions were initiated by adding formaldehyde (20 μM) from a sidearm to an anaerobic solution (1.0 mL) containing the same components as described above except that sarcosine oxidase was omitted in one of the reactions. An initial rapid decrease in A_{340} was not observed in either of these two reactions.

mixtures containing sarcosine oxidase (20.7 μM), H_4folate, $NADP^+$, and 5,10-CH_2-H_4folate dehydrogenase. Following an initial rapid decrease in absorption at 340 nm due to reduction of sarcosine oxidase, we observed a slower increase in absorbance at this wavelength due to NADPH formation (Figure 3). The amount of 5,10-CH_2-H_4folate detected in this assay is nearly stoichiometric with enzyme flavin (1.2 mol/mol of flavin). A similar kinetic trace for NADPH formation was obtained when 5,10-CH_2-H_4folate (23 μM) was added to reaction mixtures containing only 5,10-CH_2-H_4folate dehydrogenase plus $NADP^+$ (data not shown). On the other hand, a much slower reaction was observed if formaldehyde (20 μM) was added to reaction mixtures containing H_4folate, 5,10-CH_2-H_4folate dehydrogenase, and $NADP^+$ (Figure 3). The results show that free formaldehyde does react slowly with tetrahydrofolate under the assay conditions to form 5,10-CH_2-H_4folate. However, this slow reaction cannot account for the 5,10-CH_2-H_4folate generated from sarcosine in the presence of H_4folate and sarcosine oxidase. The results indicate that 5,10-CH_2-H_4folate is formed in a reaction catalyzed by sarcosine oxidase rather than via reaction of free H_4folate with formaldehyde released into solution.

It was of interest to determine whether sarcosine oxidase would also catalyze the formation of 5,10-CH_2-H_4folate using exogenous formaldehyde instead of formaldehyde (or a precursor) generated during sarcosine oxidation. In one experiment, formaldehyde (20 μM) was added to reaction mixtures containing sarcosine oxidase (20 μM with respect to flavin), H_4folate, 5,10-CH_2-H_4folate dehydrogenase, and $NADP^+$. (For comparison with previous experiments, the formaldehyde added was comparable to the amount that would be generated from the anaerobic reaction of the same amount of enzyme with sarcosine.) A slow rate of NADPH formation was observed. The rate was unaffected by omitting sarcosine oxidase (Figure 3). Since H_4folate might react with the imine formed from the oxidation of sarcosine, the experiment was repeated in the presence of glycine. Addition of glycine (20 mM) did not affect the rate of NADPH formation (data not shown). The results indicate that, under the conditions tested, sarcosine oxidase can catalyze the formation of 5,10-CH_2-H_4folate only when formaldehyde (or a precursor) is generated during sarcosine oxidation.

Discussion

Although bacterial sarcosine oxidase binds 2 mol of H_4-folate/mol of enzyme, the oxidation of sarcosine does not depend on the presence of H_4folate nor does H_4folate affect the rate of sarcosine oxidation. On the other hand, H_4folate blocks the formation of formaldehyde as a final product of sarcosine oxidation. In the presence of H_4folate, sarcosine oxidation is accompanied by the formation of 5,10-CH_2-H_4-folate at a rate that is considerably faster than that observed for the nonenzymic reaction of free formaldehyde with H_4-folate. This rate acceleration is not observed if sarcosine is replaced by exogenous formaldehyde in the presence or absence of glycine. The results indicate that in the sarcosine-initiated reaction H_4folate reacts with formaldehyde (or a precursor) at a rate which exceeds the rate at which formaldehyde can be released into solution. In this regard H_4folate appears to act simply to trap formaldehyde as it is formed at the active site. A similar role has been suggested for H_4folate in the case of serine hydroxymethylase (Chen & Schirch, 1972).

The effect of H_4folate on catalysis by sarcosine oxidase is very similar to that reported for sarcosine dehydrogenase from rat liver (Wittwer & Wagner, 1981; Steenkamp & Husain, 1982; Porter et al., 1985). The bacterial enzyme exhibits a complex quaternary structure and contains two nonequivalent flavins that have different roles in catalysis. The mammalian enzyme is a much simpler protein containing only one subunit and one flavin. The results suggest that the ability to transfer a one-carbon unit from sarcosine to H_4folate is a general property retained by sarcosine oxidizing enzymes despite major differences in protein structure and coenzyme composition.

Studies with various folate derivatives suggest that the affinity of sarcosine oxidase for pteroylmonoglutamates is quite sensitive to structural changes in the pteridine ring. This is evidenced by the fact that H_4folate and 5-HCO-H_4folate are tightly bound to the enzyme, whereas no binding is detectable with 5-CH_3-H_4folate or folate. This sensitivity is not apparent with pteroyltetraglumates where similar binding is observed with $H_4PteGlu_4$ and $PteGlu_4$. This difference between the monoglutamates and the tetraglutamates is puzzling since $H_4PteGlu_4$ and H_4folate exhibit the same binding stoichiometry and exhibit dissociation constants that differ only by a factor of 2. An explanation for this behavior must await studies with a wider range of folate derivatives.

That H_4folate and $H_4PteGlu_4$ bind to sarcosine oxidase with a 2:1 stoichiometry is noteworthy since, although 2 mol of sarcosine are required for complete reduction, only one of the two nonequivalent flavins in the enzyme appears to act as an input site for electrons from sarcosine (Jorns, 1985; Kvalnes-Krick & Jorns, 1986). The enzyme appears to have only a single binding site for sarcosine, as suggested by the fact that complete inactivation is observed upon modification of a single histidine residue with diethyl pyrocarbonate in a reaction that is blocked by acetate, a competitive inhibitor with respect to sarcosine (Hayashi et al, 1983). Although binding data suggest that the two folate sites are equivalent, it is conceivable that only one site is functional as a formaldehyde trap. On the other hand, the presence of two functional folate sites, flanking the sarcosine site, would eliminate the need to displace 5,10-CH_2-H_4folate in the half-reduced enzyme with fresh H_4folate. This might be an advantage with respect to the efficiency of formaldehyde trapping during the conversion of half-reduced to fully reduced enzyme. Studies are currently in progress to determine which of the four nonidentical sub-

units in sarcosine oxidase is involved in the binding of folates and sarcosine.

ACKNOWLEDGMENTS

We thank Juanita Yee-Foon Peck for excellent technical assistance.

REFERENCES

Ackers, G. K. (1973) *Methods Enzymol. 27*, 441–455.
Chen, M. S., & Schirch, L. (1972) *J. Biol. Chem. 248*, 3631–3635, 7979–7984.
Cook, R. J., Misono, K. S., & Wagner, C. (1984) *J. Biol. Chem. 259*, 12475–12480.
Foo, S. K., Cichowicz, D. S., & Shane, B. (1980) *Anal. Biochem. 107*, 109–115.
Hayashi, S., Nakamura, S., & Suzuki, M. (1980) *Biochem. Biophys. Res. Commun. 96*, 924–930.
Hayashi, S., Suzuki, M., & Nakamura, S. (1982) *Biochem. Int. 4*, 617–620.
Hayashi, S., Suzuki, M., & Nakamura, S. (1983) *J. Biochem. (Tokyo) 94*, 551–558.
Hummel, J. P., & Dreyer, W. J. (1962) *Biochim. Biophys. Acta 63*, 530–532.
Jorns, M. S. (1985) *Biochemistry 24*, 3189–3194.
Jorns, M. S., & Hersh, L. B. (1975) *J. Biol. Chem. 250*, 3620–3628.
Kallen, R. G. (1971) *Methods Enzymol. 18*, 705–716.
Kvalnes-Krick, K., & Jorns, M. S. (1986) *Biochemistry 25*, 6061–6069.
Matthews, R. G., & Baugh, C. M. (1980) *Biochemistry 19*, 2040–2045.
Patek, D. R., & Frisell, W. R. (1972) *Arch. Biochem. Biophys. 150*, 347–354.
Porter, D. H., Cook, R. J., & Wagner, C. (1985) *Arch. Biochem. Biophys. 243*, 396–407.
Ramasastri, B. V., & Blakley, R. L. (1964) *J. Biol. Chem. 239*, 106–111.
Sato, M., Ohnishi, N., & Yagi, K. (1981) *Biochem. Int. 3*, 89–92.
Scatchard, G. (1949) *Ann. N.Y. Acad. Sci. 51*, 660–672.
Scrimgeour, K. G. (1980) *Methods Enzymol. 66*, 517–523.
Steenkamp, D. J., & Husain, M. (1982) *Biochem. J. 203*, 707–715.
Suzuki, M. (1981) *J. Biochem. (Tokyo) 89*, 599–607.
Wittwer, A. J., & Wagner, C. (1981) *J. Biol. Chem. 256*, 4102–4108.

Infrared Studies of Fully Hydrated Unsaturated Phosphatidylserine Bilayers. Effect of Li^+ and Ca^{2+} †

H. L. Casal,* A. Martin, and H. H. Mantsch
Division of Chemistry, National Research Council of Canada, Ottawa, Canada K1A 0R6
F. Paltauf
Institut für Biochemie und Lebensmittelchemie, Technische Universität Graz, A-8010 Graz, Austria
H. Hauser
Laboratorium für Biochemie, ETH-Zentrum, CH-8092 Zürich, Switzerland
Received March 18, 1987; Revised Manuscript Received June 24, 1987

ABSTRACT: Infrared spectroscopy has been used to characterize the thermal-phase behavior of fully hydrated 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine (POPS) and 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine (DOPS) as well as their interaction with Li^+ and Ca^{2+}. The order–disorder transition of POPS–NH_4^+ is at 17 °C; in the presence of Li^+ a POPS–Li^+ complex is formed, and the transition temperature of this complex is 40 °C. DOPS–NH_4^+ has an order–disorder transition at −11 °C, and unlike POPS the addition of Li^+ has no effect on the thermal behavior of DOPS–NH_4^+. This indicates that the binding of Li^+ to DOPS is negligible or very weak. Li^+ binds to the phosphate and carboxylate groups of POPS, and as a result these groups lose their water of hydration. Li^+ binding induces a conformational change, probably in the glycerol backbone of POPS; however, the conformation of the two P–O ester bonds remains gauche–gauche as in POPS–NH_4^+. Both POPS and DOPS form crystalline complexes with Ca^{2+}. As a result of Ca^{2+} binding to the phosphate, this group loses its water of hydration and there is a conformational change in the P–O ester bonds from gauche–gauche to antiplanar–antiplanar. In contrast to the POPS–Li^+ complex, the carboxylate group remains hydrated in the Ca^{2+} complexes. Furthermore, in these PS–Ca^{2+} complexes a new hydrogen bond is formed between one of the ester C=O groups and probably water. Such a situation is not found in the NH_4^+ and Li^+ salts of phosphatidylserine.

The study of the interaction of metal ions with membrane lipids continues to be a subject of considerable interest. Such studies are generally aimed at advancing our understanding of the basic properties of lipid assemblies and, in the case of Li^+ and Ca^{2+} binding to PS,[1] possible physiological implications are of interest (Schou, 1976; Papahadjopoulos, 1978; Wilschut et al., 1981; Ohki, 1982). The macroscopic properties of both Li^+ and Ca^{2+} complexes with different saturated phosphatidylserines (PS) have been investigated by physical techniques such as calorimetry and X-ray diffraction (Jacobson

† Issued as NRCC Publication No. 28178. This work was supported by the Swiss National Science Foundation (Grant 3,579-0.84).

[1] Abbreviations: PS, phosphatidylserine; DMPS, 1,2-dimyristoyl-*sn*-glycero-3-phospho-L-serine; POPS, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-L-serine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phospho-L-serine; GPS, *sn*-glycero-3-phospho-L-serine; Tes, 2-[[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino]ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

0006-2960/87/0426-7395$01.50/0 Published 1987 by the American Chemical Society