Kinetics of Electron Entry, Exit, and Interflavin Electron Transfer during Catalysis by Sarcosine Oxidase†

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ABSTRACT: Sarcosine oxidase contains 1 mol of covalently bound plus 1 mol of noncovalently bound FAD per active site. The first phase of the anaerobic reduction of the enzyme with sarcosine converts oxidized enzyme to an equilibrium mixture of two-electron-reduced forms (EH_2) and occurs at a rate (2700 min^{-1}, pH 8.0) similar to that determined for the maximum rate of aerobic turnover in steady-state kinetic studies (2600 min^{-1}). The second phase of the anaerobic half-reaction converts EH_2 to the four-electron-reduced enzyme (EH_4) and occurs at a rate (k = 350 min^{-1}) which is 7-fold slower than aerobic turnover. Reaction of EH_2 with oxygen is 1.7-fold faster (k = 4480 min^{-1}) than aerobic turnover and 13-fold faster than the anaerobic conversion of EH_2 to EH_4. The results suggest that the enzyme cycles between fully oxidized and two-electron-reduced forms during turnover with sarcosine. The long wavelength absorbance observed for EH_2 is attributable to a flavin biradical ($FADH^{\bullet}$ $FAD^{\bullet-}$) which is generated in about 50% yield at pH 8.0 and in nearly quantitative yield at pH 7.0. The rate of biradical formation is determined by the rate of electron transfer from sarcosine to the noncovalent flavin since electron equilibration between the two flavins (k = 750 s^{-1} or 45000 min^{-1}, pH 8.0) is nearly 20-fold faster, as determined in pH-jump experiments. Only two of the three possible isoelectronic forms of EH_2 are likely to transfer electrons to oxygen since the reaction is known to occur at the covalent flavin. However, equilibration among EH_2 forms is probably maintained during reoxidation, consistent with the observed monophasic kinetics, since interflavin electron transfer is 10-fold faster than electron transfer to oxygen.

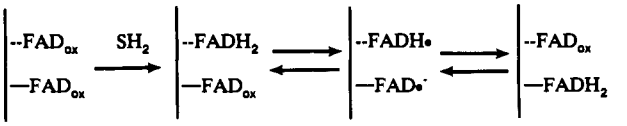
Sarcosine oxidase catalyzes the oxidative demethylation of sarcosine

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

The enzyme from *Corynebacterium* sp. P-1 contains covalent [(8α-N^3-histidyl)FAD][1] and noncovalent FAD, four different subunits, a single binding site for sarcosine near the noncovalent flavin, and two sites for tetrahydrofolate (H_4folate) (Kvalnes-Krick & Jorns, 1986, 1987; Zeller et al., 1989). H_4folate does not affect the rate of sarcosine oxidation, but the reaction in the presence of H_4folate yields 5,10-methylenetetrahydrofolate instead of formaldehyde (Kvalnes-Krick & Jorns, 1987). Various studies indicate that the noncovalent flavin accepts electrons from sarcosine which are then transferred to the covalent flavin which reduces oxygen to hydrogen peroxide (Kvalnes-Krick & Jorns, 1986; Jorns, 1985; Zeller et al., 1989).

Several heterocyclic amines (e.g., L-proline) have recently been identified as poor substrates for the enzyme (Zeller et al., 1989). Anaerobic reduction of the enzyme with L-proline or other heterocyclic amine substrates at pH 8.0 results in the formation of a long-wavelength intermediate which decays in a slower second phase to yield 1,5-dihydro-FAD. The slow phase is not kinetically significant during aerobic turnover at pH 8.0 and is not detected during anaerobic reduction experiments at pH 7.0. The long-wavelength intermediate has been identified as a two-electron-reduced form of the enzyme

Scheme I: Two-Electron Reduction of Sarcosine Oxidase



--FAD = noncovalent FAD —FAD = covalent FAD

(EH_2) containing one electron per flavin. The spectral properties of this biradical intermediate suggest that it consists of a equimolar mixture of red anionic and blue neutral flavin radicals. The biradical exists in equilibrium with other two-electron-reduced forms of the enzyme (see Scheme I). At pH 7.0 the equilibrium lies strongly in favor of the biradical, whereas at pH 8.0 only 50% of EH_2 is present as the biradical (Zeller et al., 1989).

The results obtained with L-proline suggested that the enzyme cycles between the fully oxidized (E_{ox}) and various two-electron-reduced (EH_2) forms and that interflavin electron transfer during catalysis occurs via one-electron steps. However, turnover with L-proline is more than two orders of magnitude slower than with the natural substrate, a feature which prompted studies to evaluate the mechanism of sarcosine oxidation. Steady-state and transient kinetic studies presented in this paper show that the biradical functions as a catalytically significant intermediate during turnover with sarcosine and that the enzyme cycles between E_{ox} and EH_2, similar to that observed with poor substrates. The rate of biradical formation

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[1] Abbreviations: FAD, flavin adenine dinucleotide; H_4folate, tetrahydrofolate; E_{ox}, oxidized enzyme; EH_2, two-electron-reduced enzyme; EH_4, four-electron-reduced enzyme.

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during sarcosine oxidation is limited by the rate of electron transfer from substrate to the noncovalent flavin and not by the rate of interflavin electron transfer which is faster than turnover.

Experimental Procedures

Materials. Catalase, L-proline, cytochrome *c* (bovine heart, type V), and superoxide dismutase (blood erythrocyte) were purchased from Sigma. Sarcosine was obtained from Aldrich. Milk xanthine oxidase was a gift from Dr. Russ Hille.

Isolation and Assay of Sarcosine Oxidase. Sarcosine oxidase was purified from *Corynebacterium* sp. P-1 as previously described (Kvalnes-Krick & Jorns, 1986). Total flavin concentration was determined by using the extinction coefficient (ϵ_{450} = 12.7 × 10^3 M^{-1} cm^{-1}) reported for the enzyme by Kvalnes-Krick and Jorns (1986). Total enzyme concentration was calculated on the basis of the flavin concentration (2 mol of FAD/mol of enzyme). Sarcosine oxidase preparations typically contain 70–90% substrate-reducible flavin (Zeller et al., 1989), a feature which is assumed to reflect the presence of 10–30% inactive enzyme. Unless otherwise specified, all calculations were made on the basis of the concentration of substrate-reducible flavin. For example, two-electron-reduced enzyme (EH_2) refers to enzyme containing two electrons per substrate-reducible flavin. Steady-state kinetics studies were conducted by monitoring oxygen consumption with a Clark-type oxygen electrode (Yellow Springs Instruments, model 53). Reactions were initiated by adding enzyme to 3.0 mL of 10 mM potassium phosphate buffer, pH 8.0, at 25 °C containing various concentrations of sarcosine and oxygen plus excess catalase (19.5 μg/mL). Oxygen consumption was corrected for the effect of catalase. Turnover numbers were calculated on the basis of active enzyme concentration. The data were analyzed by linear regression analysis, as described under Results, or by fitting to the rate equation for an intersecting initial velocity pattern using fitting routines developed by Cleland (1979) and software purchased from Dr. R. Viola.

Stopped-Flow Studies. Unless otherwise stated, all measurement were made at 25 °C in 10 mM potassium phosphate buffer at pH 8.0 or 7.0. Experiments were conducted using a stopped-flow spectrophotometer equipped with a Kinetics Instruments Flow System (2-cm observation cell). All other components, including the light source, photometer subsystem, and software for data acquisition and analysis, were from On-Line Instruments Systems. The dead time of the instrument was determined by a procedure similar to that described by Harvey (1969). In these studies the reduction of 2,6-dichlorophenolindophenol by ascorbate (Tonomura et al., 1978) (in 0.025 M sodium citrate, pH 3.0, containing 0.1 M sodium chloride) was used as the test reaction. For anaerobic experiments, oxygen was scrubbed from the driving syringes and the flow cell by flushing repeatedly with an anaerobic dithionite solution (5 mg/mL in 50 mM sodium pyrophosphate buffer, pH 8.0) for 24 h. Dithionite was removed just before use by flushing the system with anaerobic reaction buffer. Anaerobic solutions were transferred to the driving syringes using tonometers designed by Dr. Larry Schopfer. Solutions were made anaerobic by at least five cycles of tonometer evacuation followed by filling with oxygen-scrubbed argon.

The kinetics of the reductive half-reaction were monitored at 450 and 550 nm after mixing oxidized enzyme with buffer (10 mM potassium phosphate, pH 8.0, unless otherwise noted) containing various concentrations of sarcosine. The kinetics of the oxidative half-reaction at pH 8.0 were monitored at 450 nm after mixing two-electron-reduced enzyme (EH_2, prepared by reduction with sarcosine as described under Results) with buffer saturated with various oxygen/nitrogen mixtures. For pH-jump experiments, the enzyme was converted to the biradical form by anaerobic reduction with 0.25 mM L-proline in 10 mM potassium phosphate, pH 7.0. Extinction coefficients for the biradical (ϵ_{550} = 3.3 × 10^3 M^{-1} cm^{-1}, ϵ_{450} = 4.2 × 10^3 M^{-1} cm^{-1}), oxidized enzyme (ϵ_{450} = 12.7 × 10^3 M^{-1} cm^{-1}), and four-electron reduced enzyme (ϵ_{450} = 1.5 × 10^3 M^{-1} cm^{-1}) (Zeller et al., 1989; Kvalnes-Krick & Jorns, 1986) were used to correlate spectral changes observed at 550 and 450 nm in pH-jump experiments. Extinction coefficients for the biradical were estimated on the basis of the absorption spectrum previously reported (Zeller et al., 1989) (see Figure 3, curve 2, in this paper), assuming quantitative conversion of substrate-reducible flavin to the biradical, as suggested by room temperature electron spin resonance studies (86 ± 13%) (Zeller et al., 1989).

Superoxide Formation. Superoxide formation during aerobic turnover with sarcosine was estimated by measuring the rate of cytochrome *c* reduction in the presence and absence of superoxide dismutase (6.2 × 10^{-7} M) under assay conditions (air-equilibrated 0.1 M sodium pyrophosphate, pH 8.5, containing 3.33 × 10^{-5} M cytochrome *c*, 10 μg/mL catalase, 120 mM sarcosine, and 1.6 × 10^{-8} M sarcosine oxidase at ambient temperature) similar to those used in a previous survey of various flavoprotein oxidases (Massey et al., 1969a). For some experiments, the pyrophosphate buffer component was replaced by 10 mM potassium phosphate, pH 8.0. Superoxide formation with xanthine oxidase (1.2 × 10^{-8} M) was measured under the same conditions except that sarcosine was replaced by xanthine (1.0 × 10^{-4} M). The concentration of xanthine oxidase was calculated on the basis of its absorbance at 450 nm (ϵ_{450} per FAD = 37 800 M^{-1} cm^{-1}, 2 mol of FAD/mol of enzyme) (Massey et al., 1969b). Reduction of cytochrome *c* was monitored at 550 nm (ϵ_{red-ox} = 2.1 × 10^4 M^{-1} cm^{-1} (Massey, 1959). Turnover numbers for xanthine oxidase were calculated on the basis of flavin concentration. Turnover numbers for sarcosine oxidase were calculated on the basis of active enzyme concentration, as described above.

Results

Steady-State Kinetics. Turnover with sarcosine at pH 8.0 was monitored by measuring oxygen uptake with an oxygen electrode. At sarcosine concentrations below 3.4 mM, double-reciprocal plots of reaction rate versus sarcosine concentration are linear as judged by linear regression analysis of the data at each oxygen concentration (correlation coefficients ≥0.995) (Figure 1). Similar results were obtained when the same set of data was replotted versus 1/[oxygen] (not shown). The slopes of the lines in each of the primary plots decreased by 25–30% as the concentration of the second substrate was increased. Secondary plots of *Y* intercepts versus 1/[sarcosine] (Figure 1) or 1/[oxygen] (not shown) are linear and were used to determine values for steady-state kinetic parameters. Similar values were obtained when the data were fitted to the rate equation for an intersecting initial velocity pattern (Table I). Although the constants appear to be well determined in the rate equation fit, as judged by standard errors less than 25% of the estimated values, the distribution of residuals was random only at the highest oxygen but not at the middle (all negative) or lowest (all positive) oxygen concentrations. At sarcosine concentrations above 3.4 mM, the reaction velocity becomes independent of the oxygen concentration, and the lines in the primary plot curve downward and appear to converge at a point near the maximum velocity (TN_{max} = 2600 min^{-1}) as estimated from data obtained at lower sarcosine concentrations (Figure 1). The steady-state kinetics of sarcosine

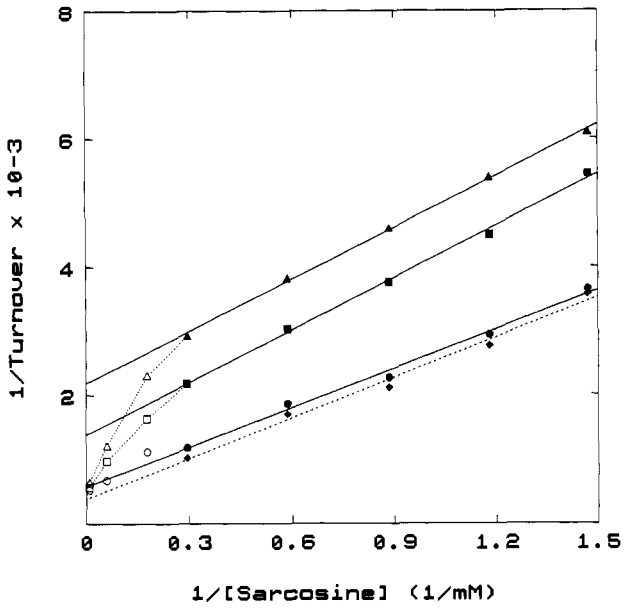

FIGURE 1: Steady-state kinetics. Turnover numbers [mol of oxygen consumed/(min·mol of active enzyme)] were measured in 10 mM potassium phosphate, pH 8.0 at 25 °C at various oxygen concentrations [139 μM (triangles); 260 μM (squares); 1250 μM, (circles)] and sarcosine concentrations as indicated in the double-reciprocal plot. Data obtained at sarcosine concentrations ≤3.4 mM (solid symbols) were analyzed by linear regression analysis (solid lines). Turnover rates at infinite oxygen (diamonds) were estimated by extrapolation of a plot of 1/turnover versus 1/[oxygen] (not shown) and analyzed by linear regression analysis (dashed line). Data points at sarcosine concentrations >3.4 mM (open symbols) are connected by dotted lines.

Table I: Steady-State Kinetic Parameters

parameter	regression analysis[a]	sequential fit[b]
turnover number (min^{-1})	2550	2600 ± 400
$K_{sarcosine}$ (mM)	5.37	5.02 ± 1.07
K_{oxygen} (μM)	636	664 ± 170
Φ_1[c] (M min)	2.10×10^{-6}	1.93×10^{-6}
Φ_2[c] (M min)	2.49×10^{-7}	2.55×10^{-7}

[a] Values were determined by linear regression analysis as described in the text. [b] Values were determined by fitting the data to the rate equation for an intersecting initial velocity pattern [$v = V_{max}[A][B]/(K_{ia}K_b + K_a[B] + K_b[A] + [A][B])$] as described under Experimental Procedures. [c] Kinetic parameters according to Dalziel terminology (Dalziel, 1969), $\Phi_1 = K_{sarcosine}$/turnover number; $\Phi_2 = K_{oxygen}$/turnover number.

oxidase are clearly complex and may reflect a branching mechanism (Segal, 1975). However, the results provide a good estimate of the maximum turnover rate which may be used to evaluate the kinetic competency of intermediates detected in stopped-flow half-reaction studies.

Anaerobic Reduction of the Enzyme with Sarcosine at pH 8.0. The disappearance of the initial oxidized enzyme upon mixing with sarcosine was monitored in a stopped-flow spectrophotometer by following the decrease in absorbance at 450 nm (Figure 2A). Absorbance changes were also monitored at 550 nm, a wavelength where the biradical absorbs but where fully oxidized or fully reduced enzyme (EH_4, four electrons/enzyme) exhibits negligible absorbance (Zeller et al., 1989). The results show that reduction of the enzyme with sarcosine is accompanied by the formation of a transient intermediate that absorbs at 550 nm. The observed spectral changes at 550 nm gave a good fit when compared with that expected for a biphasic reaction involving rapid formation, followed by a slower decay, of a long-wavelength-absorbing intermediate (Figure 2B). (Similar rates of intermediate

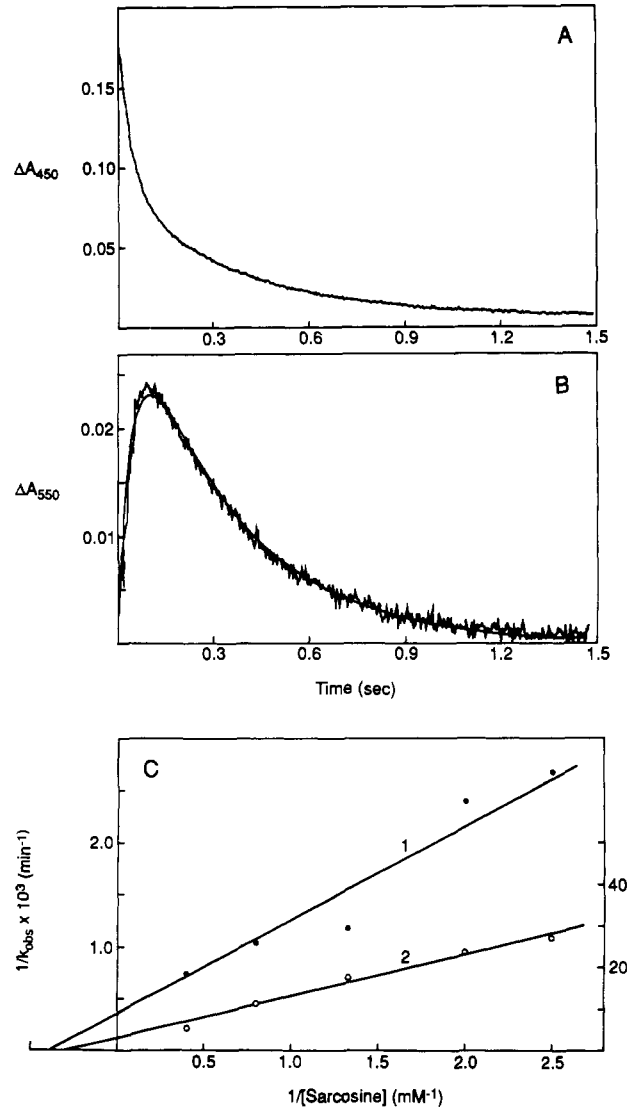

FIGURE 2: Anaerobic reduction of sarcosine oxidase with sarcosine. Reactions were conducted in 10 mM potassium phosphate, pH 8.0 at 25 °C. Reaction traces of absorbance changes at 450 nm (panel A) or 550 nm (panel B) versus time were obtained after mixing the enzyme with 2.5 mM sarcosine (concentration after mixing). The smooth curve in panel B was obtained when the data (noisy trace) were fitted to an equation for two sequential first-order processes (A → B → C) (Frost & Pearson, 1961) using software from On-Line Instrument Systems. [Similar values for rate constants were obtained when the data were analyzed using semilogarithmic plots as described by Fersht (1977)]. Panel C is a double-reciprocal plot of the observed rates of formation (line 1) and decay (line 2) of the species absorbing at 550 nm, as estimated by the fitting procedure, versus sarcosine concentration. The data in lines 1 and 2 were plotted according to the scales on the left and right, respectively.

formation and decay were obtained by analysis of the biphasic absorbance changes at 450 nm.) The observed rates of intermediate formation and decay increased as the sarcosine concentration was increased in the range from 0.4 to 2.5 mM. Double-reciprocal plots of the apparent first-order rate constants for intermediate formation or decay versus the sarcosine concentration are linear with finite intercepts on the Y axis (Figure 2C). The results suggest that the reduction of the enzyme with sarcosine involves the formation of two enzyme–substrate complexes. The complex formed with fully oxidized enzyme (eq 2) reacts to yield the long-wavelength intermediate (eq 3). The long-wavelength intermediate then forms a complex with a second molecule of sarcosine (eq 4), which reacts to yield fully reduced enzyme (eq 5).

$$E_{ox} + Sar \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E_{ox}\cdot Sar \quad (2)$$

$$E_{ox}\cdot Sar \xrightarrow{k_2} E_{INT} \quad (3)$$

$$E_{INT} + Sar \underset{k_{-1s}}{\overset{k_{1s}}{\rightleftharpoons}} E_{INT}\cdot Sar \quad (4)$$

$$E_{INT}\cdot Sar \xrightarrow{k_{2s}} EH_4 \quad (5)$$

Linear double-reciprocal plots with finite intercepts are expected when equilibrium conditions apply ($k_{-1} \gg k_2$, $k_{-1s} \gg k_{2s}$). Also, no lag phase was detected when primary semilogarithmic plots were used to determine k_{obs} for intermediate formation, a property expected when equilibrium, but not steady-state, conditions apply. The results suggest that equilibrium conditions can be reasonably assumed, although rigorous proof would require testing a larger range of substrate concentrations, as discussed by Strickland (1975). Under equilibrium conditions the ratio of slope to intercept of the double-reciprocal plots can be used to estimate the dissociation constant of the complexes (Table II). The value obtained for the complex with oxidized enzyme (k_{-1}/k_1 = 2.5 mM) is not very different from that observed for the complex with the long-wavelength intermediate (k_{-1s}/k_{1s} = 3.6 mM). The extrapolated first-order rate constant for intermediate formation at infinite sarcosine (k_2 = 2700 min^{-1}) is similar to the value obtained for the maximum rate of aerobic turnover in steady-state kinetic studies (TN_{max} = 2600 min^{-1}). However, the extrapolated first-order rate constant for intermediate decay at infinite sarcosine (k_{2s} = 350 min^{-1}) is more than 7-fold slower than the maximum rate of aerobic turnover. The results suggest that reaction of the long-wavelength intermediate with oxygen may be faster than conversion of the intermediate to fully reduced enzyme and that the slow phase of the anaerobic half-reaction is not likely to be kinetically significant during aerobic turnover.

Identification of the Long-Wavelength Intermediate. Dissociation constants for enzyme–substrate complexes may increase when the pH is lowered from 8.0 to 7.0 since higher concentrations of sarcosine are required at pH 7.0 to achieve comparable anaerobic reduction rates. For example, at pH 8.0 apparent first-order rate constants for formation (k_{obs} = 22.6 s^{-1}) and decay (k_{obs} = 3.1 s^{-1}) of the intermediate were obtained with 2.5 mM sarcosine, whereas fairly similar reaction rates are observed at pH 7.0 with 12.5 mM sarcosine (28.1 and 1.2 s^{-1}, respectively). As judged by absorbance changes at 550 nm, the maximum yield of the long-wavelength intermediate in the pH 7.0 reaction with 12.5 mM sarcosine was 2.3-fold greater than for the pH 8.0 reaction with 2.5 mM sarcosine. The maximum yield of an intermediate (B) in a two-step, irreversible, consecutive reaction (A → B → C) is normally determined by the relative rates of its formation versus decay (Frost & Pearson, 1961). On this basis, it is calculated that the maximum yield of the long-wavelength intermediate in the pH 7.0 reaction (87%) should be 1.2-fold greater than for the pH 8.0 reaction (73%). However, if the long-wavelength intermediate exists in a pH-dependent equilibrium with other isoelectronic species that do not absorb at 550 nm, as observed for the biradical (see Scheme I), the intermediate yield in the pH 7.0 reaction should be 2.4-fold larger than for the pH 8.0 reaction since the equilibrium lies strongly in favor of the biradical at pH 7.0, whereas at pH 8.0 the biradical is formed in only 50% yield (Zeller et al., 1989). The intermediate formed in the reaction with 12.5 mM sarcosine at pH 7.0 reached a maximum concentration at 90 ms after mixing. Its absorption spectrum was constructed by measuring absorbance values at various wavelengths at 90 ms after mixing and was corrected for the presence of residual oxidized flavin (see Experimental Procedures) that is not reducible by substrate. The calculated spectrum is similar to the absorption spectrum of the stable biradical formed by mixing the enzyme with 0.25 mM L-proline at pH 7.0 (Figure 3). The results show that the same biradical intermediate is formed with both sarcosine and L-proline.

Table II: Half-Reaction Kinetic Parameters

parameter[a]	stopped-flow value
k_{-1}/k_1 (mM)	2.5
k_2 (min^{-1})	2700
k_{-1s}/k_{1s} (mM)	3.6
k_{2s} (min^{-1})	350
k_{-3}/k_3 (μM)	230
k_4 (min^{-1})	4480
Φ_1 (M min)	0.93×10^{-6}

[a] Rate constants are defined as shown in eq 2–5, 7, and 8. The value for Φ_1 was calculated using the relationship $\Phi_1 = k_{-1}/k_1k_2$.

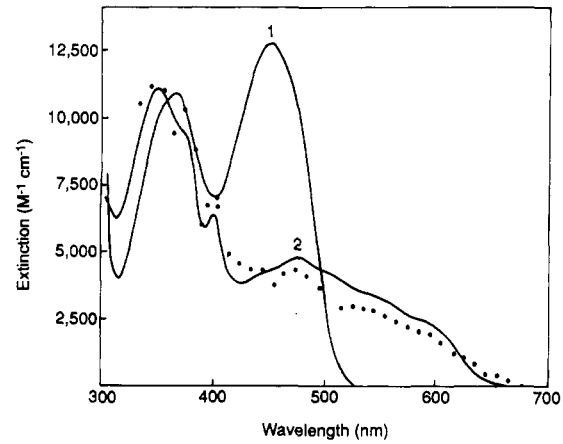

FIGURE 3: Absorption spectrum of the long-wavelength intermediate formed during reduction with sarcosine. All spectral data were obtained under anaerobic conditions in 10 mM potassium phosphate buffer, pH 7.0, at 25 °C. Curve 1 is the spectrum of oxidized enzyme. The absorption spectrum of the biradical formed with L-proline (0.25 mM), as reported by Zeller et al. (1989), is shown in curve 2 and has been corrected for the presence of residual oxidized flavin. Curve 3 is the calculated absorption spectrum of the long-wavelength intermediate formed at 90 ms after mixing with 12.5 mM sarcosine and has been corrected for the presence of residual oxidized flavin.

Rate of Interflavin Electron Transfer. No lag was detected in the formation of the biradical intermediate during reduction of the enzyme with sarcosine at pH 8.0 or 7.0. This suggested that interflavin electron transfer might be faster than the rate of electron transfer from sarcosine to the noncovalent flavin. To test this hypothesis, interflavin electron transfer was blocked by reacting the enzyme with sulfite (20 mM in 10 mM potassium phosphate buffer, pH 8.0) which selectively complexes the covalent flavin (Kvalnes-Krick & Jorns, 1986; Jorns, 1985). The enzyme–sulfite complex was then mixed with 2.5 mM sarcosine under anaerobic conditions at pH 8.0. Biradical formation cannot occur under these conditions, and a transient increase in absorbance at 550 nm was not observed. However, electron transfer from sarcosine to the noncovalent flavin did occur and could be monitored by following the decrease in absorbance at 450 nm. More than 90% of the absorbance decrease at 450 nm occurred at a rate (k = 21.4 s^{-1}) similar to that observed for the formation of the biradical with sulfite-free enzyme under otherwise identical conditions (k = 22.6 s^{-1}). [It is unclear why 9% of the reaction with the enzyme–sulfite complex occurred at a slower rate (k = 1.2 s^{-1})]. The

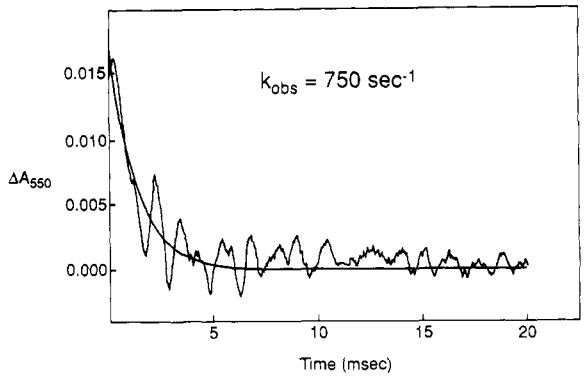

FIGURE 4: Effect of pH on the electron distribution in two-electron-reduced sarcosine oxidase. The biradical form of the enzyme was prepared by anaerobic reduction with 0.25 mM L-proline in 10 mM potassium phosphate, pH 7.0, at 25 °C. The noisy trace shows the time course of absorbance changes at 550 nm observed after mixing with anaerobic buffer (75 mM potassium phosphate, pH 9.0) to jump the pH to 8.0. The smooth curve is the fit for a first-order process (k_{obs} = 750 s^{-1}).

results are consistent with the hypothesis that electron transfer from sarcosine to the noncovalent flavin is slow compared to interflavin electron transfer and limits the rate of biradical formation during reduction of sulfite-free enzyme.

At pH 7.0 the equilibrium of the biradical with other two-electron-reduced forms (EH_2) lies strongly in favor of the biradical, whereas at pH 8.0 only 50% of EH_2 is present as the biradical (Zeller et al., 1989). This suggested that, if the pH of a solution of EH_2 was jumped from pH 7.0 to 8.0, a rapid disappearance of 50% of the biradical would occur owing to an electron redistribution between the two flavins (eq 6). [The fully reduced flavin (unknown protonation state) is arbitrarily shown as an anion (FlH^-).]

$$FlH^{\bullet} + Fl^{\bullet -} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} Fl_{ox} + FlH^- \qquad (6)$$

The approach to the new equilibrium position at pH 8.0 should be a first-order process with an observed rate equal to the sum of the forward and reverse rate constants ($k_{obs} = k_f + k_r$). To test this hypothesis, the enzyme was converted to the biradical by anaerobic reduction with 0.25 mM L-proline at pH 7.0. [Further reduction of the enzyme is not observed under these conditions (Zeller et al., 1989).] The pH of the solution was rapidly jumped from pH 7.0 to 8.0 by mixing with buffer at a higher pH (75 mM potassium phosphate, pH 9.0) in an anaerobic stopped-flow experiment. As judged by absorbance changes at 550 nm, the pH jump resulted in a very rapid disappearance of 55% of the biradical in a reaction exhibiting apparent first-order kinetics (k_{obs} = 750 s^{-1} or 45 000 min^{-1}) (Figure 4). The reaction was so fast that 58% of the total spectral change accurred during mixing, as would be expected on the basis of the observed rate constant and the measured dead time (1.2 ms) of the instrument. The observed disappearance of the biradical at 550 nm was accompanied by an increase in absorption at 450 nm consistent with the disproportionation reaction shown in eq 6. Biradical disappearance due to reaction with residual oxygen would be much slower (vide infra) and was not detected in a control stopped-flow experiment when the biradical solution at pH 7.0 was mixed with anaerobic buffer at the same pH. Conversion of EH_2 to EH_4 is observed with L-proline at pH 8.0, but the reaction is very slow and insignificant in the time scale of a pH-jump experiment (Zeller et al., 1989). The pH-jump data show that electrons equilibrate between the two flavins in sarcosine oxidase at a rate which is nearly 20-fold faster than the rate of biradical formation at infinite sarcosine (k_2 = 2700 min^{-1}). This clearly demonstrates that biradical formation during reduction with sarcosine is limited by the rate of electron transfer from substrate to the noncovalent flavin and not by the rate of interflavin electron transfer.

Reaction of Reduced Enzyme with Oxygen. Comparison of rate constants determined for the biphasic anaerobic reduction of the enzyme with sarcosine (k_2 = 2700 min^{-1}, k_{2s} = 350 min^{-1}) with the maximum turnover rate (TN_{max} = 2600 min^{-1}) observed at pH 8.0 suggested that two-electron-reduced enzyme (EH_2) would probably react faster with oxygen than with a second molecule of sarcosine. To test this hypothesis, the enzyme was partially converted to EH_2 by reduction with 0.68 mol of sarcosine/mol of enzyme at pH 8.0. Under these conditions, formation of EH_2 is directly proportional to the amount of sarcosine added whereas formation of EH_4 is negligible, as judged by results obtained in titration experiments (Zeller et al., 1989). Although substrate was used to reduce the enzyme, it is assumed that free EH_2 is actually generated because formation of a stable EH_2·P species under the experimental conditions ($[EH_2]_{total} = [P]_{total}$ = 6.0 μM) would require tight product binding (e.g., $K_d \approx 20$ nM for 95% formation of EH_2·P at equilibrium) whereas relatively weak binding is predicted, as judged by the stability estimated for EH_2·sarcosine (K_d = 3.6 mM). The reaction of EH_2 with oxygen is a monophasic process as judged by analysis of absorbance increases at 450 nm. The observed oxidation rate increased as the oxygen concentration was increased in the range from 130 to 625 μM. A double-reciprocal plot of apparent first-order rate constants versus oxygen concentration was linear with a finite intercept on the Y axis (plot not shown) and was used to determine the dissociation constant of the EH_2·O_2 complex ($k_{-3}/k_3 = 2.3 \times 10^{-4}$ M) (eq 7). The extrapolated rate constant at infinite oxygen (k_4 = 4480 min^{-1}) (eq 8) is 1.7-fold faster than aerobic turnover and 13-fold faster than anaerobic reduction of EH_2 to EH_4 (k_{2s} = 350 min^{-1}).

$$EH_2 + O_2 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} EH_2{\cdot}O_2 \qquad (7)$$

$$EH_2{\cdot}O_2 \xrightarrow{k_4} E_{ox} \qquad (8)$$

The results show that formation of EH_4 is not likely to occur during aerobic turnover with sarcosine, suggesting that the enzyme cycles between fully oxidized and two-electron-reduced forms.

Superoxide Formation during Turnover. Because other mechanisms involve spin-forbidden steps (Hamilton, 1971), it is generally believed that reduction of oxygen to hydrogen peroxide by flavoprotein oxidases involves initial transfer of one electron from fully reduced flavin to oxygen, forming a flavin/superoxide radical pair. Little or no superoxide formation is actually detectable during aerobic turnover of most flavoprotein oxidases, presumably because further reduction to hydrogen peroxide is much faster than release of the superoxide intermediate from the active site. In this regard, xanthine oxidase is a notable exception. This complex flavoprotein contains several redox-active cofactors in addition to FAD (site of oxygen reduction) and can accept a total of six electrons. Superoxide formation, observed only during reoxidation of the two-electron-reduced enzyme, is attributed to slow electron transfer from the one-electron-reduced enzyme (Massey et al., 1969a; Hille & Massey, 1981; Porras et al., 1981). The rate of superoxide formation with sarcosine oxidase was estimated by measuring the rate of cytochrome *c* reduction in the presence and absence of superoxide dismutase at pH 8.5 under conditions similar to those used in a previous survey

Table III: Superoxide Formation During Turnover

assay buffer	enzyme	turnover number[a] cytochrome c reduction no SOD	cytochrome c reduction plus SOD	superoxide formation
0.1 M sodium pyrophosphate, pH 8.5	sarcosine oxidase	30.3	6.0	24.3
	xanthine oxidase[b]	551 (336)	22 (4)	529 (332)
	other oxidases[c]	0.01–0.68	0.01–0.50	0.0–0.18
10 mM potassium phosphate, pH 8.0	sarcosine oxidase	21.7	0.9	20.8
	xanthine oxidase	260	20	240

[a] Turnover numbers for cytochrome *c* reduction = moles of cytochrome *c* reduced per minute per mole of active site. The difference in cytochrome reduction rates observed in the absence or presence of superoxide dismutase (SOD) was used to estimate turnover numbers for superoxide formation. [b] Literature values are shown in parentheses (Massey et al., 1969a). [c] Data obtained by Massey et al. (1969a) with glucose oxidase, D-amino acid oxidase, L-amino acid oxidase, glycolate oxidase, and lactate oxidase.

of various flavoprotein oxidases (Massey et al., 1969a). The rate of superoxide formation with sarcosine oxidase (TN = 24.3 min^{-1}) is quite low compared to that with xanthine oxidase [TN = 529 min^{-1} (this study) or 332 min^{-1} (Massey et al., 1969a)] but considerably higher than observed in a previous study with five simple flavoprotein oxidases (TN = 0.0–0.18 min^{-1}) (Massey et al., 1969a) (Table III). A similar rate of superoxide formation was observed with sarcosine oxidase when measurements were made in 10 mM potassium phosphate at pH 8.0 (TN = 20.8 min^{-1}). Comparison of this value with the rate of sarcosine oxidation under the same conditions (TN = 1800 min^{-1}, see Figure 1) shows that superoxide formation can account for only 1% of turnover with sarcosine.

Discussion

The anaerobic reduction of sarcosine oxidase with sarcosine occurs as a biphasic reaction. In the first phase, oxidized enzyme is converted to an equilibrium mixture of two-electron-reduced forms (EH_2) (see Scheme I). The rate of EH_2 formation (2700 min^{-1}, pH 8.0) is similar to the maximum rate of aerobic turnover determined in steady-state kinetic studies (2600 min^{-1}). The long wavelength absorbance observed for EH_2 is attributable to a flavin biradical ($FADH^{\bullet}$ $FAD^{\bullet-}$) which is generated in about 50% yield at pH 8.0. Nearly quantitative conversion to the biradical is observed at pH 7.0, consistent with previous studies with EH_2 which show that the equilibrium lies strongly in favor of the biradical at pH 7.0 (Zeller et al., 1989). The rate of biradical formation is determined by the rate of electron transfer from sarcosine to the noncovalent flavin since electron equilibration between the two flavins is nearly 20-fold faster, as determined in pH-jump experiments. EH_2 is converted to four-electron-reduced enzyme (EH_4) in the second phase of the anaerobic half-reaction at a rate which is slower (7-fold) than aerobic turnover. Reaction of EH_2 with oxygen is somewhat faster (1.7-fold) than aerobic turnover and considerably faster (13-fold) than the anaerobic conversion of EH_2 to EH_4. The results suggest that the enzyme cycles between fully oxidized and two-electron-reduced forms during turnover with sarcosine, similar to that previously observed with a poor substrate, L-proline (Zeller et al., 1989). Only two of the three possible isoelectronic forms of EH_2 are likely to transfer electrons to oxygen since the reaction is known to occur at the covalent flavin (Kvalnes-Krick & Jorns, 1986; Jorns, 1985; Zeller et al., 1989). However, equilibration among EH_2 forms is likely to be maintained during reoxidation, consistent with the observed monophasic kinetics, since interflavin electron transfer is 10-fold faster than electron transfer to oxygen. A correlation is expected between steady-state parameters and kinetic parameters determined for the first phase of the anaerobic half-reaction with sarcosine ($\Phi_1 = K_{sarcosine}/TN_{max} = k_{-1}/k_1k_2$) (Strickland et al., 1975; Bright & Porter, 1975). The value obtained for Φ_1 from steady-state parameters (1.9×10^{-6} M min) is in fair agreement with the value estimated from transient kinetics (0.93×10^{-6} M min). The steady-state kinetic data obtained at sarcosine concentrations ≤3.4 mM are consistent with a sequential mechanism, suggesting that EH_2·P is the species that reacts with oxygen during turnover. In this case, agreement is not expected between the value determined for Φ_2 from steady-state data ($\Phi_2 = K_{oxygen}/TN_{max} = 2.5 \times 10^{-7}$ M min) and a value calculated on the basis of oxidative half-reaction data obtained with free EH_2 ($k_{-3}/k_3k_4 = 0.51 \times 10^{-7}$ M min). For the reaction of the enzyme with sarcosine, oxidative half-reaction studies with EH_2·P are difficult because the suspected imine product ($H_2C{=}NH^+$-$CH_2CO_2^-$) is unstable in solution, where the equilibrium lies strongly in favor of its hydrolysis products.

In studies with sarcosine oxidase from a different corynebacterium (*Corynebacterium* sp. U-96), Kawamura-Konishi and Suzuki (1987) reported that anaerobic reduction with sarcosine at pH 8.0 occurred as a triphasic reaction, as judged by analysis of absorbance changes at 450 nm, and did not involve formation of a long-wavelength intermediate. The apparent discrepancy between these studies and our results with sarcosine oxidase from *Corynebacterium* sp. P-1 was puzzling since previous side-by-side comparison studies showed that the two enzymes exhibit very similar physical and catalytic properties (Kvalnes-Krick & Jorns, 1986; Zeller et al., 1989). Unlike Kawamura-Konishi and Suzuki (1987), we find that anaerobic reduction of the enzyme from *Corynebacterium* sp. U-96 with sarcosine (2.5 mM) is a biphasic reaction (k_{fast} = 27.1 min^{-1}, k_{slow} = 3.3 min^{-1}, pH 8.0) involving transient formation of an intermediate that absorbs at 550 nm (Ali and Jorns, unpublished results), similar to that observed with the *Corynebacterium* sp. P-1 enzyme under the same conditions (k_{fast} = 22.6 min^{-1}, k_{slow} = 3.1 min^{-1}).

The mechanism suggested by our studies with sarcosine oxidase exhibits similarities with mechanisms proposed for NADPH cytochrome P450 reductase (Vermillion et al., 1981; Oprian & Coon, 1982) and NADPH sulfite reductase (Siegel et al., 1971; Ostrowski et al., 1989). These enzymes contain two flavins (FAD, FMN) per active site which have different roles in catalysis, similar to sarcosine oxidase, except that both flavins in the reductases are noncovalently bound. In each of the reductases, FAD serves as an entry port for electrons from NADPH which are then transferred in one-electron steps to FMN, which serves as an exit port, to either cytochrome P450 or the hemoprotein subunit in sulfite reductase. Also similar to sarcosine oxidase, no lag is observed in the formation of a biradical intermediate ($FADH^{\bullet}$ $FMNH^{\bullet}$) during reduction of cytochrome P450 reductase with NADPH, suggesting that interflavin electron transfer in the reductase is fast compared to electron transfer from NADPH to FAD (k = 28 s^{-1}) (Oprian & Coon, 1982). Tollin and co-workers (Bhattacharyya et al., 1991) claim that electron transfer from $FADH^{\bullet}$ to FMN in cytochrome P450 reductase occurs at a rate of 70 s^{-1}, but ambiguities in the interpretation of the laser flash photolysis data suggest that further studies are needed. Unlike cytochrome P450 reductase, which has a single active site per molecule of enzyme (Iyanagi & Mason, 1973), sulfite re-

ductase contains four FAD–FMN pairs per molecule of enzyme (Siegel & Davis, 1974). The rate of electron equilibration between FAD–FMN pairs has been measured ($t_{1/2}$ = 100 ms) and may be fast enough to be important during catalysis, but the rate of electron transfer within a FAD–FMN pair is unknown (Siegel et al., 1971; Ostrowski et al., 1989).

Rates of electron transfer between flavin and other redox centers in the same active site have been measured for several metalloflavoenzymes. Rapid, pH-dependent electron transfer is observed between FAD and the iron–sulfur centers in xanthine oxidase [k = 160 s^{-1} (pH 6.0); k = 330 s^{-1} (pH 8.5)] (Hille & Massey, 1986) and between FMN and the iron–sulfur center in trimethylamine dehydrogenase [k = 200 s^{-1} (pH 6.0); k = 1000 s^{-1} (pH 10.0)] (Rohlfs & Hille, 1991). In each case, the rate of electron transfer is fast compared to enzyme turnover under the same conditions, similar to sarcosine oxidase. Rates have also been reported for electron transfer between FAD and heme c in p-cresol methylhydroxylase (k = 220 s^{-1} at pH 7.6) (Bhattacharyya et al., 1985) and between FMN and heme b in yeast flavocytochrome b_2 (k = 400–1200 s^{-1}, depending on ionic strength) (Tollin & Hazzard, 1991). The rate observed for flavin-to-flavin electron transfer with sarcosine oxidase (k = 750 s^{-1}, pH 8.0) falls within the range observed for flavin-to-metal center electron transfer in the metalloflavoenzymes.

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