Reynolds, M. A., Oppenheimer, N. J., & Kenyon, G. L. (1983) *J. Am. Chem. Soc. 105*, 6663–6667.
Rubio, V., Britton, H. G., Grisolia, S., Sproat, B. S., & Lowe, G. (1981) *Biochemistry 20*, 1969–1974.
Samuel, C. E., D'Ari, L., & Rabinowitz, J. C. (1970) *J. Biol. Chem. 245*, 5115–5121.
Scott, J. M. (1980) *Methods Enzymol. 66*, 657–660.
Shafer, J. A., Chiancone, E., Yielding, K. L., & Antonini, E. (1972) *Eur. J. Biochem. 28*, 528–532.
Sly, W. S., & Stadtman, E. R. (1963) *J. Biol. Chem. 238*, 2936–2647.
Smithers, G. W., Jahansouz, H., Kofron, J. L., Himes, R. H., & Reed, G. H. (1987) *Biochemistry 26*, 3943–3948.
Staben, C., Whitehead, T. R., & Rabinowitz, J. C. (1987) *Anal. Biochem. 162*, 257–264.
Stewart, J. M., & Young, J. D. (1984) *Solid Phase Peptide Synthesis*, 2nd ed., pp 1–107, Pierce Chemical Co., Rockford, IL.
Strong, W., Joshi, G., Lura, R., Muthukumaraswamy, N., & Schirch, V. (1987) *J. Biol. Chem. 262*, 12519–12525.
Temple, C., & Montgomery, J. A. (1984) in *Folates and Pterins* (Blakley, R. L., & Benkovic, S. J., Eds.) Vol. 1, pp 62–86, Wiley, New York.
Tipton, P. A., & Cleland, W. W. (1988a) *Biochemistry 27*, 4317–4325.
Tipton, P. A., & Cleland, W. W. (1988b) *Biochemistry 27*, 4325–4331.
Trams, E. G. (1967) *J. Lipid Res. 8*, 698.
Villafranca, J. J., & Raushel, F. M. (1980) *Annu. Rev. Biophys. Bioeng. 9*, 363–392.
von der Saal, W., Anderson, P. M., & Villafranca, J. J. (1985) *J. Biol. Chem. 260*, 14993–14997.
Wendland, M. F., Stevens, T. H., Buttlaire, D. H., Everett, G. W., & Himes, R. H. (1983) *Biochemistry 22*, 819–826.
Williams, S. P., & Bridger, W. A. (1987) *Biochemistry 26*, 4483–4487.
Wimmer, M. J., Rose, I. A., Powers, S. G., & Meister, A. (1979) *J. Biol. Chem. 254*, 1854–1859.
Yeh, Chih-hsun, Y., Hanna, D. H., Everett, G. W., & Himes, R. H. (1988) *Biochem. J. 251*, 89–93.

# Bacterial Sarcosine Oxidase: Identification of Novel Substrates and a Biradical Reaction Intermediate†

Hans-Dieter Zeller,‡ Russ Hille,§ and Marilyn Schuman Jorns*,‡

*Department of Biological Chemistry, Hahnemann University School of Medicine, Philadelphia, Pennsylvania 19102, and Department of Physiological Chemistry, The Ohio State University, Columbus, Ohio 43210*

*Received December 21, 1988; Revised Manuscript Received March 15, 1989*

ABSTRACT: Corynebacterial sarcosine oxidase contains both covalently and noncovalently bound FAD and forms complexes with various heterocyclic carboxylic acids (D-proline and 2-furoic, 2-pyrrolecarboxylic, and 2-thiophenecarboxylic acids). 2-Furoic acid, a competitive inhibitor with respect to sarcosine, selectively perturbs the absorption spectrum of the noncovalent flavin, suggesting that the enzyme has a single sarcosine binding site near the noncovalent flavin. Several heterocyclic amines have been identified as new substrates for the enzyme. Similar reactivity is observed with L-proline and L-pipecolic acid whereas L-2-azetidinecarboxylic acid is less reactive. Turnover with L-proline is slow (TN = 4.4 $min^{-1}$) as compared with sarcosine (TN = 1000 $min^{-1}$). Anaerobic reduction of the enzyme with heterocyclic amine substrates at pH 8.0 occurs as a biphasic reaction. A similar long-wavelength intermediate is formed in the initial fast phase of each reaction and then decays in a slower second phase to yield 1,5-dihydroFAD. The slow phase is not kinetically significant during aerobic turnover at pH 8.0 and is absent when the anaerobic reactions are conducted at pH 7.0. EPR and other studies at pH 7.0 show that the long-wavelength species is a half-reduced form of the enzyme (1 electron/substrate-reducible flavin) containing 0.9 mol of flavin radical/mol of substrate-reducible flavin. This biradical intermediate exhibits an absorption spectrum similar to that expected for a 50:50 mixture of red anionic and blue neutral flavin radicals. A similar long-wavelength species is observed during titration of the enzyme with sarcosine and other reductants. Studies with L-proline suggest that reduction of the enzyme involves initial transfer of two electrons to the noncovalent flavin. The covalent flavin is not required and can be complexed with sulfite without affecting the rate of electron transfer. The initial half-reduced form of the enzyme appears to be rapidly converted to the biradical form via comproportionation of the reduced noncovalent flavin with the oxidized covalent flavin.

Sarcosine oxidase from *Corynebacterium* sp. P-1 is an inducible enzyme that is synthesized in large amounts (3% of total protein) when the organism is grown with sarcosine as the source of carbon and nitrogen (Kvalnes-Krick & Jorns, 1986). The enzyme catalyzes the oxidative demethylation of sarcosine to yield formaldehyde, glycine, and hydrogen peroxide. The enzyme binds tetrahydrofolate (2 mol/mol of enzyme). The presence of tetrahydrofolate does not affect the rate of sarcosine oxidation, but 5,10-methylenetetrahydrofolate is formed as a reaction product in place of formaldehyde (Kvalnes-Krick & Jorns, 1987). Sarcosine oxidase contains both covalently bound flavin adenine dinucleotide (FAD)[1]

† This work was supported in part by Grants GM 31704 (M.S.J.) and AR 38917 (R.H.) from the National Institutes of Health.
* To whom correspondence should be addressed.
‡ Hahnemann University School of Medicine.
§ The Ohio State University.

0006-2960/89/0428-5145$01.50/0 

[8α-($N^3$-histidyl)FAD] and noncovalently bound FAD. The enzyme is composed of four nonidentical subunits ($M_r$ 100000, 42000, 20000, and 6000). The covalent flavin is attached to the subunit with a molecular weight of 42000 (Kvalnes-Krick & Jorns, 1986). The presence of both covalent and noncovalent flavin distinguishes this enzyme from most other known flavoproteins. Our previous studies have suggested that the noncovalent flavin acts as an entry port for electrons from sarcosine which are then transferred to the covalent flavin. The covalent flavin appears to function as an exit port for electrons to oxygen (Jorns, 1985; Kvalnes-Krick & Jorns, 1986).

In this paper, we identify new inhibitors and substrates for sarcosine oxidase which have proved useful in probing the reaction mechanism of the enzyme. Studies with inhibitors provide evidence for a single sarcosine binding site near the noncovalent flavin. Studies with novel heterocyclic amine substrates provide evidence to support the proposal that interflavin electron transfer occurs via one-electron steps, involving a biradical intermediate.

## Experimental Procedures

*Materials.* Acetic acid, L-2-azetidinecarboxylic acid, 2-furoic acid, L-pipecolic acid, D-proline, L-proline, 2-pyrrolecarboxylic acid, 2-thiophenecarboxylic acid, and bovine liver catalase were purchased from Sigma. Flavodoxin from *Desulfovibrio vulgaris* was a gift from Dr. Richard P. Swenson.

*Isolation and Assay of Sarcosine Oxidase.* Sarcosine oxidase was purified from *Corynebacterium* sp. P-1 as previously described (Kvalnes-Krick & Jorns, 1986). Flavin concentration was determined by using the extinction coefficient ($\epsilon_{450}$ = 12.7 × $10^3$ $M^{-1}$ $cm^{-1}$) reported for the enzyme by Kvalnes-Krick and Jorns (1986). Enzyme concentration was calculated on the basis of flavin concentration (2 mol of FAD/mol of enzyme). For inhibition studies with 2-furoic acid, enzyme activity was measured at 25 °C using sarcosine as substrate and an NADH peroxidase coupled assay as described by Kvalnes-Krick and Jorns (1987). Steady-state kinetic studies with L-proline were conducted by monitoring oxygen consumption with a Clark-type oxygen electrode (Yellow Springs Instruments, Model 53). Reactions were initiated by adding sarcosine oxidase (16 μM) to 3.0 mL of 10 mM potassium phosphate buffer, pH 8.0 at 25 °C, containing various concentrations of L-proline plus excess catalase (3320 units). Oxygen consumption was corrected for the effect of catalase. Buffer solutions were saturated with 100% oxygen. More extensive studies at lower oxygen concentrations were not feasible owing to the large amount of enzyme required for measurements with a poor substrate using the relatively insensitive oxygen electrode technique. Turnover numbers were calculated on the basis of enzyme concentration.

*Binding Studies with Various Carboxylic Acid Derivatives.* Difference spectra were recorded with a Perkin-Elmer Lambda 3B spectrophotometer. Except where noted, binding constants were measured on the basis of perturbation of the absorption spectrum of the enzyme flavin and were conducted in 10 mM potassium phosphate buffer, pH 8.0 at 25 °C. Dissociation constants were determined by plotting absorbance changes (near maxima or minima in the difference spectra) according to the method described by Benesi and Hildebrand (1949).

*Anaerobic Reduction Experiments.* For anaerobic reduction experiments with L-proline, L-2-azetidinecarboxylic acid, and L-pipecolic acid, a specially constructed cuvette was made anaerobic as described previously (Jorns & Hersh, 1975). A similar procedure was used to achieve anaerobiosis in titrations with dithionite, sarcosine, and dithiothreitol. The titrations were performed by using an apparatus similar to that described by Burleigh et al. (1969). Dithionite solutions were standardized by titration with lumiflavin-$N^3$-acetic acid, as described by Foust et al. (1969). All reduction experiments were performed in 10 mM potassium phosphate buffer, pH 8.0 or pH 7.0, at 25 °C. Absorption spectra were recorded with a scan speed of 120 nm/min using a Perkin-Elmer Lambda 3B spectrophotometer. In the case of reactions showing biphasic kinetic behavior, rate constants were determined by analyzing absorbance changes at 450 nm, as described by Fersht (1977).

*EPR Studies.* X-band EPR experiments were carried out by using a Bruker ER300 spectrometer equipped with a rectangular $TE_{102}$ cavity. Microwave frequency was independently monitored by using a Hewlett-Packard 5352B microwave frequency counter. Room temperature EPR samples were prepared in nitrogen-flushed flat cells (Wilmad Glass Co.) and, where appropriate, monitored spectrophotometrically by inserting the flat cell into the sample compartment of a modified Cary 14 spectrophotometer. Double integrations of the EPR signals obtained by reaction of sarcosine oxidase with L-proline were done with the Bruker ESP 300 version 2.0 software package. To determine the spin concentration in the sarcosine oxidase samples, the double integrations were normalized to signals obtained with known concentrations of the semiquinone form of flavodoxin from *Desulfovibrio vulgaris*. The concentration of the flavodoxin radical was determined on the basis of its absorption at 580 nm ($\epsilon_{580}$ = 4.7 × $10^3$ $M^{-1}$ $cm^{-1}$) (Dubardieu & LeGall, 1970).

Table I: Stability of Complexes Formed with Sarcosine Oxidase and Various Carboxylic Acid Derivatives

| compound | dissociation constant[a] (mM) |
|---|---|
| 2-furoic acid | 0.34 (0.09)[b] |
| 2-pyrrolecarboxylic acid | 0.38 |
| 2-thiophenecarboxylic acid | 3.3 |
| acetic acid | 0.72 |
| D-proline | 22 |
| L-proline | 5.0[c] |
| L-2-azetidinecarboxylic acid | 23 |

[a] Except as otherwise noted, dissociation constants were measured at 25 °C in 10 mM potassium phosphate, pH 8.0. Complex formation was estimated on the basis of the perturbation of the visible absorption spectrum of the enzyme. [b] The value in parentheses was obtained in 0.33 M potassium phosphate, pH 7.0. [c] The dissociation constant was calculated according to the method of Strickland et al. (1975).

## Results

*Interaction of Sarcosine Oxidase with Carboxylic Acids.* Titration of sarcosine oxidase with 2-furoic acid resulted in the formation of a complex, as evidenced by a perturbation of the visible absorption spectrum of the enzyme. Difference spectra, recorded during the titration, showed isosbestic points at 382 and 408 nm (Figure 1A). Other carboxylic acids (D-proline, acetic acid, 2-pyrrolecarboxylic acid, and 2-thiophenecarboxylic acid) were also found to bind to the enzyme. The dissociation constants of the various complexes varied in the range 0.09–23 mM (Table I). A unique spectral perturbation was observed for each compound tested (Figure 1B), but the perturbations observed with different compounds also shared certain common features. For example, all difference spectra showed a prominent positive peak in the 486–500-nm region, accompanied by a less intense positive peak in the 455–467-nm region. An additional prominent peak was seen in the 385–404-nm region, but for some ligands (D-proline, 2-thiopehenecarboxylic acid, and acetic acid), the perturbation in this region was positive whereas for the other

[1] Abbreviations: FAD, flavin adenine dinucleotide; DTT, dithiothreitol; EPR, electron paramagnetic resonance.

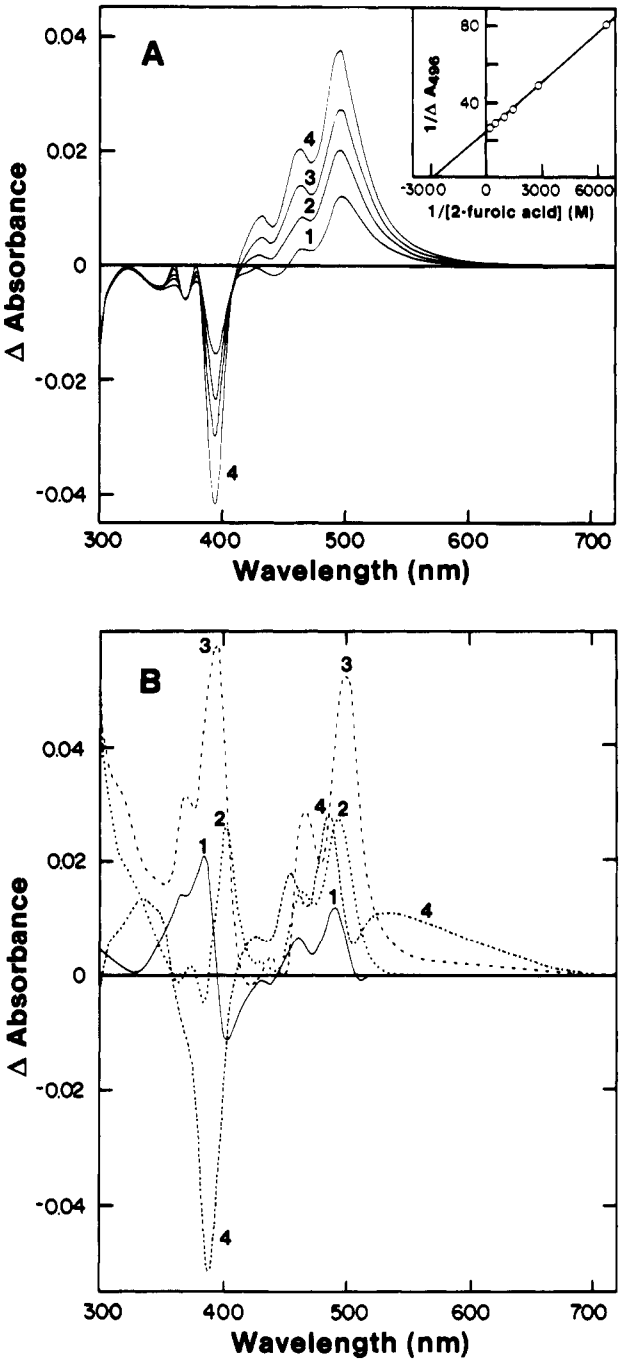



FIGURE 1: Perturbation of the absorption spectrum of sarcosine oxidase with various carboxylic acids. Difference spectra were recorded in 10 mM potassium phosphate buffer, pH 8.0 at 25 °C. Panel A shows a titration of the enzyme (25 $\mu$M) with 2-furoic acid. Curves 1–4 were recorded after adding 0.154, 0.358, 0.698, and 6.05 mM 2-furoic acid, respectively. The inset shows a Benesi–Hildebrand plot of absorbance changes at 496 nm. (Panel B) Curves 1–4 show difference spectra recorded for complexes formed with sarcosine oxidase (25 $\mu$M) and acetate (9.34 mM), 2-thiophenecarboxylic acid (15.2 mM), D-proline (321 mM), or 2-pyrrolecarboxylic acid (2.11 mM), respectively.

ligands (2-furoic acid and 2-pyrrolecarboxylic acid) a negative band was observed.

An initial survey showed that the enzyme was inhibited by each of the carboxylic acids described above. More detailed studies with 2-furoic acid showed that the compound acted as a competitive inhibitor with respect to sarcosine (data not shown). The value obtained for the inhibition constant ($K_i$ = 0.14 mM) is in reasonably good agreement with the value observed for the complex dissociation constant ($K_d$ = 0.09 mM) under the same conditions (0.33 M potassium phosphate, pH 7.0). Our previous studies have suggested that sarcosine oxidase contains a single binding site for sarcosine near the noncovalent flavin which acts as an electron acceptor from sarcosine and that sarcosine does not directly interact with the covalent flavin (Jorns, 1985; Kvalnes-Krick & Jorns, 1986). In this case, it might be expected that a competitive inhibitor with respect to sarcosine would also exhibit a single binding site near the noncovalent flavin. Evidence to evaluate this hypothesis was sought by determining whether the spectral change observed with 2-furoic acid could be attributed to a selective perturbation of the absorption due to the noncovalent flavin. The latter is unaffected by sulfite whereas the visible absorption due to the covalent flavin can be selectively eliminated by reaction with sulfite to form a covalent adduct (Jorns, 1985; Kvalnes-Krick & Jorns, 1986). Titration of the enzyme–sulfite complex with 2-furoic acid resulted in a perturbation of the residual absorption due to the noncovalent flavin (Figure 2). The observed perturbation was similar to that obtained upon complexation of the sulfite-free enzyme with 2-furoic acid, as judged by the overall magnitude of the perturbation and by the positions of positive and negative peaks in the difference spectrum, although the relative intensities of the three positive peaks in the 400–500-nm region were somewhat affected. The affinity of the enzyme–sulfite complex for 2-furoic acid ($K_d$ = 0.41 mM) was virtually identical with that observed for sulfite-free enzyme ($K_d$ = 0.34 mM) under the same conditions (10 mM potassium phosphate, pH 8.0). In a separate experiment, it was found that complexation of the enzyme with 2-furoic acid did not affect the affinity of the enzyme for sulfite, as judged by comparison of the values obtained for the dissociation constant of the enzyme–sulfite complex in the absence ($K_d$ = 0.26 mM) or presence ($K_d$ = 0.25 mM) of 2-furoic acid (14.2 mM) under otherwise identical conditions (10 mM potassium phosphate, pH 8.0). The results are consistent with the hypothesis that the enzyme has a single binding site for 2-furoic acid near the noncovalent flavin.

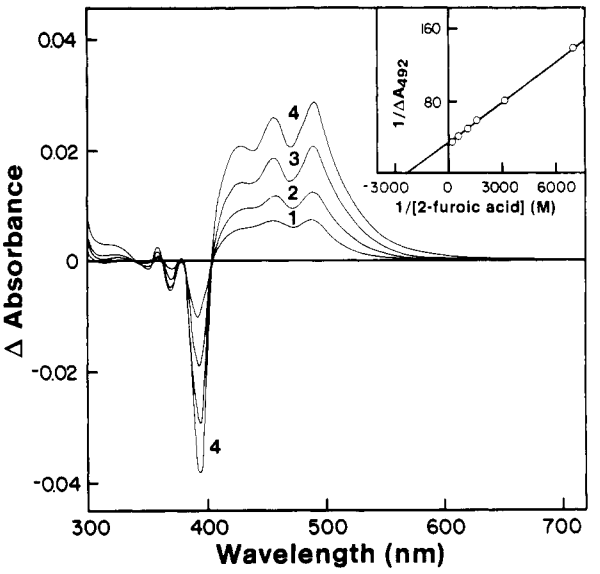



FIGURE 2: Titration of the sarcosine oxidase–sulfite complex with 2-furoic acid. Except for the presence of sulfite (44.2 mM), reaction conditions are the same as shown for the titration in Figure 1A. Curves 1–4 were recorded after adding 0.143, 0.333, 0.964, and 5.64 mM 2-furoic acid, respectively. The inset shows a Benesi–Hildebrand plot of absorbance changes at 492 nm.

*Reaction of Sarcosine Oxidase with* L-*Proline as Substrate.* That sarcosine oxidase would bind various heterocyclic car-

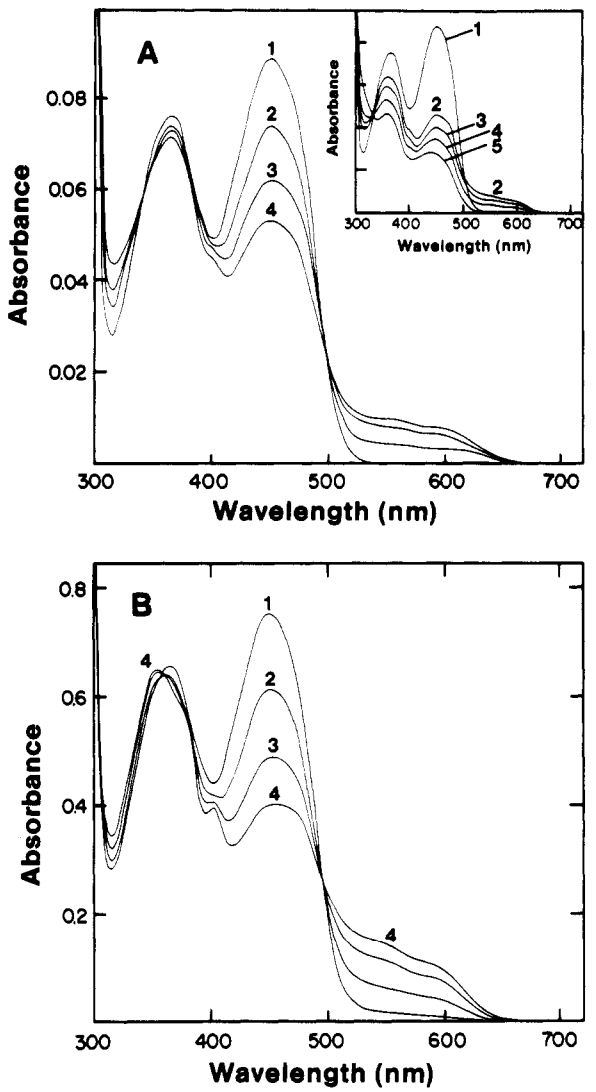



FIGURE 3: Reduction of sarcosine oxidase with L-proline. Reactions were conducted in 10 mM potassium phosphate at 25 °C under anaerobic conditions at pH 8.0 (panel A) or pH 7.0 (panel B). (Panel A) The starting enzyme (curve 1) was mixed with 0.2 mM L-proline at pH 8.0, and curves 2–4 were recorded after 2, 8, and 20 min, respectively. Inset: The starting enzyme (curve 1) was mixed with 1.0 mM L-proline at pH 8.0, and curves 2–5 were recorded after 3, 9, 24, and 130 min, respectively. (Panel B) The starting enzyme (curve 1) was mixed with 0.25 mM L-proline at pH 7.0, and curves 2–4 were recorded after 11, 41, and 146 min, respectively.

boxylic acid derivatives prompted studies to determine whether related cyclic secondary amines might function as substrates for the enzyme. Potential substrates were screened by determining whether the compounds could reduce the enzyme flavin under anaerobic conditions. Although D-proline could bind to the enzyme, as described above, the compound did not function as a substrate. On the other hand, reduction of the enzyme with L-proline was readily detectable and proceeded as a biphasic reaction at pH 8.0. The rates of both phases of the reaction depended on the concentration of L-proline. The spectral course of the first phase of the reaction could be observed most easily at lower L-proline concentrations whereas the second, slower phase was more conveniently monitored at a higher substrate concentration (Figure 3A). A long-wavelength-absorbing species was formed in the first phase of the reaction, accompanied by an increase in absorption at $\lambda > 500$ nm, a decrease in absorption at 450 nm, and the appearance of isosbestic points at 346 and 500 nm. The latter were lost during the second phase of the reaction but were replaced by a new isosbestic point at 332 nm. The long-wavelength-absorbing species decayed during the second phase of the reaction, accompanied by a further decrease in absorption at 450 nm. The spectrum observed after reduction with L-proline was similar to that observed after reduction of the enzyme with dithionite, except for a somewhat higher residual absorbance at 450 nm (vide infra).

The simplest explanation for results obtained with L-proline at pH 8.0 is that reduction of the enzyme proceeds via the formation of a single intermediate that absorbs at $\lambda > 500$ nm. However, the data are also compatible with a reaction that involves *two or more intermediates*, provided that the intermediates are formed as an *equilibrium mixture*, e.g., I, II, and III in eq 1 where $E_{ox}$ and $E_{red}$ refer to the oxidized and the

$$E_{ox} \xrightarrow{\text{proline}} I \rightleftharpoons II \rightleftharpoons III \rightarrow E_{red} \quad (1)$$

fully reduced forms of the enzyme, respectively. In fact, the mechanism shown in eq 1 is favored by data which will be presented later. In subsequent discussion, *long-wavelength species* will be used to specifically refer to the partially reduced form of the enzyme (II in eq 1) that absorbs at $\lambda > 500$ nm. The *long-wavelength intermediate* will be used more broadly to refer to the long-wavelength species plus any isoelectronic form of the enzyme which exists in equilibrium with the long-wavelength species.

The rate of formation and decay of the long-wavelength intermediate at pH 8.0 was measured at various concentrations of L-proline. For each phase, a plot of $1/k_{obsd}$ versus $1/[\text{L-proline}]$ was linear with a finite intercept on the $y$ axis (data not shown). The results are consistent with a mechanism involving formation of two distinct enzyme–substrate complexes. An initial complex ($E_{ox}$·Pro, eq 2), is converted to the long-wavelength intermediate ($E_{INT}$, eq 3). The latter forms a second complex with substrate (eq 4) which then reacts to form the fully reduced enzyme (eq 5). Values for the dis-

$$E_{ox} + Pro \overset{K_d}{\rightleftharpoons} E_{ox}\cdot Pro \quad (2)$$

$$E_{ox}\cdot Pro \xrightarrow{k_1} E_{INT} \quad (3)$$

$$E_{INT} + Pro \overset{K_d'}{\rightleftharpoons} E_{INT}\cdot Pro \quad (4)$$

$$E_{INT}\cdot Pro \xrightarrow{k_2} E_{red} \quad (5)$$

sociation constants of the two enzyme–substrate complexes ($K_d$ = 5.0 mM, $K_d'$ = 0.78 mM) and rate constants for complex reduction ($k_1$ = 3.0 min$^{-1}$, $k_2$ = 7.7 × 10$^{-2}$ min$^{-1}$) were calculated according to the method described by Strickland et al. (1975).

Oxidized enzyme was immediately regenerated upon exposure of the fully reduced enzyme or the long-wavelength intermediate to air. The latter observation suggested that the slow anaerobic reduction of the long-wavelength intermediate might not be competitive with respect to its rapid reoxidation under aerobic conditions. Evidence to evaluate this hypothesis was sought in steady-state kinetic studies. In these experiments, the rate of oxygen consumption was measured by using 100% oxygen-saturated solutions and a range of L-proline concentrations similar to that used in anaerobic reduction experiments. The oxygen concentration used in these studies was found to be nearly saturating with sarcosine as substrate (Zeller and Jorns, unpublished observations). Comparison of the turnover number (TN) observed with L-proline (TN = 4.4 min$^{-1}$) with the value obtained with sarcosine under the same conditions [TN = 1000 min$^{-1}$ (Zeller and Jorns, unpublished

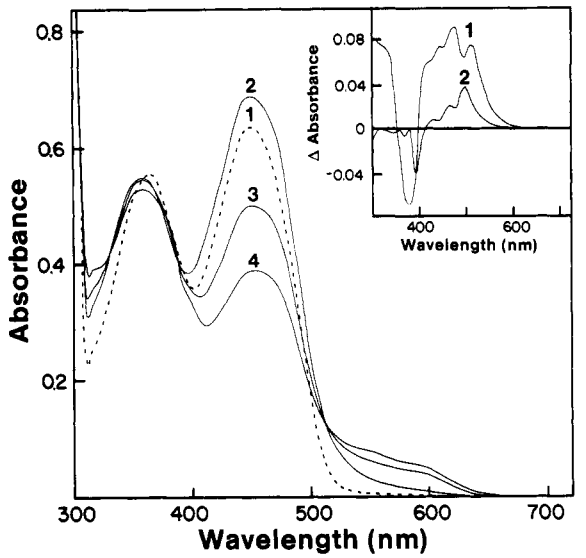



FIGURE 4: Reduction of sarcosine oxidase with L-2-azetidinecarboxylic acid at pH 8.0. Curve 1 is the spectrum of the enzyme in 10 mM potassium phosphate buffer, pH 8.0, under anaerobic conditions at 25 °C. Curve 2 was recorded immediately after adding 40 mM L-2-azetidinecarboxylic acid. Curves 3 and 4 were recorded 80 and 200 min after mixing, respectively. The intermediate shown in curve 4 was converted to fully reduced enzyme (data not shown) in a reaction which was not complete when the sample was made aerobic after 460 min. The spectrum recorded after reoxidation superimposed with the spectrum shown in curve 2. Inset: Difference spectra recorded for complexes formed with oxidized enzyme and L-2-azetidinecarboxylic acid (92.9 mM) or 2-furoic acid (6.05 mM) are shown in curves 1 and 2, respectively. Enzyme concentration (25 μM) and other reaction conditions are the same as those shown for the titrations in Figure 1.

observations)] indicates that L-proline is a rather poor substrate for the enzyme. The turnover number observed with L-proline is more than 50-fold greater than the rate constant obtained for the slow phase of the anaerobic reduction of the enzyme with L-proline ($k_2 = 7.7 \times 10^{-2}$ min$^{-1}$) but fairly comparable to the value obtained for the rate constant associated with the formation of the long-wavelength intermediate ($k_1 = 3.0$ min$^{-1}$). The results indicate that the slow phase of the reduction of the enzyme with L-proline at pH 8.0 is not significant under aerobic conditions.

The preceding studies were all conducted in 10 mM potassium phosphate buffer at pH 8.0. Further studies with L-proline showed that the relative rate of formation versus decay of the long-wavelength intermediate could be altered by changing the pH or the buffer concentration. The reaction observed in 10 mM potassium phosphate, pH 7.0, was of particular interest since the slow decay of the long-wavelength intermediate was not detectable and the intermediate was stable under anerobic conditions (Figure 3B). The rate of intermediate formation with 0.25 mM L-proline was about 5-fold slower at pH 7.0, but the yield of the long-wavelength species was twice that observed for the reaction at pH 8.0.

*Reaction of Sarcosine Oxidase with L-2-Azetidinecarboxylic Acid and L-Pipecolic Acid as Substrates.* Evidence regarding the effect of ring size on the ability of the enzyme to oxidize cyclic secondary amines was sought by studying proline analogues that contained a four-membered ring (L-2-azetidinecarboxylic acid) or a six-membered ring (L-pipecolic acid).

A spectral perturbation was observed immediately after mixing oxidized enzyme with L-2-azetidinecarboxylic acid under anaerobic conditions at pH 8.0 (Figure 4, curve 2). This initial perturbation is due to the formation of an oxidized enzyme–substrate complex (vide infra). The complex reacts to form a long-wavelength intermediate (Figure 4, curves 2–4), similar to that observed with L-proline, except that the reaction with L-2-azetidinecarboxylic acid is considerably slower. Formation of the long-wavelength intermediate with L-2-azetidinecarboxylic acid exhibits isosbestic points (333, 389, and 512 nm) that are not intersected by the initial spectrum recorded for uncomplexed, oxidized enzyme. Similar to results obtained with L-proline, the reaction with L-2-azetidinecarboxylic acid exhibits a slower second phase at pH 8.0 (data not shown), whereas at pH 7.0 the second phase is absent and the yield of the long-wavelength species is twice that observed at pH 8.0.

Immediate reoxidation of enzyme reduced with excess L-2-azetidinecarboxylic acid at pH 8.0 occurred upon exposure to air. The spectrum of the reoxidized enzyme superimposed with the spectrum observed immediately after anaerobic mixing of the enzyme with substrate (Figure 4, curve 2). That the enzyme flavin remained in the oxidized state in the presence of excess L-2-azetidinecarboxylic acid under aerobic conditions suggested that it might be possible to determine the dissociation constant of the oxidized enzyme–substrate complex by spectral titration. Substrate concentrations suitable for titration studies were found to be 2–3 orders of magnitude higher than the enzyme concentration. This observation, coupled with the slow rate of enzymic oxidation, meant that the substrate concentration would not be significantly altered by turnover during the time required for a titration experiment. The spectral course of the titration experiment, monitored by difference spectroscopy, exhibited isosbestic points at 354 and 398 nm. The magnitude of the spectral perturbation observed with L-2-azetidinecarboxylic acid is considerably larger than that observed for any of the other compounds tested in these studies [see Figure 4 (inset) and Figure 1]. A Benesi–Hildebrand plot of the titration data was linear over a wide range of L-2-azetidinecarboxylic acid concentrations (1.8–92 mM) (data not shown) and was used to calculate a value for the dissociation constant of the complex ($K_d = 23$ mM). This value is about 5-fold larger than that determined for the complex formed with the oxidized enzyme and L-proline ($K_d = 5.0$ mM). The rate constant observed for the formation of the long-wavelength intermediate ($k_{obsd} = 9.4 \times 10^{-3}$ min$^{-1}$) at a concentration of L-2-azetidinecarboxylic acid (40 mM) which converts 63% of the enzyme to the enzyme–substrate complex was used to estimate the rate constant for formation of the long-wavelength intermediate at saturating substrate. Comparison of the estimated value ($k = 1.5 \times 10^{-2}$ min$^{-1}$) with results obtained with L-proline ($k_1 = 3.0$ min$^{-1}$) suggests that conversion of the enzyme–substrate complex to the long-wavelength intermediate is about 200-fold slower with L-2-azetidinecarboxylic acid.

Reduction of sarcosine oxidase with L-pipecolic acid under anaerobic conditions at pH 8.0 occurred as a biphasic reaction that involved the formation and decay of a long-wavelength intermediate (data not shown), similar to that observed with L-proline or L-2-azetidinecarboxylic acid. In comparison studies with 0.22 mM L-pipecolic acid or L-proline at pH 8.0, it was found that the rate of formation of the long-wavelength intermediate was 30% slower with L-pipecolic acid whereas the rate of intermediate decay was 65% faster. The results suggest that L-pipecolic acid is fairly comparable to L-proline as a substrate for sarcosine oxidase. A spectral perturbation, due to formation of an oxidized enzyme–substrate complex, was not detected prior to electron transfer with L-proline or L-pipecolic acid, which are oxidized more rapidly than L-2-azetidinecarboxylic acid.

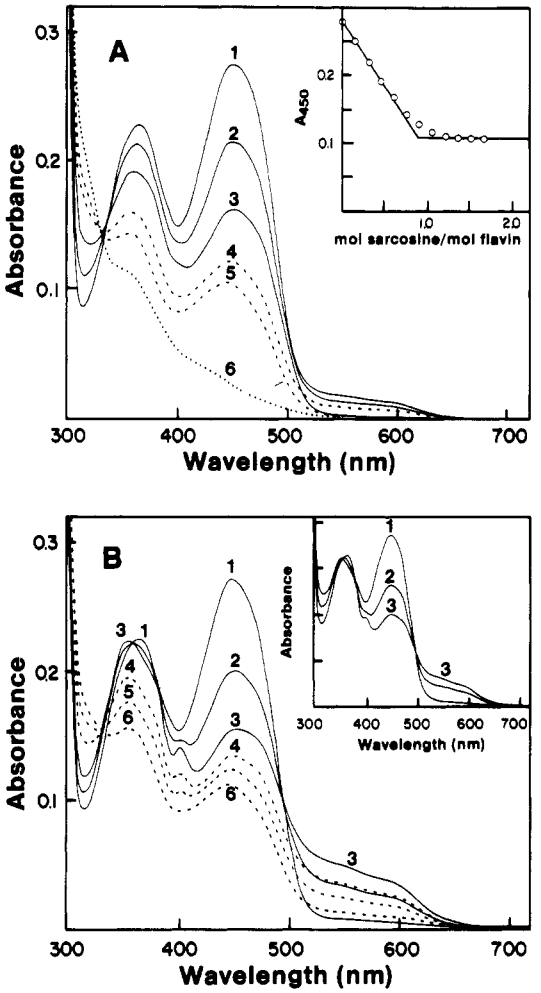



FIGURE 5: Titration of sarcosine oxidase with sarcosine. Titrations were conducted in 10 mM potassium phosphate at 25 °C under anaerobic conditions at pH 8.0 (panel A) or pH 7.0 (panel B). (Panel A) Curve 1 is the initial spectrum of the enzyme (10.9 μM) at pH 8.0. Curves 2–5 were recorded after adding 4.59, 9.14, 13.6, and 22.5 μM sarcosine, respectively. For comparison, curve 6 shows the absorption spectrum of enzyme reduced with a stoichiometric amount of dithionite, under otherwise identical conditions. The inset shows a plot of the absorbance at 450 nm versus the amount of substrate added. Flavin refers to substrate-reducible flavin, as discussed in the text. (Panel B) Curve 1 is the initial spectrum of the enzyme (10.7 μM) at pH 7.0. Curves 2–6 were recorded after adding 4.43, 8.83, 17.5, 23.9, and 36.5 μM sarcosine, respectively. Inset: A titration of sarcosine oxidase with DTT is shown. Curve 1 is the initial spectrum of the enzyme (7.1 μM) at pH 7.0. Curves 2 and 3 were recorded after complete reaction (60–80 min) with 5.39 and 10.7 μM DTT, respectively. Further reduction of the enzyme, accompanied by a decrease in the long-wavelength region, was observed at higher DTT concentrations (data not shown).

*Reaction of Sarcosine Oxidase with Sarcosine and Other Reductants.* Titration studies with sarcosine were conducted to determine whether a long-wavelength intermediate, similar to that observed with L-proline and other cyclic amines, could be formed with a good substrate. As shown in Figure 5A, a long-wavelength intermediate was observed during titration of the enzyme with sarcosine at pH 8.0. The yield of the long-wavelength species was maximal near the midpoint of the titration and was doubled when the pH of the titration was decreased from pH 8.0 to pH 7.0 (Figure 5B). The spectral course of the titration of the enzyme with sarcosine at pH 8.0 or 7.0 is similar to that observed during reduction of the enzyme with excess L-proline at the corresponding pH value (see Figures 3 and 5), except that the long-wavelength intermediate is reducible by sarcosine at pH 7.0 but not by L-proline.

As compared with dithionite-reduced enzyme (Figure 5A, curve 6), the absorption spectrum observed for sarcosine- or L-proline-reduced enzyme at pH 8.0 exhibits a larger amount of residual absorbance at 450 nm. The residual absorbance at 450 nm observed for dithionite-reduced enzyme (11.8%) is typical of that expected for the conversion of FAD to 1,5-dihydroFAD and was used to calculate a value for the difference in extinction coefficient between oxidized versus fully reduced enzyme ($\epsilon_{450} = 11.2 \times 10^3\ M^{-1}\ cm^{-1}$). On the basis of this value and the residual absorbance observed for substrate-reduced enzyme, it is estimated that 70% of the flavin in this enzyme preparation was reducible by either sarcosine or L-proline. The extent of reduction of the enzyme with sarcosine at pH 8.0 was nearly stoichiometric with respect to the amount of added substrate (Figure 5A, inset) whereas at pH 7.0 stoichiometric reduction was observed only during the initial 60% of the titration and complete reduction required more than a 2-fold excess of sarcosine (plot not shown). At either pH, the same final extent of reduction was observed, and the extrapolated end point of the titration [0.9 mol (pH 8.0) or 0.8 mol (pH 7.0) of sarcosine/mol of substrate-reducible flavin] agrees reasonably well with that expected for a two-electron flavin reduction (four electrons per enzyme). The results suggest that the addition of the second pair of electrons to the enzyme with sarcosine at pH 7.0 is more difficult than at pH 8.0.

Titration of the enzyme with dithiothreitol (DTT) at pH 7.0 resulted in the formation of a long-wavelength intermediate (Figure 5B, inset), similar to that observed during reduction with sarcosine or L-proline at this pH. A long-wavelength intermediate was also observed during titration of the enzyme with dithionite at pH 8.0 (data not shown). Except for a difference in the extent of reduction, noted above, the spectral course of the titration with dithionite and sarcosine at pH 8.0 was virtually identical.

*Characterization of the Long-Wavelength Intermediate.* The stability of the long-wavelength intermediate formed with L-proline at pH 7.0 prompted studies to directly evaluate the reduction level and the electron distribution in this form of the enzyme. In one experiment, the intermediate was formed with L-proline (0.23 mM) at pH 7.0 and then denatured under anaerobic conditions with sodium dodecyl sulfate (0.5%). Treatment with sodium dodecyl sulfate eliminated the long-wavelength absorption band and converted the enzyme flavin to a mixture of oxidized (65%) plus fully reduced (35%) flavin, which corresponds to 1.4 mol of electron/mol of enzyme. After correction for flavin that was not reducible by substrate (30%), it is evident that the reactive flavin in the intermediate was released as an equimolar mixture of oxidized and fully reduced flavin.

The development of an absorption band in the long-wavelength region in flavoenzyme reactions is generally attributable to the formation of a charge transfer complex or to the formation of flavin radicals. In the case of sarcosine oxidase, initial reduction of the noncovalent flavin (*nc*FAD) by substrate, followed by a rapid comproportionation of the reduced noncovalent flavin with the oxidized covalent flavin (*c*FAD), could yield a biradical intermediate (eq 6). An EPR signal

$$ncFADH_2 + cFAD \rightleftharpoons ncFADH\cdot + cFADH\cdot \quad (6)$$

was observed for the intermediate formed with L-proline at pH 7.0 throughout the temperature range 5–300 K. The EPR spectrum, recorded at room temperature for a sample containing 28.5 μM enzyme (70% substrate-reducible flavin), is indicative of the flavin semiquinone with a *g* value very near that of the free electron and a line width of 20 G (Figure 6,

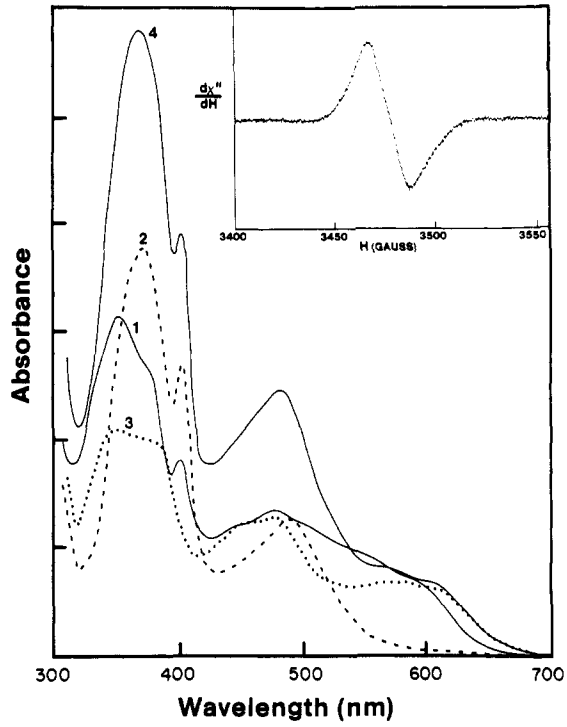



FIGURE 6: Spectral properties of the biradical intermediate formed with sarcosine oxidase and L-proline at pH 7.0. The absorption spectrum of the biradical (curve 1) was calculated by correcting curve 4 in Figure 3B for the presence of residual oxidized flavin (30%). Residual oxidized flavin was assumed to have spectral properties identical with that observed for the starting enzyme. Spectra reported by Massey and Palmer (1966) for the red and blue radical forms of the flavoenzyme glucose oxidase are shown in curves 2 and 3, respectively. Curves 2 and 3 were added to generate curve 4, which corresponds to a 50:50 mixture of both radical forms. The inset shows the EPR spectrum of the biradical. The biradical was formed by mixing enzyme with L-proline (200 $\mu$M) in 10 mM potassium phosphate, pH 7.0, under anaerobic conditions. Absorbance changes were monitored, as described under Experimental Procedures, and the EPR spectrum was recorded at room temperature when the reaction was complete. The instrument settings were the following: microwave frequency, 9.772 GHz; microwave power, 10 mW; magnetic field modulation, 5 G.

inset). The radical concentration of the sample (33 ± 5 $\mu$M) was estimated on the basis of the integrated room temperature signal and corresponds to 1.2 mol of radical/mol of enzyme. The results are in satisfactory agreement with the value (1.4 mol of radical/mol of enzyme) expected in the case where the equilibrium in eq 6 lies completely to the right and all of the reactive flavin in the intermediate is present as flavin radical, especially given the uncertainties in the integration of room temperature signals. Although less accurate as compared with low-temperature measurements, room temperature integration was preferred since a change in temperature might affect the electron distribution between the two flavins and all other studies were conducted at 25 °C. At low temperatures, there was no evidence in the $g \approx 2$ region for spin–spin interaction between covalent and noncovalent flavin radicals attached to the same enzyme molecule since the observed isotropic signal was not split into a pair of overlapping doublets.

The absorption spectrum observed for the long-wavelength intermediate formed with L-proline at pH 7.0 was corrected for the presence of 30% oxidized flavin to yield a difference spectrum which should correspond to the absorption spectrum of the biradical intermediate. The calculated spectrum (Figure 6, curve 1) exhibits absorption maxima at 350, 400, and 476 nm. The peak at 400 nm is sharp whereas the peak at 476 nm is broad and extends to nearly 700 nm. A sharp peak at 400 nm is a characteristic feature in spectra observed for various red anionic flavin radicals (Massey & Palmer, 1966). However, unlike the sarcosine oxidase intermediate, red radicals do not exhibit significant absorption at $\lambda > 600$ nm (Figure 6, curve 2). On the other hand, blue neutral radicals do absorb at $\lambda > 600$ nm but do not show a sharp peak at 400 nm (Figure 6, curve 3). An absorption spectrum corresponding to that expected for a 50:50 mixture of red and blue radicals was estimated by using data reported by Massey and Palmer (1966) for the blue and red radical forms of glucose oxidase. This composite spectrum (Figure 6, curve 4) is similar to the spectrum (Figure 6, curve 1) calculated for the biradical intermediate formed with sarcosine oxidase. An EPR signal due to a mixture of anionic and neutral radicals, which typically exhibit line widths of 12–15 and 18–23 G, respectively (Ehrenberg et al., 1967; Massey & Palmer, 1966; Palmer et al., 1971; Edmondson et al., 1981), is expected to exhibit a non-Gaussian line shape. We have examined both the absorption and second-derivative spectra (not shown) of the sarcosine oxidase biradical signal, and each appears by inspection to be non-Gaussian, consistent with the presence of two similar but distinct signals.

Previous studies have suggested that the covalent flavin is not required for electron transfer from substrate (Jorns, 1985; Kvalnnes-Krick & Jorns, 1986). However, if the mechanism shown in eq 6 is correct, the covalent flavin would be required for the formation and stability of the biradical intermediate. Evidence regarding this prediction was sought in studies involving reaction of the enzyme with L-proline plus sulfite. In one experiment, the biradical intermediate was formed with L-proline at pH 7.0 and then mixed with excess sulfite. Addition of sulfite resulted in the disappearance of the long-wavelength absorption band and the appearance of a species with an absorption spectrum (Figure 7, curve 3) similar to the final spectrum observed after reduction of the enzyme with L-proline or sarcosine at pH 8.0. In a separate experiment, the oxidized enzyme was mixed with sulfite (20 mM), and then the enzyme–sulfite complex was reacted with L-proline (0.2 mM) at pH 7.0 under anaerobic conditions. The enzyme–sulfite complex was reduced by L-proline to yield a final spectrum (data not shown) similar to that observed when the order of addition of L-proline and sulfite was reversed, as in the experiment shown in Figure 7. No long-wavelength species was detected during reduction of the enzyme–sulfite complex, but the rate of the reaction ($k_{obsd} = 3.3 \times 10^{-2}$ min$^{-1}$) was similar to that observed for the formation of the biradical intermediate with L-proline and sulfite-free enzyme under otherwise identical conditions ($k_{obsd} = 2.6 \times 10^{-2}$ min$^{-1}$). The results show that complexation of the covalent flavin with sulfite does not affect electron transfer from substrate to the noncovalent flavin but does prevent formation of the biradical and also destabilizes the biradical.

## Discussion

A variety of carboxylic acids, including 2-furoic acid, were found to bind to sarcosine oxidase, causing a perturbation of its visible absorption spectrum and an inhibition of catalytic activity. Studies with 2-furoic acid showed that the spectral perturbation could be attributed to a selective effect on the absorption spectrum of the noncovalent flavin and that the compound acted as a competitive inhibitor with respect to sarcosine. The results are consistent with the proposal that the enzyme has a single binding site for sarcosine or 2-furoic acid near the noncovalent flavin. Several heterocyclic amines were identified as new substrates for sarcosine oxidase. Similar reactivity was observed with nitrogen heterocycles that con-

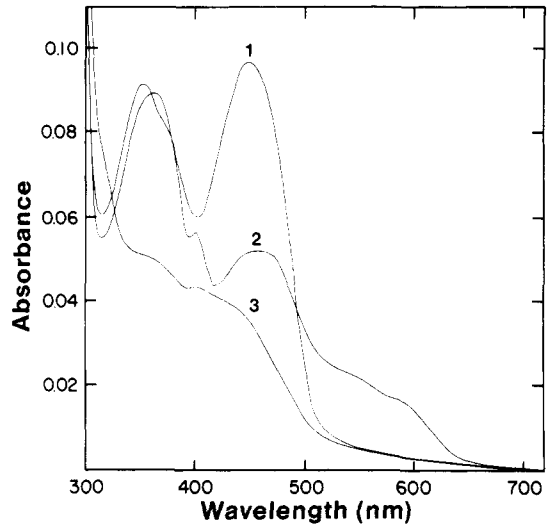
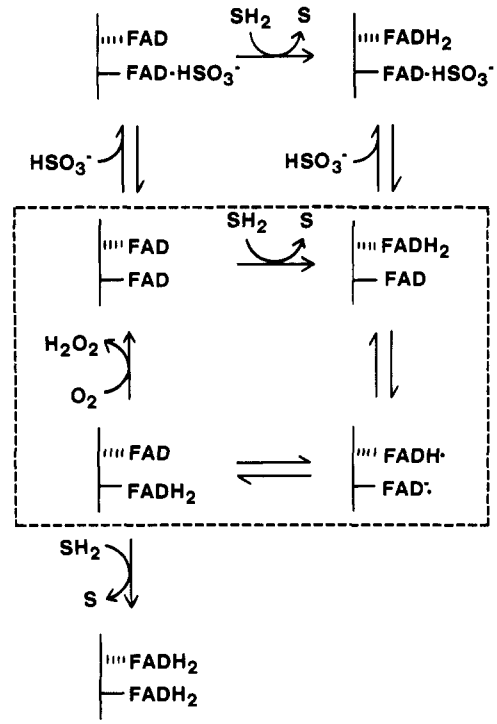



FIGURE 7: Effect of sulfite on the long-wavelength intermediate formed with sarcosine oxidase and L-proline. Curve 1 is the initial spectrum of the enzyme in 10 mM potassium phosphate buffer, pH 7.0, under anaerobic conditions at 25 °C. Curve 2 is the spectrum of the intermediate formed with 0.2 mM L-proline. Curve 3 was recorded after reaction of the intermediate with sulfite (11.8 mM).

tained a five-membered (L-proline) or a six-membered (L-pipecolic acid) ring whereas a derivative that contained a four-membered ring (L-2-azetidinecarboxylic acid) was more than 2 orders of magnitude less reactive. Substrate stereospecificity was shown by the fact that, although D-proline could bind to the enzyme, it did not function as a substrate. Reduction of the enzyme with L-proline, L-pipecolic acid, or L-2-azetidinecarboxylic acid occurred as a biphasic reaction under anaerobic conditions at pH 8.0. In each case, a similar long-wavelength intermediate was formed in the initial phase of the reaction and then decayed in a slower, second phase to yield enzyme that contained 1,5-dihydroFAD plus residual oxidized flavin. The latter was reducible with dithionite but not with any of the substrates tested in these studies, including sarcosine. The second phase was absent when the reduction of the enzyme was conducted at pH 7.0, and the yield of the long-wavelength species was doubled as compared with results obtained at pH 8.0. A long-wavelength intermediate was also observed during titration of the enzyme with sarcosine at pH 7.0 or 8.0. At each pH, the spectral course of the sarcosine titration was similar to that observed during the reduction of the enzyme with heterocyclic amines, except that the long-wavelength intermediate was reducible with excess sarcosine at pH 7.0. Further studies are planned to determine whether a similar long-wavelength intermediate is also formed during turnover of the enzyme with sarcosine, which occurs at a much faster rate (TN = 1000 $min^{-1}$) than that observed with L-proline (TN = 4.4 $min^{-1}$) under the same conditions (10 mM potassium phosphate, pH 8.0, and 1.25 mM oxygen, 25 °C.).

The studies described in this paper were conducted largely with an enzyme preparation that contained 70% substrate-reducible flavin, a feature which is assumed to reflect the presence of 30% inactive enzyme. (Anaerobic reduction experiments with L-proline and L-pipecolic acid at pH 8.0 were repeated with a different preparation that contained 88% substrate-reducible flavin. The observed spectral course and the reaction kinetics were similar to those observed with enzyme that contained 70% substrate-reducible flavin.) In the following discussion, half-reduced enzyme is used to refer to enzyme containing one electron per substrate-reducible flavin, independent of the electron distribution between the two flavins. Fully reduced enzyme refers to enzyme containing two electrons per substrate-reducible flavin.

Scheme I: Proposed Mechanism of Oxidation of L-Proline by Sarcosine Oxidase[a]




[a] The noncovalent flavin is indicated by a dashed line whereas a solid line is used for the covalent flavin. $SH_2$ and S are used to represent L-proline and $\Delta^1$-pyrroline-5-carboxylate, respectively. The fully reduced enzyme is formed under anaerobic conditions at pH 8.0. Under turnover conditions, the enzyme cycles between the fully oxidized state and half-reduced forms, as indicated by the reactions within the box. Reoxidation may occur as shown or via reaction of oxygen with the biradical. The effect of sulfite on the reaction is shown in the upper portion of the scheme.

A mechanism for the reaction of the enzyme with L-proline is suggested in Scheme I. The saturation kinetics observed with L-proline indicate that an initial enzyme–substrate complex (not shown) is formed. In the case of L-2-azetidinecarboxylic acid, formation of an enzyme–substrate complex was detected, prior to electron transfer, as an initial perturbation of the absorption spectrum of the oxidized enzyme. The *long-wavelength intermediate* formed with L-proline at pH 7.0 is a half-reduced form of the enzyme since denaturation of the intermediate released the substrate-reducible flavin as a 50:50 mixture of oxidized FAD plus 1,5-dihydroFAD. Formation of this intermediate involves the initial transfer of two electrons from substrate to the noncovalent flavin. The covalent flavin is not required for this initial step since the rate of enzyme reduction with L-proline at pH 7.0 is unaffected by complexation of the covalent flavin with sulfite. This initial, half-reduced form of the enzyme is converted to a biradical via a rapid comproportionation of the reduced noncovalent flavin with the oxidized covalent flavin. The biradical is the only form of half-reduced enzyme that absorbs at $\lambda > 500$ nm and, therefore, corresponds to the *long-wavelength species*. A third half-reduced form can be generated by disproportionation of the biradical to generate enzyme containing oxidized noncovalent flavin plus fully reduced covalent flavin. The biradical appears to be the major species generated by reaction of the enzyme with L-proline at pH 7.0, as judged by quantitative EPR studies. That the various half-reduced forms of the enzyme are in equilibrium is suggested by the fact that the electron distribution in the intermediate formed with L-proline at pH 7.0 can be shifted in favor of the noncovalent flavin by

complexation of the covalent flavin with sulfite. As judged by the intensity of the long-wavelength absorption band observed at the end of the fast phase of the L-proline reaction at pH 8.0, the yield of the biradical at this pH is approximately 50% lower than that observed at pH 7.0. A pH-dependent change in the redox properties of the two flavins could affect the electron distribution in the half-reduced enzyme. Alternatively, the yield of the biradical might be lower at pH 8.0 because the yield of half-reduced enzyme is lower due to a slow conversion to fully reduced enzyme which does not occur at pH 7.0. Although further studies are needed, the redox hypothesis could explain why the yield of the biradical is pH dependent but not significantly affected by the nature of the enzyme reductant. Transfer of a second pair of electrons from L-proline to the enzyme at pH 8.0 most likely involves reaction with half-reduced enzyme where the noncovalent flavin is in the oxidized state. However, this reaction is not kinetically significant during turnover with L-proline where the enzyme cycles between the oxidized and half-reduced states. Since electron transfer to oxygen occurs at the covalent flavin, reoxidation might involve reaction of oxygen with half-reduced enzyme where the covalent flavin is fully reduced, as shown in Scheme I, or with the biradical. No reoxidation was detected when half-reduced or fully reduced enzyme (generated by titration with sarcosine at pH 7.0) was mixed with excess $\Delta^1$-pyrroline-5-carboxylate (Zeller and Jorns, unpublished results). $\Delta^1$-Pyrroline-5-carboxylate is the relatively stable imine formed as a product during L-proline oxidation with sarcosine oxidase. The results suggest that electron transfer from L-proline to the enzyme is not readily reversible.

The biradical intermediate formed with sarcosine oxidase and L-proline appears to contain two different radicals, as evidenced by the similarity of its absorption spectrum with that expected for a 50:50 mixture of red and blue radicals and by the non-Gaussian shape of its EPR signal. In order to account for the lack of a pronounced effect on the line width of the EPR signal observed at $g \approx 2$, any spin–spin interaction that exists between the flavin radicals can give rise to no greater than a 10-G split in the signal. On the basis of this upper limit (and assuming a totally dipolar interaction), then it appears unlikely that the two flavins in sarcosine oxidase are closer than 10 Å to one another. Further work is required to establish whether this is in fact the case. In Scheme I, the red radical is attributed to the covalent flavin [8$\alpha$-($N^3$-histidyl)FAD] which reacts with oxygen and sulfite whereas the blue radical is assigned to the noncovalent flavin. This assignment is based, in part, on the fact that thermodynamic stabilization of a red, anionic radical is observed with other flavoprotein oxidases which typically contain a single flavin per active site that reacts with sulfite and oxygen (Massey et al., 1969; Massey & Hemmerich, 1980). A red radical is also observed with enzymes other than the oxidases when the flavin is covalently attached and substituted at the 8$\alpha$-position (Edmondson et al., 1981).

Sarcosine oxidase from *Corynebacterium* sp. P-1, used for the studies described in this paper, exhibits many similarities with sarcosine oxidase from *Corynebacterium* sp. U-96 (Jorns, 1985; Kvalnes-Krick & Jorns, 1986). The latter enzyme will be referred to as sarcosine oxidase J. We previously reported that a long-wavelength intermediate is formed during titration of sarcosine oxidase J with dithionite or sarcosine (Jorns, 1985). Recent studies show that L-proline is a substrate for sarcosine oxidase J and that a long-wavelength intermediate is formed during anaerobic reduction of the enzyme (Zeller and Jorns, unpublished results). A long-wavelength intermediate was not detected by other workers during reduction of sarcosine oxidase J with sarcosine in titration or rapid reaction studies (Hayashi, 1984; Kawamura-Konishi & Suzuki, 1987). Kawamura-Konishi and Suzuki suggested that the discrepancy might be related to a difference in reaction temperature. However, a long-wavelength intermediate was formed when we titrated sarcosine oxidase J with sarcosine under the same buffer and temperature conditions used in the Kawamura-Konishi and Suzuki studies (Zeller and Jorns, unpublished observations). The basis for the difference in results is unclear but may stem from differences in anaerobic procedures. The procedure used in our studies leaves no detectable residual oxygen (<1 $\mu$M) (Jorns et al., 1984). Kawamura-Konishi and Suzuki attributed complications in reaction kinetics to the presence of residual oxygen. The reduction stoichiometry reported by Hayashi (2.4 mol of sarcosine/mol of flavin) also suggests the presence of residual oxygen.

Recent studies show that sarcosine oxidase from *Arthrobacter ureafaciens* also contains both covalent and noncovalent flavin and is composed of four nonidentical subunits (Ogushi et al., 1988), similar to that observed for the two corynebacterial enzymes. With respect to the presence of both covalent and noncovalent flavin, this group of three enzymes is unique as compared with all other known flavoproteins. Among other flavoproteins, only NADPH–cytochrome P-450 reductase (Vermilion et al., 1981; Oprian & Coon, 1982; Iyanagi et al., 1981), NADPH–sulfite reductase (Siegel et al., 1972), and dihydroorotate dehydrogenase (Aleman et al., 1966) contain two nonequivalent flavins. In each case, the two flavins (FAD and FMN) are noncovalently bound; FAD serves as an entry port for electrons from NADPH which are transferred to FMN which serves as an exit port. Studies with NADPH–cytochrome P-450 reductase indicate that interflavin electron transfer occurs via a series of one-electron steps and involves a biradical intermediate, similar to that observed with sarcosine oxidase and L-proline, except that the biradical formed with the reductase contains two blue, neutral radicals.

## Acknowledgments

We thank Dr. Richard P. Swenson for his generous gift of flavodoxin.

## References

Aleman, V., Smith, S. T., Rajagopalan, K. V., & Handler, P. (1966) in *Flavins and Flavoproteins* (Slater, E. C., Ed.) pp 99–114, Elsevier Publishing Co., Amsterdam.

Benesi, H. A., & Hildebrand, J. H. (1949) *J. Am. Chem. Soc. 71*, 2703–2707.

Burleigh, B. D., Foust, G. P., & Williams, C. H. (1969) *Anal. Biochem. 27*, 536–544.

Dubardieu, M., & LeGall, J. (1970) *Biochem. Biophys. Res. Commun. 38*, 965–972.

Edmondson, D. E., Ackrell, B. A. C., & Kearney, E. B. (1981) *Arch. Biochem. Biophys. 208*, 69–74.

Ehrenberg, A., Müller, F., & Hemmerich, P. (1967) *Eur. J. Biochem. 2*, 286–295.

Fersht, A. (1977) *Enzyme Structure and Mechanism*, pp 163–164, W. H. Freeman, Reading, MA.

Foust, G. P., Burleigh, B. D., Mayhew, S. G., Williams, C. H., & Massey, V. (1969) *Anal. Biochem. 27*, 530–535.

Hayashi, S. (1984) *J. Biochem. 95*, 1201–1207.

Iyanagi, T., Makino, R., & Anan, F. K. (1981) *Biochemistry 20*, 1722–1730.

Jorns, M. S. (1985) *Biochemistry 24*, 3189–3194.

Jorns, M. S., & Hersh, L. B. (1975) *J. Biol. Chem. 250*, 3620-3628.
Jorns, M. S., Sancar, G. B., & Sancar, A. (1984) *Biochemistry 23*, 2673-2679.
Kawamura-Konishi, Y., & Suzuki, H. (1987) *Biochim. Biophys. Acta 915*, 346-356.
Kvalnes-Krick, K., & Jorns, M. S. (1986) *Biochemistry 25*, 6061-6069.
Kvalnes-Krick, K., & Jorns, M. S. (1987) *Biochemistry 26*, 7391-7395.
Massey, V., & Palmer, G. (1966) *Biochemistry 5*, 3181-3189.
Massey, V., & Hemmerich, P. (1980) *Biochem. Soc. Trans. 8*, 246-257.
Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G., & Foust, G. P. (1969) *J. Biol. Chem. 244*, 3999-4006.
Ogushi, S., Nagao, K., Emi, S., Ando, M., & Tsuru, D. (1988) *Chem. Pharm. Bull. 36*, 1445-1450.
Oprian, D. D., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 8935-8944.
Palmer, G., Müller, F., & Massey, V. (1971) in *Flavins and Flavoproteins* (Kamin, H., Ed.) pp 123-137, University Park Press, Baltimore, MD.
Siegel, L. M., Faeder, E. G., & Kamin, H. (1972) *Z. Naturforsch., B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol. 27B*, 1087-1089.
Strickland, S., Palmer, G., & Massey, V. (1975) *J. Biol. Chem. 250*, 4048-4052.
Vermilion, J. L., Ballou, D. P., Massey, V., & Coon, M. J. (1981) *J. Biol. Chem. 256*, 266-277.

# A Subfamily of Bovine Prolactin-Related Transcripts Distinct from Placental Lactogen in the Fetal Placenta†

Mark A. Kessler, Mira Milosavljevic, Claus G. Zieler, and Linda A. Schuler*
*Department of Comparative Biosciences, School of Veterinary Medicine, University of Wisconsin, Madison, Wisconsin 53706*
*Received October 11, 1988; Revised Manuscript Received February 28, 1989*

ABSTRACT: The placentae of many species express genes homologous to the pituitary hormones. In the bovine, two transcripts distinct from the pituitary hormones have been previously described: bovine placental lactogen (bPL) and bovine prolactin-related cDNA I (bPRCI). Here we provide evidence for a subfamily of prolactin-related transcripts quite different from bPL and the rodent placental homologues, which include proliferin and rat prolactin-related proteins. Bovine prolactin-related cDNAs II and III (bPRCII and bPRCIII) are about 75% similar in nucleotide sequence to one another and bPRCI, but only 56% similar to bPL, and about 45% to the rodent placental transcripts. The deduced amino acid sequences follow a similar pattern: they are about 60% similar to one another, but only about 35% similar to bPL as well as the predicted rodent placental proteins. mRNA levels corresponding to bPL, bPRCI, and bPRCIII in the fetal placenta show distinct patterns. The role of these predicted hormones during pregnancy remains to be determined.

Little is understood of the complex processes which control the growth and development of the fetus and placenta, as well as the modifications in maternal physiology that result in a successful pregnancy. While many of the activities observed during this period, including growth of the fetus and placenta, modulations in maternal energy metabolism, development of maternal mammary tissue, and maintenance of steroidogenesis, are related to functions regulated by prolactin (Prl)[1] and growth hormone (GH) postnatally, the pituitary hormones do not seem to be involved in these activities during gestation. Circulating levels of Prl are relatively low during most of pregnancy in nonprimates, including the cow (Kelly et al., 1976), and despite the presence of circulating GH, no receptors have been observed for this hormone in the ovine fetus (Gluckman et al., 1983; Parkes & Hill, 1985).

The placenta, which synthesizes homologues of a number of pituitary hormones, has been found to express members of the prolactin-growth hormone gene family. These placental products may play roles in fetal development analogous to those performed by GH and Prl in the adult. In several species, including ruminants, rodents, and primates, binding assays using lactogenic and somatogenic receptors have permitted the isolation of some of the placental hormones, referred to as placental lactogens (PLs). A single PL more closely related to Prl than to GH has been isolated in ruminant species (Beckers et al., 1980; Eakle et al., 1982; Murthy et al., 1982; Chan et al., 1976). However, additional hormones related to prolactin have been predicted in the cow, as well as the rat and mouse, by isolation of cDNAs from placental libraries. We have described bovine prolactin-related cDNA I (bPRCI), which predicts a secreted product quite different (35% amino acid homology) from bovine placental lactogen (bPL) and the pituitary hormones (Schuler & Hurley, 1987; Schuler et al., 1988). It is also quite unlike all proteins deduced from the

† This work was supported by NSF Grant DCB8608739, USDA Grant 85-CRCR-1-1837, and University of Wisconsin Graduate School Project 880525.
* To whom correspondence should be addressed.

[1] Abbreviations: Prl, prolactin; GH, growth hormone; PL, placental lactogen; bPRCI, bovine prolactin-related cDNA I; bPRCII, bovine prolactin-related cDNA II; bPRCIII, bovine prolactin-related cDNA III; SDS, sodium dodecyl sulfate.

0006-2960/89/0428-5154$01.50/0 