Properties and Catalytic Function of the Two Nonequivalent Flavins in Sarcosine Oxidase†

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ABSTRACT: Sarcosine oxidase from *Corynebacterium* sp. U-96 contains 1 mol of noncovalently bound flavin and 1 mol of covalently bound flavin per mole of enzyme. Anaerobic titrations of the enzyme with either sarcosine or dithionite show that both flavins are reducible and that two electrons per flavin are required for complete reduction. Absorption increases in the 510–650-nm region, attributed to the formation of a blue neutral flavin radical, are observed during titration of the enzyme with dithionite or substrate, during photochemical reduction of the enzyme, and during reoxidation of substrate-reduced enzyme. Fifty percent of the enzyme flavin forms a reversible, covalent complex with sulfite (K_d = 1.1 × 10^{-4} M), accompanied by a complete loss of catalytic activity. Sulfite does not prevent reduction of the sulfite-unreactive flavin by sarcosine but does interfere with the reoxidation of reduced enzyme by oxygen. The stability of the sulfite complex is unaffected by excess acetate (an inhibitor competitive with sarcosine) or by removal of the noncovalent flavin to form a semiapoprotein preparation where 75% of the flavin reacts with sulfite (K_d = 9.4 × 10^{-5} M) while only 3% remains reducible with sarcosine. The results indicate that oxygen and sulfite react with the covalently bound flavin and suggest that sarcosine is oxidized by the noncovalently bound flavin.

Sarcosine oxidase catalyzes the oxidative demethylation of sarcosine (eq 1). The enzyme has recently been isolated from

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

Corynebacterium sp. U-96 and shown to contain 1 mol of covalently bound flavin [8α-(N^3-histidyl)-FAD] plus 1 mol of noncovalently bound flavin adenine dinucleotide (FAD)[1] per mole of enzyme (M_r 174000). The enzyme contains four nonidentical subunits (A, M_r 110000; B, M_r 44000; C, M_r 21000; D, M_r 10000). The covalent flavin is attached to subunit B (Hayashi et al., 1980, 1982; Suzuki, 1981). The presence of both covalent and noncovalent flavin distinguishes the *Corynebacterium* sarcosine oxidase from all other known flavoproteins including sarcosine-oxidizing enzymes (oxidases or dehydrogenases) from other sources (Patek & Frisell, 1972; Sato et al., 1981; Patek et al., 1972; Pinto & Frisell, 1975; Mori et al., 1980). On the other hand, a couple of flavoenzymes are known that contain two nonequivalent flavins: NADPH-cytochrome P-450 reductase (Vermilion et al., 1981) and NADPH-sulfite reductase (Siegel et al., 1972) contain 1 mol each of noncovalently bound FAD and FMN per active site. In these dehydrogenase/electron transferase enzymes FAD serves as an entry port for electrons from NADPH which are then transferred to FMN which serves as an exit port. In this paper we present evidence for a similar mechanism with sarcosine oxidase from *Corynebacterium* sp. U-96. This enzyme appears to be the first example of a flavoprotein oxidase where separate flavins are required for substrate dehydrogenation and for reduction of oxygen.

Experimental Procedures

Materials. Sarcosine oxidase from *Corynebacterium* sp. U-96 was a gift from Dr. Masaru Suzuki (Noda Institute for Scientific Research). The enzyme preparation exhibited spectral properties (A_{280}/A_{450} = 12.9) and a specific activity [10.3 μmol of sarcosine oxidized min^{-1} (mg of enzyme)$^{-1}$] similar to those reported by Suzuki (1981) for electrophoretically homogeneous enzyme. Chymotrpsin and trypsin were purchased from Sigma. All other reagents were the best commercially available grade and were used as received.

Methods. Sarcosine oxidase assays were conducted in 0.06 M glycylglycine buffer, pH 8.3, containing 0.12 M sarcosine at 37 °C. Activity was measured by monitoring oxygen consumption with an oxygen electrode (Yellow Springs Instruments Model 53) or by measuring the amount of formaldehyde formed. In the latter assay formaldehyde was determined by the Nash procedure (Nash, 1953), similar to that described by Suzuki (1981) except that the sarcosine oxidase reaction was quenched after a 3-min incubation. Protein concentration was determined from the absorbance of the enzyme at 280 mm by using the extinction coefficient ($E^{1\%}$ = 13.1) reported by Suzuki (1981).

To determine the relative composition of covalent vs. noncovalent flavin, sarcosine oxidase was precipitated with 5% trichloroacetic acid. The sample was centrifuged, and the precipitate was washed with an aliquot of 5% trichloroacetic acid. The initial supernatant and the wash were combined and neutralized, and the absorbance at 450 nm was recorded as a measure of the amount of noncovalent flavin in the enzyme. The protein precipitate was resuspended in 0.3 M potassium phosphate, pH 8.5, to yield a mixture with a pH of 7.7. Chymotrypsin and trypsin (0.1 mg of each protease/mg of sarcosine oxidase) were added to digest the protein. After 20 h at 37 °C a clear solution was obtained, and the amount of covalent flavin was determined from the absorbance at 450 nm.

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[1] Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance.

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For anaerobic photoreduction experiments a specially constructed cuvette was made anaerobic as previously described (Jorns & Hersh, 1975). A similar procedure was used to achieve anaerobiosis in dithionite and substrate titrations which were performed by using an apparatus similar to that described by Burleigh et al. (1969). In photoreduction experiments the sample was illuminated at 4 °C by using a halogen lamp as previously described (Jorns, 1979). Absorption spectra were obtained by using a Beckman 25 or a Perkin-Elmer Lambda 3 spectrophotometer.

For the preparation of semiapoprotein, sarcosine oxidase (200 μL, 20 mg/mL) was mixed with 200 μL of 3.0 M KBr and 200 μL of saturated ammonium sulfate, pH 4.0. A precipitate formed after adding 300 μL of saturated ammonium sulfate, pH 3.0, and was removed by centrifugation. The pellet was redissolved in 200 μL of 0.1 M sodium pyrophosphate, pH 7.5, and then precipitated by adding 100 μL of saturated ammonium sulfate, pH 4.0, followed by 200 μL of saturated ammonium sulfate, pH 3.0. The pellet obtained after centrifugation was dissolved in 200 μL of 0.1 M sodium pyrophosphate, pH 7.5, and precipitated with acid ammonium sulfate as in the previous step. The pellet obtained after centrifugation was redissolved as before and precipitated by adding 200 μL of neutral saturated ammonium sulfate. The pellet obtained after centrifugation was dissolved in 200 μL of 0.1 M sodium pyrophosphate, pH 8.5, and spun to remove a small amount of debris.

Results

Nearly half (43%) of the absorbance observed for sarcosine oxidase at 450 nm is released into solution upon denaturation with 5% trichloroacetic acid. The remainder (48%) is associated with a yellow protein precipitate that can be solubilized by treating with a mixture of chymotrypsin and trypsin. The results serve to verify that the enzyme contains both covalent and noncovalent flavin, in agreement with previous studies (Suzuki, 1981). The somewhat less than quantitative recovery of absorbance at 450 nm (91%) can be attributed to the fact that the extinction coefficient of flavin bound to sarcosine oxidase (E_{450} = 12.7 × 10^3 M^{-1} cm^{-1}) is significantly higher than that observed for free FAD (E_{450} = 11.3 × 10^3 M^{-1} cm^{-1}). A average extinction coefficient for both the covalent and the noncovalent flavin is readily determined from spectral changes observed after heat denaturation which results in decreases in absorbance in both the 450- and 370-nm regions. Surprisingly, protein precipitation does not interfere with absorbance measurements in experiments using moderately dilute enzyme solutions (2 mg/mL). A protein precipitate does form when the heat-denatured sample is mixed with trypsin and chymotrypsin. Proteolytic digestion occurs during subsequent incubation of the sample at 23 °C to yield a clear solution with the same absorbance at 450 nm and a small increase in absorbance at 370 nm as compared with the initial heat extract.

Titration of the enzyme with dithionite results in decreases in absorbance at 450 nm accompanied by a small transient increase in absorbance at λ > 510 nm (Figure 1). A plot of absorbance at 450 nm vs. moles of dithionite per mole of flavin (Figure 1, inset) shows that the titration is linear for 85% of the reaction when a small deviation from linearity is observed. Extrapolation of the linear portion to the A_{450} value observed for the end point of the titration yields a value of 1.1 mol of dithionite per flavin required for complete reduction. This value is only slightly less than that determined from the actual amount of dithionite added (1.2 mol of dithionite/mol of flavin). In either case the results suggest that flavin is the only component in the enzyme that reacts with dithionite. Further

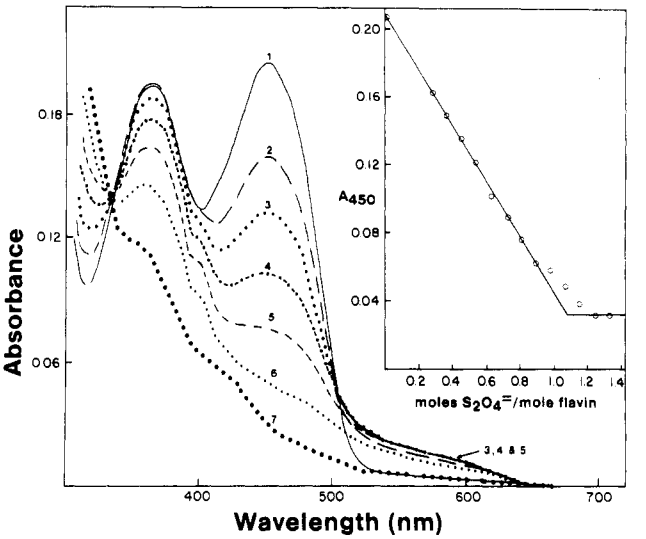

FIGURE 1: Titration of sarcosine oxidase with sodium dithionite. Curve 1 is the spectrum of the starting enzyme under anaerobic conditions in 0.01 M potassium phosphate buffer, pH 8.0 at 5 °C. Curves 2–7 were recorded after adding 0.267, 0.445, 0.623, 0.801, 1.07, and 1.33 mol of dithionite/mol of flavin, respectively. The inset shows a plot of absorbance at 450 nm vs. the amount of titrant added.

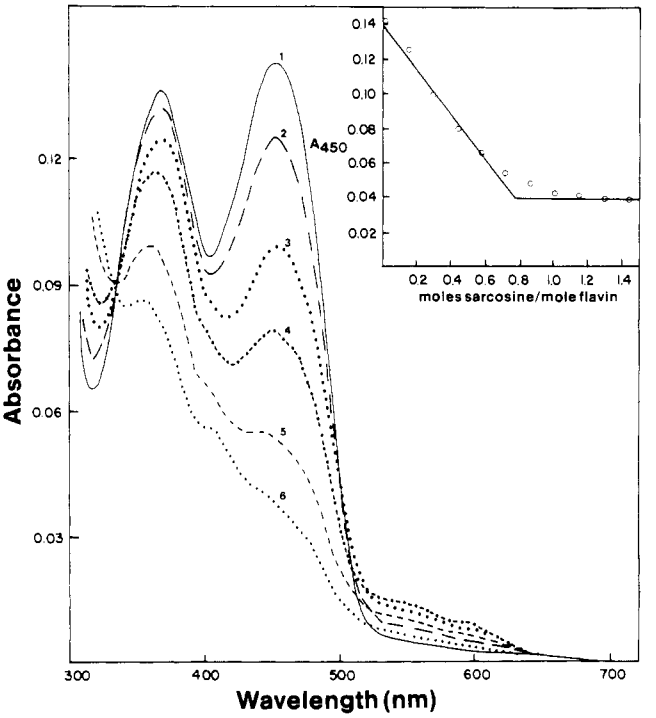

FIGURE 2: Titration of sarcosine oxidase with sarcosine. Curve 1 is the spectrum of the enzyme in 0.01 M potassium phosphate buffer, pH 8.0, under anaerobic conditions at 5 °C. Curves 2–6 were recorded after adding 0.142, 0.286, 0.429, 0.716, and 1.43 mol of sarcosine/mol of flavin. The inset shows a plot of absorbance at 450 nm vs. the amount of substrate added.

evidence that flavin is the only redox-active group is obtained by titration of the enzyme with sarcosine (Figure 2). That both flavins in sarcosine oxidase are reducible (either directly or indirectly) by substrate is suggested by the fact that most of the absorbance of the enzyme at 450 nm is bleached upon addition of stoichiometric amounts of substrate. As observed with dithionite, reduction with sarcosine is accompanied by a transient increase in absorbance in the long-wavelength region. The extent of bleaching at 450 nm with excess sarcosine (72%) is somewhat less than that observed with excess

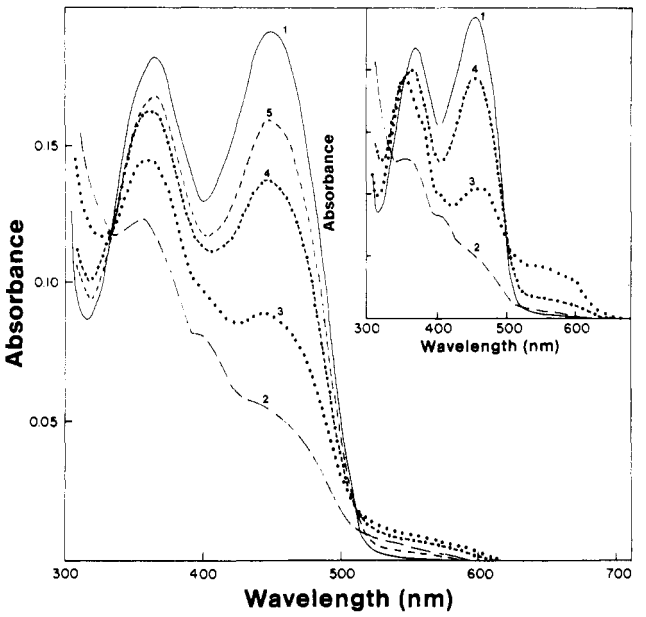

FIGURE 3: Reaction of sarcosine oxidase with sarcosine under aerobic conditions. Curve 1 is the spectrum of the enzyme in 0.01 M potassium phosphate pH 8.0 at 5 °C. Curve 2 was recorded immediately after adding 4.0 mM sarcosine under aerobic conditions. Curves 3–5 were recorded after 20, 28, and 36 min, respectively. The spectrum recorded after 91 min is superimposable with curve 1. The inset shows data from the reaction of native enzyme (curve 1) with 40 mM sarcosine under otherwise similar conditions. Curve 2 was recorded immediately after substrate addition. Curves 3 and 4 were recorded after 24 and 48 h, respectively. The reaction could not be followed to completion owing to the onset of turbidity.

dithionite (85%). Assuming that the residual absorbance in the presence of excess dithionite can be used to calculate an extinction coefficient for fully reduced enzyme, it is estimated that the extent of bleaching observed with excess sarcosine corresponds to 85% reduction of the enzyme flavin. A plot of absorbance at 450 nm vs. moles of substrate added (Figure 2, inset) yields an extropolated end point of 0.78 mol of sarcosine/mol of total flavin. Correcting for the fact that substrate appears to reduce 85% of the enzyme flavin, the results correspond to 0.91 mol of sarcosine/mol of flavin reduced. The transient increase in absorbance at long wavelengths which we observe during titration with either substrate or dithionite was not detected in a recent study by Hayashi (1984) where 2.4 mol of sarcosine/mol of flavin was reported for enzyme reduction (79% bleaching at 450 nm). The basis for the difference in flavin reduction stoichiometry between the Hayashi study and our results is not clear but may stem from differences in anaerobic procedures. The procedure used in our studies leaves no detectable residual oxygen (<1 μM) (Jorns et al., 1984). Residual oxygen was not reported for the Hayashi procedure where samples were rendered anaerobic by treating with glucose plus glucose oxidase under a layer of parafilm.

The spectrum observed immediately after addition of 4.0 mM sarcosine under aerobic conditions (Figure 3) is similar to that observed in anaerobic experiments with stoichiometric amounts of sarcosine. In both cases a small increase in absorbance at $\lambda > 510$ nm is observed for the reduced enzyme. A small further increase in this region is observed transiently during reoxidation of enzyme reduced with 4.0 mM sarcosine in a reaction that is complete within 90 min. At a 10-fold higher substrate concentration (40 mM sarcosine) the reaction is quite slow and cannot be followed to completion owing to denaturation of the enzyme. However, under these conditions the species that absorbs at longer wavelengths accumulates in larger amounts (Figure 3, inset). That this species is a blue, neutral flavin radical is suggested, in part, on the basis of the similarity of its spectral properties with those observed for other neutral radicals (Müller et al., 1972). Further evidence is provided by the fact that a similar long-wavelength intermediate, observed by Hayashi (1984) during reduction of the enzyme with dithiothreitol, exhibits an ESR signal with a line width in the range observed for other neutral flavin radicals (Hayashi, 1984; Palmer et al., 1971; Edmonson et al., 1981). The amount of radical formed during reoxidation of enzyme reduced with 40 mM sarcosine has been estimated by assuming an extinction coefficient in the range observed for other blue flavoprotein radicals ($E_{580} = 3.4 \times 10^3$–$5.4 \times 10^3$ M^{-1} cm^{-1}) (Müller et al., 1972), and corresponds to 35–50% conversion of oxidized flavin to the radical form.

Quantitative radical formation has been observed with many flavoproteins during photoreduction in the presence of an external electron donor (Massey & Palmer, 1966; Massey & Hemmerich, 1978). To further investigate the nature of the radical that is formed with sarcosine oxidase, photoreduction of the enzyme in the presence of 5-deazariboflavin was studied in the pH range 6–9 with EDTA or oxalate as electron donor. Under all conditions tested the photoreactions appear to proceed via a blue radical intermediate as judged by the increase in absorbance in the range 510–650 nm. The radical yield at pH 8.0 and 9.0 is similar to that observed during reduction of the enzyme with dithionite. A progressive increase in radical yield is observed at lower pH values. At pH 6.0 the maximum amount of radical observed approaches the level seen during reoxidation of enzyme reduced with 40 mM sarcosine. Attempts to increase radical yield by conducting the photoreduction at more acidic pH values were prevented by the instability of the enzyme at low pH.

Formation of a reversible covalent complex involving nucleophilic attack of sulfite at the 5-position of enzyme-bound flavin is a property characteristically observed with flavoprotein oxidases (Massey et al., 1969; Jorns & Hersh, 1976). That sarcosine oxidase might react with sulfite is suggested by the somewhat less than quantitative recovery of absorbance at 450 nm (94%) observed after reoxidation of dithionite-reduced enzyme. The apparent small loss of absorbance might reflect reaction of the enzyme with sulfite formed during dithionite oxidation. Evidence in support of this hypothesis is provided by results obtained during titration of the enzyme with sulfite (Figure 4) where a progressive loss of absorbance at 450 nm is observed, as expected for formation of a sulfite complex. That the reaction with sarcosine oxidase is reversible is evidenced by the return to the original spectrum of native enzyme observed upon mixing the complex (1.6×10^{-5} M enzyme-bound flavin plus 8.8×10^{-4} M sulfite) with a sulfite scavenger such as formaldehyde (10 mM). A value of 1.1×10^{-4} M was obtained for the dissociation constant of the complex from a Benesi–Hildebrand plot (Benesi & Hildebrand, 1949) (Figure 4, inset).

The feature that distinguishes the sulfite reaction with sarcosine oxidase from that observed with all other flavoprotein oxidases is that only half (48%) of the flavin in sarcosine oxidase reacts with sulfite as evidenced by the residual absorbance at 450 nm observed in the presence of excess sulfite. That the enzyme–sulfite complex is catalytically inactive is evidenced by the nearly complete inhibition (93%) of activity observed when assays are conducted in the presence of excess sulfite. That the loss of activity is reversible is supported by the nearly quantitative return of activity (97%) observed when the enzyme is mixed with excess sulfite but then diluted in

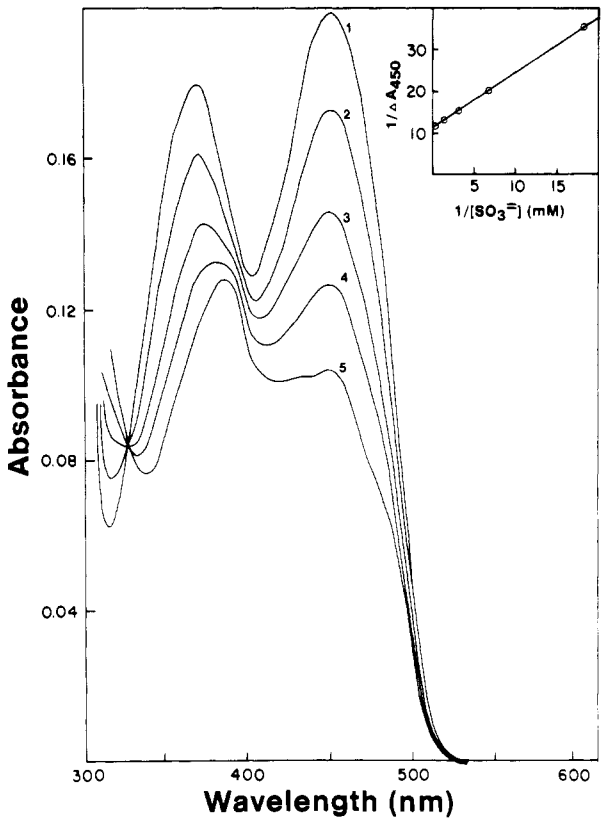

FIGURE 4: Titration of sarcosine oxidase with sulfite. Curve 1 is the spectrum of the starting enzyme in 0.01 M potassium phosphate buffer, pH 8.0, at 5 °C. Curves 2–5 were recorded after adding 5.5×10^{-5}, 1.5×10^{-4}, 3.3×10^{-4}, and 1.6×10^{-2} M sulfite, respectively. The inset shows a Benesi–Hildebrand plot (Benesi & Hildebrand, 1949) of absorbance changes at 450 nm.

order to dissociate the complex under assay conditions. That complexation of 50% of the flavin causes nearly complete loss of catalytic activity might reflect the fact that only one of the two nonequivalent flavins in sarcosine oxidase reacts with sulfite whereas both flavins are required for catalysis. In this case it seems reasonable to consider that one flavin might function as a "dehydrogenase" flavin, accepting reducing equivalents from sarcosine and then transferring them to the "oxidase" flavin which in turn would reduce oxygen to hydrogen peroxide. Since sulfite reactivity is observed only with flavoprotein oxidases, it might be expected that only the oxidase flavin in sarcosine oxidase would react with sulfite. In this case is expected that sulfite would not directly interfere with sarcosine oxidation but would rather inhibit the enzyme by blocking the reoxidation reaction. Consistent with this hypothesis, addition of sarcosine (4.0 mM) in the presence of excess sulfite results in rapid reduction of the sulfite-unreactive flavin (Figure 5). That sulfite interferes with the oxygen reaction is suggested by the fact that no reoxidation is detectable for 3 h after substrate addition under aerobic conditions whereas reoxidation is complete in 90 min for the comparable reaction in the absence of sulfite. Reoxidation in the presence of sulfite does occur but only after prolonged incubation. Further evidence that sarcosine and sulfite do not interact with the same flavin is suggested by the fact that the stability of the sulfite complex is unaffected by addition of excess acetate (2.9×10^{-2} M), a known competitive inhibitor with respect to sarcosine ($K_i = 2.9 \times 10^{-3}$ M) (Hayashi et al., 1983a). This result should be contrasted with data obtained with other flavoprotein oxidases where substrate, competitive inhibitors, and sulfite interact with a single flavin at the active site. In these enzymes it has been found that competitive

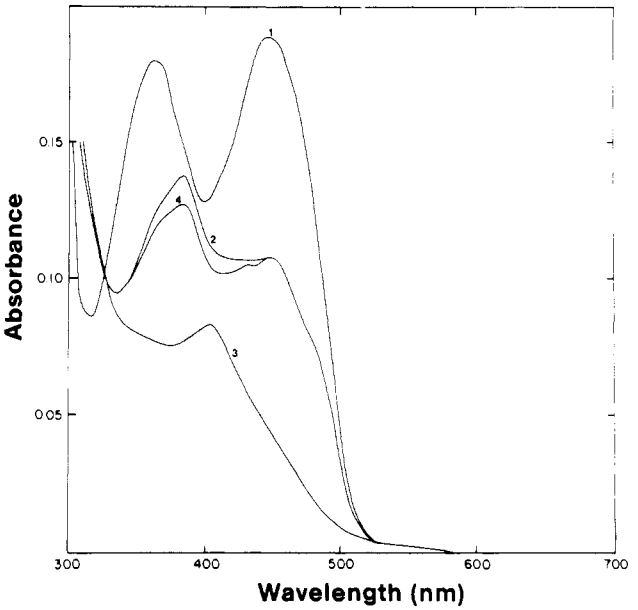

FIGURE 5: Effect of sulfite on the reaction of sarcosine oxidase with sarcosine. Curve 1 is the spectrum of the enzyme in 0.01 M potassium phosphate buffer, pH 8.0, at 5 °C. Curve 2 was recorded after adding 1.6×10^{-2} M sulfite. Curve 3 was recorded immediately after adding 4.0 mM sarcosine. The spectrum remained unchanged for 3 h. Curve 4 was recorded after 20 h.

inhibitors interfere with formation of the sulfite complex (Massey et al., 1969; Jorns & Hersh, 1976; Schuman & Massey, 1971).

In an effort to determine which flavin reacts with sulfite and oxygen, the noncovalent flavin was removed by precipitation of the enzyme with acid ammonium sulfate. The resulting semiapoprotein preparation exhibited 2% of the activity observed for native enzyme. The covalent flavin in the preparation is not reducible by substrate as evidenced by the small decrease in absorbance at 450 nm (3%) observed upon addition of excess sarcosine under conditions where most of the flavin absorbance is bleached with native enzyme. On the other hand, the covalent flavin in semiapoprotein does react with sulfite in a reaction that results in the bleaching of 75% of the absorbance of the preparation at 450 nm (Figure 6). That sulfite does not bleach an even larger percentage of the flavin absorption might reflect a certain amount of denaturation during the preparation of semiapoprotein. The dissociation constant determined for the complex formed with semiapoprotein ($K_d = 9.4 \times 10^{-5}$ M) is very similar to that observed with native enzyme ($K_d = 1.1 \times 10^{-4}$ M).

The spectrum of the semiapoprotein in the presence of excess sulfite exhibits a peak at 410 nm. A similar peak is observed with native enzyme in the presence of sulfite and sarcosine (Figure 5). A shoulder in this region is observed for enzyme reduced with sarcosine in the absence of sulfite. A similar shoulder is observed during titration of the enzyme with dithionite but disappears toward the end of the titration. The nature of the 410-nm absorbance is currently under investigation.

Discussion

That the loss of activity observed with sarcosine oxidase in the presence of sulfite is due to formation of a complex with only the covalently bound, oxidase flavin is supported by the following observations: (1) Only 50% of the absorbance of the enzyme at 450 nm is bleached in the presence of excess sulfite. (2) The sulfite-unreactive flavin remains readily reducible by sarcosine. (3) Reoxidation of enzyme reduced in

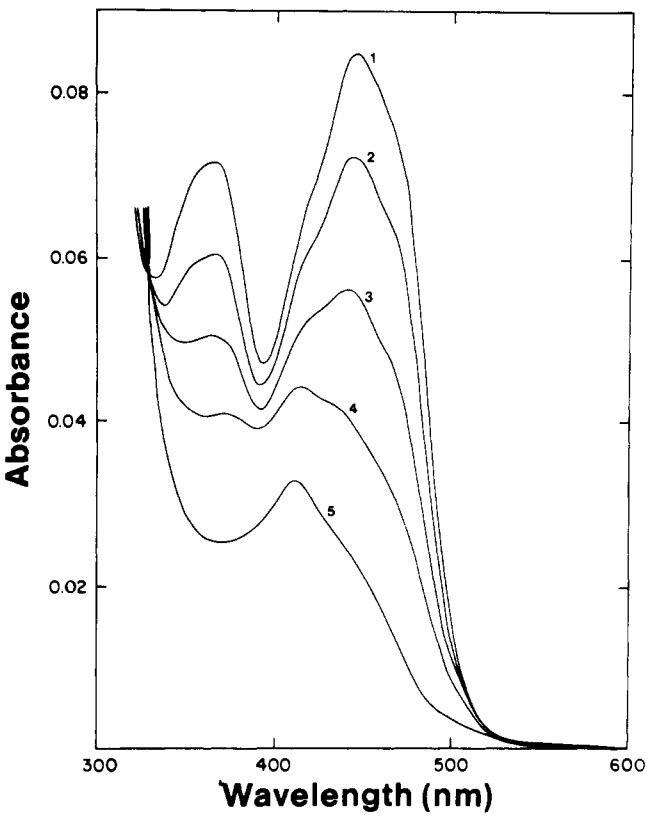

FIGURE 6: Titration of semiapoenzyme with sulfite. Curve 1 is the spectrum of semiapoenzyme in 0.01 M potassium phosphate buffer, pH 8.0, at 5 °C. Curves 2–5 were recorded after adding 2.75×10^{-5}, 7.35×10^{-5}, 4.39×10^{-4}, and 4.59×10^{-2} M sulfite, respectively.

the presence of sulfite is strongly inhibited. (4) No effect on sulfite complex stability is observed with excess acetate, an inhibitor competitive with sarcosine. (5) Sulfite reactivity is not appreciably affected by removal of the noncovalent flavin. That the covalent flavin in semiapoprotein is not reducible by sarcosine suggests that the noncovalent flavin is responsible for substrate dehydrogenation. Subsequent transfer of electrons from the dehydrogenase to the oxidase flavin would explain why both flavins are reducible by sarcosine in native enzyme. In this case it is expected that the enzyme would have only a single binding site for sarcosine. This hypothesis is supported by the fact that complete inactivation of the enzyme is observed upon modification of a single histidine residue with diethyl pyrocarbonate in a reaction that is strongly inhibited by acetate (Hayashi et al., 1983b). On the other hand, it might be argued that reduction of the covalent flavin in semiapoprotein with sarcosine is not observed because this property is destroyed during the acid ammonium sulfate precipitation. While retention of sulfite reactivity and secondary structure (Hayashi et al., 1980) similar to those observed with native enzyme argues against extensive denaturation, further studies are clearly needed since reconstitution attempts with FAD have thus far yielded only poor (~10%) return of catalytic activity.

In addition to reacting with sulfite, most flavoprotein oxidases show thermodynamic stabilization of the red, anionic semiquinone (Massey et al., 1969; Massey & Hemmerich, 1980). Massey & Hemmerich (1980) have suggested that oxidase enzymes contain a protonated base near the N-1 position of the flavin that facilitates formation of a sulfite complex and also stabilizes the red radical. A red radical is also observed with enzymes other than the oxidases when the flavin is covalently attached and substituted at the 8α-position (Edmondson et al., 1981). Since the flavin in sarcosine oxidase that reacts with oxygen and sulfite is also covalently attached to the protein at the 8α-position, it is somewhat surprising that we do not observe a red radical with this enzyme. On the other hand, blue radicals are frequently observed with flavoproteins other than the oxidases. That the blue radical observed with sarcosine oxidase is formed with the dehydrogenase rather than the oxidase flavin is suggested by the fact that the radical observed during reduction of native enzyme with dithiothreitol is not seen during reduction of the covalent flavin in semiapoprotein preparations (Hayashi, 1984).

To determine whether sarcosine oxidase from *Corynebacterium* sp. U-96 is unique, several Corynebacteria were isolated from soil, which produce sarcosine oxidase as an inducible enzyme. A strain (*Corynebacterium* sp. P-1) that produces the enzyme in fairly large amounts and that is easily disrupted with lysozyme was selected for further study. The enzyme isolated from this strain exhibits an electrophoretic mobility that is similar to but not identical with that observed for the enzyme from *Corynebacterium* sp. U-96. Half of the flavin in sarcosine oxidase from *Corynebacterium* sp. P-1 is present as noncovalently bound FAD whereas the other half is covalently attached to the protein (K. Kvalnes-Krick and M. Jorns, unpublished results). The results suggest that the synthesis of an enzyme containing both covalent and noncovalent flavin is not a very unusual event in Corynebacteria, at least as far as sarcosine oxidase is concerned.

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Further Characterization of an Essential Histidine Residue of Ribulose-1,5-bisphosphate Carboxylase/Oxygenase†

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ABSTRACT: Ribulose-1,5-bisphosphate carboxylase/oxygenase from spinach leaves was previously shown to have one essential histidine residue per active site [Bhagwat, A. S., & Ramakrishna, J. (1981) *Biochim. Biophys. Acta 662*, 181-189; Saluja, A. K., & McFadden, B. A. (1982) *Biochemistry 21*, 89-95]. Here, the pH dependence of the reactivity of the enzyme toward diethyl pyrocarbonate was used to delineate the pK_a value of this putatively essential histidine residue. A value of 6.85 at 15 °C and ionic strength I = 0.10 was experimentally obtained. Corrected for temperature and ionic strength, the pK_a became 6.54, in excellent agreement with a pK_a of 6.55 identified in a pH profile of V_{max}/K_m for ribulose 1,5-bisphosphate at 30 °C and ionic strength I = 0.14. These findings further substantiate the hypothesis that a histidine residue is involved in the catalytic mechanism of ribulose-1,5-bisphosphate carboxylase/oxygenase.

Lack of a detailed three-dimensional structure of ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBP carboxylase)[1] makes the kinetic analysis of chemical modificatin reactions still a viable option for investigating amino acid residues functional in the catalysis of carboxylation and oxygenation of RuBP. While RuBP carboxylase activity was shown to be sensitive to derivatization of lysine (Whitman & Tabita, 1976; Paech et al., 1977; Schloss et al., 1978b), arginine (Lawlis & McFadden, 1978; Schloss et al., 1978a; Chollet, 1981), histidine (Bhagwat & Ramakrishna, 1981; Saluja & McFadden, 1980, 198), tyrosine [Robison & Tabita, 1979; Bhagwat, 1982; but cf. Barnard et al. (1983)], cysteine (Rabin & Trown, 1964), and carboxyl (Valle & Vallejos, 1984) residues, only lysine-201 of the spinach enzyme was assigned a function in the mechanism of RuBP carboxylase by virtue of the fact that CO_2, as a homotropic effector, covalently binds to the ϵ-amino group of this lysine (Lorimer, 1981). To suggest the "essentiality" of other amino acid residues, one must use circumstantial evidence until such time that X-ray crystallography or other physical methods establish their presumed positions.

In the studies reported herein, we have used pH-dependent rates of ethoxyformylation of a single histidine residue of RuBP carboxylase (Bhagwat & Ramakrishna, 1981; Saluja & McFadden, 1982) to delineate its pK_a. Responses of K_m and V_{max} for RuBP to pH changes further support the notion that this histidine is involved in the catalysis of RuBP carboxylation.

Experimental Procedures

Materials

RuBP carboxylase was purified to homogeneity from freshly harvested spinach (*Spinacia oleracea* L.) leaves and stored at −100 °C as pellets formed from a precipitate with 50% saturated ammonium sulfate (McCurry et al., 1982). Prior to use, the precipitated enzyme was resuspended in 50 mM Bicine–NaOH, pH 8.0, containing 1 mmol of DTT. Dialysis for 12–16 h (overnight) against 1 L of 50 mM Bicine–NaOH and 0.2 mM EDTA, pH 8.0, at room temperature, followed by a brief centrifugation for clarification, yielded solutions with protein concentrations of 40–80 mg/mL. Activation of RuBP carboxylase (2 mg/mL) was allowed to proceed at room temperature for at least 30 min with 10 mM $NaHCO_3$ and 10 mM $MgCl_2$ in the assay buffer placed in a "Reacti-vial" equipped with a "Mininert-valve" (Pierce Chemical Co.). The specific activity [based on protein determined by the method of Gornall et al. (1948)] was 1.6–2.0 μmol of CO_2 fixed·min^{-1}·(mg of protein)$^{-1}$ in an assay buffer consisting of 10 mM $NaHCO_3$, 0.5 mM RuBP, 10 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, and 0.1 M Bicine–NaOH, pH 8.3 at 30 °C. RuBP was synthesized enzymically from ribose 5-phosphate (Horecker et al., 1958). $NaH^{14}CO_3$ was purchased from ICN Chemical and Radioisotope Division and was adjusted to 0.16 Ci/mol for standard assays or to 1.9 Ci/mol for assays in the

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[1] Abbreviations: Bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; DEPC, diethyl pyrocarbonate; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid disodium salt; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; RuBP, D-ribulose 1,5-bisphosphate; RuBP carboxylase, D-ribulose-1,5-bisphosphate carboxylase/oxygenase (EC 4.1.1.39); Da, dalton(s).

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