Preparation and Properties of Recombinant Corynebacterial Sarcosine Oxidase: Evidence for Posttranslational Modification during Turnover with Sarcosine†

Lawrence J. Chlumsky, Lening Zhang, Andrew J. Ramsey, and Marilyn Schuman Jorns*

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

Received March 16, 1993; Revised Manuscript Received July 20, 1993®

ABSTRACT: The genes encoding the four subunits of sarcosine oxidase from *Corynebacterium* sp. P-1 were isolated and overexpressed in a single step by using indicator plates to screen a genomic library for colonies that generated hydrogen peroxide in a sarcosine-dependent reaction. The genomic library was constructed by inserting size-fractionated genomic DNA, previously subjected to partial digestion by *Sau3*AI, into pBluescript II SK (+). At least 1.0 kb, but less than 4.0 kb, can be deleted from the 3′ end of the original cornyebacterial insert (7.3 kb) without affecting sarcosine oxidase expression, consistent with the estimated 5.0-kb operon size. Recombinant sarcosine oxidase is isolated as a heterotetramer containing equimolar amounts of covalent and noncovalent flavin, identical to that observed for enzyme isolated from *Corynebacterium* sp. P-1. Despite its similar flavin content, recombinant enzyme exhibits significantly different spectral properties than enzyme from *Corynebacterium* sp. P-1 (values shown in parentheses) [ϵ_{450} = 9.7 (12.7) mM^{-1} cm^{-1}; A_{368}/A_{450} = 1.0 (0.83); A_{280}/A_{450} = 16.9 (12.2)]. This difference is due to the fact that about half of the covalent flavin in recombinant enzyme forms a reversible covalent 4a-adduct with a cysteine residue (λ_{max} = 383 nm; ϵ_{383} = 7.3 mM^{-1} cm^{-1}). The equilibrium is shifted in favor of adduct dissociation by oxidizing the cysteine residue with hydrogen peroxide or by alkylation with methyl methanethiosulfonate in a reaction that is fully reversible upon addition of excess dithiothreitol. The cysteine residue is also oxidized during aerobic turnover with sarcosine. Reaction of the cysteine residue with hydrogen peroxide (or a precursor) formed during turnover partially competes with the release of hydrogen peroxide into solution, as judged by the effect of catalase on this reaction. Although the same specific activity is observed for recombinant enzyme and enzyme from *Corynebacterium* sp. P-1, the recombinant enzyme exhibits a pronounced lag in an NADH peroxidase-coupled assay. The lag is eliminated by prior disruption of the 4a-thiolate adduct via reaction with hydrogen peroxide or methyl methanethiosulfonate. The results show that the 4a-thiolate adduct is an inactive form of sarcosine oxidase that can be activated by reaction with sarcosine in what appears to be the first example of a posttranslational modification associated with turnover. Complete activation occurs *in vivo* when sarcosine oxidase is produced in *Corynebacterium* sp. P-1, where enzyme synthesis is induced by growth of the organism with sarcosine as the source of carbon and energy. The opportunity for *in vivo* activation is diminished when the recombinant enzyme is produced in *Escherichia coli* under the control of a vector-encoded *lac* promoter.

Sarcosine oxidase is produced as an inducible enzyme when *Corynebacterium* sp. P-1 is grown with sarcosine as the source of carbon and energy (Kvalnes-Krick & Jorns, 1986). The enzyme catalyzes the oxidative demethylation of sarcosine to yield glycine, formaldehyde, and hydrogen peroxide (eq 1).

$$CH_3N^+H_2CH_2CO_2^- + O_2 + H_2O \rightarrow H_3N^+CH_2CO_2^- + H_2CO + H_2O_2 \quad (1)$$

Tetrahydrofolate does not affect the rate of sarcosine oxidation, but the reaction in the presence of tetrahydrofolate yields 5,10-methylenetetrahydrofolate instead of formaldehyde (Kvalnes-Krick & Jorns, 1987), similar to the reaction observed for mammalian sarcosine dehydrogenase (Wittwer & Wagner, 1981; Porter et al., 1985; Steenkamp & Husain, 1982). Corynebacterial sarcosine oxidase has two binding sites for tetrahydrofolate and a single binding site for sarcosine (Kvalnes-Krick & Jorns, 1987; Zeller et al., 1989). The enzyme contains both covalent [(8α-N^3-histidyl)FAD] and noncovalent FAD,[1] a feature which distinguishes this enzyme from most other known flavoproteins. Corynebacterial sarcosine oxidase is composed of four nonidentical subunits (α, 100 000; β, 42 000; γ, 20 000; δ, 6000). The covalent flavin is attached to the β subunit (Kvalnes-Krick & Jorns, 1986). The noncovalent (dehydrogenase) flavin accepts electrons from sarcosine, which are then transferred in one-electron steps to the covalent (oxidase) flavin, which reduces oxygen to hydrogen peroxide (Kvalnes-Krick & Jorns, 1986; Jorns, 1985; Zeller et al., 1989; Ali et al., 1991). With respect to the presence of two flavins per active site with different roles in catalysis, corynebacterial sarcosine oxidase is similar to several other enzymes, including NADPH cytochrome P450 reductase (Vermillion et al., 1981; Oprian & Coon, 1982), NO synthase (Bredt et al., 1991; Stuehr et al., 1991; Mayer et al., 1991; Hevel et al., 1991), and NADPH sulfite reductase (Siegel et al., 1971; Ostrowski et al., 1989), except that both flavins (FAD, FMN) in these enzymes are noncovalently bound. As detailed in a recent review (Kvalnes-Krick & Jorns, 1991),

† This work was supported in part by Grant GM 31704 (M.S.J.) from the National Institutes of Health.

* To whom requests for reprints and correspondence should be addressed.

® Abstract published in *Advance ACS Abstracts,* September 15, 1993.

[1] Abbreviations: FAD, flavin adenine dinucleotide; EDTA, ethylenediaminetetraacetic acid; IPTG, isopropyl β-D-thiogalactopyranoside; SDS, sodium dodecyl sulfate; TEMED, *N,N,N′,N′*-tetramethylethylenediamine; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; TCA, trichloroacetic acid; MMTS, methyl methanethiosulfonate; DDT, dithiothreitol.

0006-2960/93/0432-11132$04.00/0

three types of microbial sarcosine oxidases can be distinguished on the basis of their quaternary structure: monomers (α, 45 000), heterodimers (α, 100 000; β, 55 000), and heterotetramers similar to the corynebacterial enzyme. Although all enzymes contain covalent flavin, only the heterotetramers contain both covalent and noncovalent flavin.

The complex quaternary structure and multiple binding sites for substrates and prosthetic groups in corynebacterial sarcosine oxidase provide a particularly intriguing target for structure–function studies. In this paper we report the cloning and overexpression of the genes that code for this enzyme. On the basis of results obtained for other multisubunit bacterial enzymes (Stewart, 1988; Cole et al., 1985; Wood et al., 1984), the genes coding for the subunits of sarcosine oxidase were likely to be organized in an operon with a coding region of about 5 kb. To clone and overexpress this operon, the individual genes might be isolated, sequenced, reassembled into an operon, and then transferred into an expression vector. In an attempt to short-circuit this multistep procedure, a corynebacterial genomic library was constructed using pBluescript II SK (+), an expression vector that can accommodate DNA fragments large enough to code for the putative sarcosine oxidase operon. A clone that overexpressed corynebacterial sarcosine oxidase under the influence of the vector-encoded *lac* promoter was isolated by using indicator plates (Sagai et al., 1989) to screen the library for colonies that generated hydrogen peroxide in a sarcosine-dependent reaction. The quaternary structure and flavin content of recombinant sarcosine oxidase are identical to those of enzyme produced in *Corynebacterium* sp. P-1. However, about half of the covalent flavin in recombinant enzyme is present as a reversible covalent adduct with a cysteine residue, a feature which affects the enzyme's visible absorption spectrum and catalytic properties. The adduct is disrupted during turnover with sarcosine in a process involving oxidation of the cysteine residue. To the best of our knowledge, the data appear to represent the first example of a posttranslational modification associated with turnover.

MATERIALS AND METHODS

Materials. Ribonuclease A, lysozyme, tetracycline, pronase E, *o*-dianisidine, iodonitrotetrazolium violet, horse radish peroxidase, methyl methanethiosulfonate (MMTS), hydrogen peroxide, and 2-furoic acid were purchased from Sigma. Ampicillin and tris(hydroxymethyl)aminomethane were from Aldrich. EDTA was from Fischer. Bacto-tryptone, Bacto-peptone, Bacto-yeast extract, Bacto-agar, and Bacto-beef extract were purchased from Difco. Acrylamide, bis-acrylamide, TEMED, and ammonium persulfate were obtained from Bio-Rad. Restriction endonucleases were purchased from Promega, New England Biolabs, or Oncogene Science and used according to the manufacturer's procedures. T4 DNA ligase and calf intestinal alkaline phosphatase were from Promega. Phenyl-Sepharose CL-4B, Ultrogel AcA 34, and DEAE-Sephacel were purchased from Pharmacia/LKB. Spectra/Por dialysis tubing (MW cutoff 3500) was from Fisher Scientific. IPTG and X-Gal were purchased from United States Biochemical. Molecular weight protein standards were obtained from Bio-Rad.

Cells Strains and Plasmids. Corynebacterium sp. P-1 was previously isolated in this laboratory (Kvalnes-Krick & Jorns, 1986). *Escherichia coli* strain XL1-Blue and pBluescript plasmids were purchased from Stratagene. *E. coli* cell cultures were grown in LB media or LB agar plates (Ausubel et al., 1989). In the case of cells carrying pBluescript II SK (+) or KS (+) and their derivatives, LB medium was supplemented with ampicillin (100 μg/mL). Competent cells were prepared according to the method of Hanahan (1983).

Activity and Protein Assays. Sarcosine oxidase activity was determined on the basis of the amount of formaldehyde formed using the Nash procedure (Nash, 1953), as described previously (Jorns, 1985). For purified preparations, activity was also measured on the basis of the amount of hydrogen peroxide produced using an NADH peroxidase-coupled assay, as described by Kvalnes-Krick & Jorns (1987). Protein concentration was determined from the absorbance at 280 nm by using the extinction coefficient ($E^{\%}$ = 13.1) reported by Suzuki (1981) or by the Bradford method (Bradford, 1976). With purified enzyme, nearly identical results were obtained with either method. With crude extracts, the value obtained by the Bradford method was about 15% lower than that obtained by the A_{280} method.

Construction of a Corynebacterium sp. P-1 Genomic Library. All common DNA manipulations were performed by standard procedures (Ausubel et al., 1989). A 500-mL culture of growth media was inoculated with a 5-mL overnight culture of *Corynebacterium* sp. P-1 and then incubated at 30 °C as previously described (Kvalnes-Krick & Jorns, 1986). After 45 h, the cells were harvested by centrifugation at 4 °C (15 min at 10 500*g*). The cell pellet was washed twice with 10 mM Tris-Cl, pH 8.0, containing 1 mM EDTA, and stored at −20 °C. Genomic DNA was isolated and then partially digested with *Sau*3AI restriction endonuclease. The digest was fractionated on a 20–40% sucrose gradient to yield a pool of DNA fragments of approximately 5.0–9.4 kb. pBluescript II SK (+) was digested with *Bam*HI, treated with calf intestinal alkaline phosphatase, and then mixed with 5.0–9.4-kb corynebacterial DNA fragments. After being treated with T4 DNA ligase, the samples were used to transform *E. coli* strain XL1-Blue to ampicillin resistance.

Screening the Corynebacterial Genomic Library. In one approach, the transformation mixture was directly plated onto indicator plates containing ampicillin to screen for ampicillin-resistant colonies that also expressed sarcosine oxidase activity. Alternatively, cells were first screened for ampicillin resistance, accompanied by the absence of β-galactosidase activity, and transformants were then selected for further screening on indicator plates. To prepare the indicator plates, 1 L of solution containing 5 g of peptone, 2 g of beef extract, 5 g of yeast extract, 1 g of NaCl, 1 g of K_2HPO_4, 0.5 g of $MgSO_4$, 9 g of sarcosine, plus 15 g of agar was sterilized by autoclaving. Solutions containing horse radish peroxidase (500 IU), *o*-dianisidine (0.01 g), or ampicillin (100 mg/mL) in 1 mL of sterile water were filter sterilized and then mixed with the agar solution just before the plates were poured. (Ampicillin was omitted for control experiments with *Corynebacterium* sp. P-1 and *E. coli* strain XL1-Blue). Except in studies with *Corynebacterium* sp. P-1, 10 μL of an IPTG solution (20 mg/mL) was spread over the surface of the plate just before use. The procedure for preparing indicator plates is similar to a method previously described (Sagai et al., 1989). As will be detailed, somewhat different temperature conditions were used for plate incubation in various experiments. However, cells expressing sarcosine oxidase activity could generally be detected as brown colonies on indicator plates after a 16–48-h incubation, whereas all other clones formed colorless colonies under these conditions. (All white colonies eventually turn brown when plates are stored for several weeks at 4 °C.) As will be described, a clone (XL1-Blue/pLJC305) expressing

sarcosine oxidase activity was isolated by this screening procedure.

Subcloning Experiments. To prepare pLJC306, plasmids pLJC305 and pBluescript II KS (+) were treated with *Xba*I and *Sac*I. The two plasmid digests were combined, treated with T4 DNA ligase, and then used to transform XL1-Blue competent cells to ampicillin resistance. To construct pLJC325, plasmid pLJC305 was treated with *Xba*I, *Hind*III and *Sca*I; the vector pBluescript II KS (+) was treated with *Xba*I and *Hind*III. The plasmid digests were combined, treated with T4 DNA ligase, and used to transform XL1-Blue competent cells to ampicillin resistance. In each case, the transformation mixtures were screened for α-complementation on plates containing IPTG and X-Gal (Ausubel et al., 1989). White colonies were further screened by restriction enzyme analysis to identify the desired constructs. These constructs were then screened for sarcosine oxidase expression on indicator plates.

Electrophoresis. Comparison of native forms of sarcosine oxidase, produced in *Corynebacterium* sp. P-1 or *E. coli* (XL1-Blue/pLJC305), was conducted using polyacrylamide slab gels (2.5% stacking gel and 14% resolving gel) at pH 8.3 (Davis, 1964). Proteins were visualized by staining with Coomassie blue (Weber & Osborn, 1969). Sarcosine oxidase activity was detected as a pink band when the gels were stained using 0.32 mM iodonitrotetrazolium violet as a redox indicator dye (Kvalnes-Krick & Jorns, 1986). To prepare crude cell extracts for the native gel electrophoretic studies shown in Figure 3, 500-mL volumes of appropriate media were inoculated with a 1-mL overnight culture of *Corynebacterium* sp. P-1, XL1-Blue/pLJC305, or XL1-Blue/pLJCXXX. All cultures were incubated for 24 h at 30 °C. In the case of XL1-Blue/pLJC305, two samples were prepared and treated identically, except that IPTG (200 μL of a solution containing 20 mg/mL) was added to one of the samples after 7 h of incubation at 30 °C. The cells were harvested by centrifugation, suspended in 20 mL of 0.02 M potassium phosphate buffer, pH 7.0, containing sucrose plus protease inhibitors and nucleases as previously described (Kvalnes-Krick & Jorns, 1986), and lysed by sonication for a total of 50 s in 10-s intervals. Cell debris was removed by centrifugation for 1 h at 27 000*g*.

Subunit composition studies were conducted by using polyacrylamide gradient (3–27% acrylamide) slab gels in the presence of phosphate buffer (100 mM sodium phosphate, pH 6.7) containing 0.1% SDS. This procedure was a modification of the system described by Swaney and Kuehl (1976). Proteins were visualized as described above. To determine the subunit composition of sarcosine oxidase in crude extracts of *Corynebacterium* sp. P-1 or *E. coli* (XL1-Blue/pLJC305), the samples were first subjected to native gel electrophoresis as described above. The band containing sarcosine oxidase was detected by staining for activity. The activity band was sliced from the gel, placed in dialysis tubing (Spectra/Por, MW cutoff 3500), and electroeluted using the native gel buffer system in a horizontal gel apparatus.

Purification of Recombinant Sarcosine Oxidase. XL1-Blue/pLJC305 was grown in an incubator shaker in 2-L Erlenmeyer flasks containing 500 mL of LB media supplemented with ampicillin. The cultures were inoculated with 10 mL of an overnight culture and incubated at 30 °C until the A_{650} of the culture was approximately 0.6. IPTG (240 μg/mL) was then added to induce sarcosine oxidase production. After 12–16 h, the cells were harvested by centrifugation, washed with phosphate-buffered saline (0.8% NaCl, 0.02% KCl, 0.115% NaH_2PO_4, 0.02% KH_2PO_4), and then stored as a pellet at −20 °C. Purification of recombinant sarcosine oxidase was carried out under yellow light at 0–4 °C using cells obtained from 10 L of culture. The cell pellets were thawed on ice and then suspended in a minimum volume of cell lysis buffer [50 mM Tris, pH 8.0, containing 100 mM NaCl, 1 mM EDTA, and 10% (w/v) sucrose] containing protease inhibitors and nucleases as described previously (Kvalnes-Krick & Jorns, 1986). The cells were lysed by sonication (Branson Model 350, power setting = 5) for a total of 120 s in 10-s intervals. The temperature during sonication did not exceed 7 °C. Cell debris was removed by centrifugation (1 h at 31 000*g*). The cell lysate was then subjected to ammonium sulfate fractionation and chromatography on Ultrogel ACA 34, as described for the purification of sarcosine oxidase from *Corynebacterium* sp. P-1 (Kvalnes-Krick & Jorns, 1986). The Ultrogel ACA 34 column eluate of recombinant sarcosine oxidase (in 10 mM potassium phosphate buffer, pH 8.0) was mixed with 150 mM potassium phosphate, pH 7.0, containing 1.5 mM EDTA plus 3.0 M ammonium sulfate, to yield a final ammonium sulfate concentration of 1.0 M. The sample was then loaded onto a Phenyl-Sepharose CL-4B column (100 mL column) equilibrated with 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA plus 1.0 M ammonium sulfate. The column was washed with the equilibration buffer and then with same buffer containing 0.4 M ammonium sulfate. The enzyme was eluted with a linear gradient formed with 500 mL each of 50 mM potassium phosphate buffer, pH 7.0, containing 0.5 mM EDTA plus 0.4 or 0.0 M ammonium sulfate. Fractions containing sarcosine oxidase were pooled and then dialyzed against 5 mM potassium phosphate buffer, pH 7.5, containing 0.3 mM EDTA. The dialyzed enzyme was applied to a DEAE-Sephacel column (3.5 × 22 cm) equilibrated with the same buffer. The column was washed with this buffer and then with a linear gradient formed with 1 L each of 5 mM potassium phosphate buffer, pH 7.5, containing 0.0 or 0.5 M potassium chloride. Sarcosine oxidase was then eluted with a linear gradient formed with 1 L each of 10 mM potassium phosphate buffer, pH 7.5, containing 0.5 or 1.0 M potassium chloride. Fractions were assayed for both sarcosine oxidase and catalase (Bergmeyer, 1974) activity. Catalase-free sarcosine oxidase eluted at approximately 0.55 M potassium chloride. The sample (184 mL) was mixed with 25 mL of 200 mM potassium phosphate, pH 7.5, and ammonium sulfate was added to 70% saturation. The precipitate was collected by centrifugation, dissolved in a minimum volume of 10 mM potassium phosphate, pH 8.0, containing 0.3 mM EDTA, and then dialyzed against the same buffer. The dialyzed enzyme was stored at −70 °C.

Purification of Sarcosine Oxidase from Corynebacterium sp. P-1. The enzyme was purified similar to a procedure previously described (Kvalnes-Krick & Jorns, 1986), except that the steps involving chromatography on DEAE-cellulose and hydroxyapatite were replaced by chromatography on Phenyl-Sepharose CL-4B. The latter was conducted similar to that described above for the isolation of recombinant enzyme.

Spectroscopy. Absorption spectra were recorded using a Perkin-Elmer Lambda-3B spectrophotometer. Unless otherwise noted, all spectral measurements were conducted in 10 mM potassium phosphate buffer, pH 8.0.

To determine the relative amount of covalent and noncovalent flavin in recombinant sarcosine oxidase, the enzyme was precipitated with 5% TCA. The supernate obtained after centrifugation was neutralized, and the absorbance at 450

nm was recorded. The pellet was resuspended in 5% TCA and processed as just described. The combined absorbance of both TCA supernates at 450 nm was used to estimate the noncovalent flavin in the enzyme. The pellet then was redissolved in 0.5 M potassium phosphate buffer, pH 7.6, containing 4 M guanidine hydrochloride, and the amount of covalent flavin was determined from the absorbance at 450 nm. Flavin concentration was estimated using the extinction coefficient of free FAD (ϵ_{450} = 11 300 M^{-1} cm^{-1}). The extinction coefficient of the intact enzyme at 450 nm was calculated on the basis of the total amount of flavin recovered after denaturation. The extinction coefficient of the intact enzyme was also determined on the basis of the absorbance change at 450 nm observed after the solution was heated under conditions where the denatured protein does not precipitate (5 min at 100 °C in 10 mM potassium phosphate, pH 7.6).

Stock solutions of hydrogen peroxide were quantified by titration with potassium permanganate solutions previously standardized against sodium oxalate (Skoog & West, 1976). Hydrogen peroxide was also determined using NADH peroxidase. Reaction conditions were identical to the NADH peroxidase assay used to measure sarcosine oxidase activity (Kvalnes-Krick & Jorns, 1987), except that sarcosine was omitted and reactions were initiated by adding an aliquot of hydrogen peroxide. Similar results were obtained by either method.

RESULTS

Isolation of a Clone Expressing Sarcosine Oxidase Activity. Preliminary studies were conducted to determine whether indicator plates could be used to screen the genomic library for colonies expressing sarcosine oxidase activity. The indicator plate assay assumes that cells expressing sarcosine oxidase activity will generate hydrogen peroxide and form brown colonies when grown on media containing sarcosine, horse radish peroxidase, and *o*-dianisidine. This approach is similar to a procedure previously described (Sagai et al., 1989). In control studies with *Corynebacterium* sp. P-1, brown colonies were observed after the plates were incubated for approximately 24–48 h at 30 °C. Only white colonies were formed when sarcosine was omitted from the plates. *E. coli* strain XL1-Blue formed white colonies when grown on standard indicator plates for 24 h at 34 °C and then left for 24 h at room temperature. This showed that the strain did not express an endogenous sarcosine oxidase that might interfere with the use of indicator plates.

The results suggested that the corynebacterial DNA library might be screened in a single step by plating transformed XL1-Blue cells directly onto sarcosine oxidase indicator plates that also contained ampicillin. In one experiment, about 1500 white, ampicillin-resistant colonies were observed after on overnight incubation at 34 °C. Five brown colonies appeared when the plates were stored at 4 °C and examined after 10 days. In a separate transformation experiment, cells were first screened for ampicillin resistance accompanied by an absence of β-galactosidase activity. Out of about 1000 transformants, 300 colonies were selected for plating onto indicator plates. The plates were incubated for 6–8 h at 34 °C and then stored at 4 °C. One dark brown colony (XL1-Blue/pLJC305) appeared after 4 days (Figure 1). The six clones identified in these studies were replated onto fresh indicator plates and incubated overnight at 34 °C and then at room temperature. With all six clones, color development was generally apparent after about 6 h at room temperature, and dark brown colonies were usually seen after 24 h.

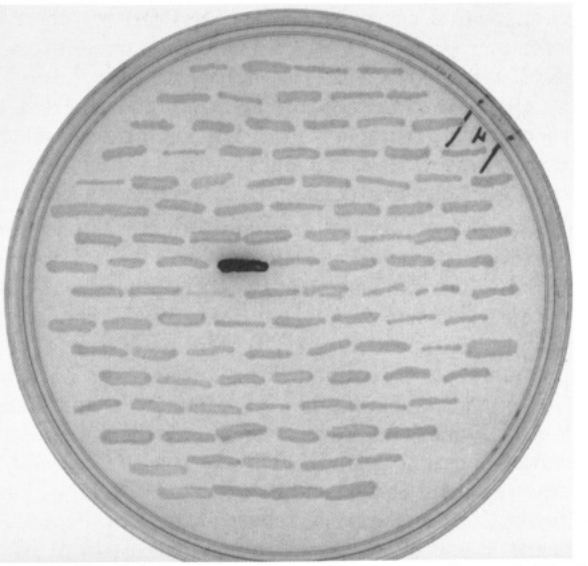


FIGURE 1: Detection of sarcosine oxidase expression on indicator plates. Colonies obtained after transformation of XL1-Blue with pBluescript II SK (+) containing fragments of corynebacterial DNA were first screened for ampicillin resistance and absence of β-galactosidase activity. About one-third of the transformants were selected for plating onto sarcosine oxidase indicator plates and then incubated as described in the text. XL1-Blue/pLJC305 formed the dark brown colony near the middle of the plate. All other colonies were white.

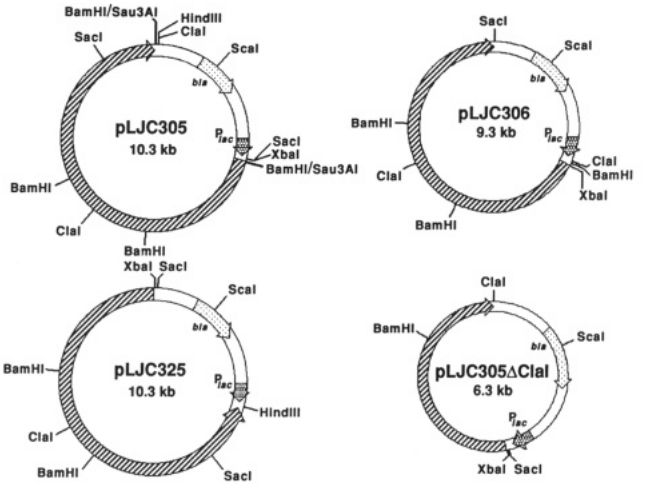


FIGURE 2: Plasmid pLJC305 was isolated as described in the legend to Figure 1. The corynebacterial insert in pLJC305 was subcloned, as described in the text, to yield pLJC325, pLJC306, and pLJC305ΔClaI. The corynebacterial insert is shown by the striped area; the arrowhead indicates the direction of transcription.

However, reproducible development of a uniform array of all brown colonies was observed only with cells carrying pLJC305, and this clone was therefore selected for further studies.

Subcloning Experiments. The fragment of corynebacterial DNA inserted into pLJC305 is approximately 7.3 kb (Figure 2). Expression of sarcosine oxidase in cells grown on standard LB media, which do not contain sarcosine, was enhanced 3-fold when XL1-Blue/pLJC305 cells were grown in the presence of IPTG (*vide infra*). This suggested that expression might be at least partially controlled by the *lac* promoter derived from pBluescript II SK (+). To test this hypothesis, pLJC305 was cut with *Xba*I, *Hind*III, and *Sca*I. The *Xba*I–*Hind*III fragment contains the entire corynebacterial DNA insert from pLJC305. This fragment was subcloned into pBluescript II KS (+) to yield pLJC325. This construct reverses the

orientation of the corynebacterial gene(s) with respect to the *lac* promoter (Figure 2). XL1-Blue cells transformed with pLJC325 formed only white colonies on indicator plates. This shows that the expression of sarcosine oxidase from the corynebacterial gene(s) in pLJC305, including the basal level observed in the absence of inducer, is completely under the control of the *lac* promoter and not a corynebacterial promoter.

The gene(s) conferring sarcosine oxidase activity were further localized by subcloning the 6.3-kb *Xba*I–*Sac*I fragment from pLJC305 into pBluescript II KS (+) to yield pLJC306. This construct deletes 1.0 kb from the 3′ end of the corynebacterial insert in pLJC305 but maintains the same orientation of the sarcosine oxidase gene(s) with respect to the *lac* promoter (Figure 2). XL1-Blue cells transformed with pLJC306 formed brown colonies on indicator plates. In a separate experiment, pLJC305 was cut with *Cla*I, and the digest was diluted 16-fold and then treated with DNA ligase to yield pLJC305ΔClaI. This construct deletes about 4.0 kb from the 3′ end of the corynebacterial insert in pLJC305 (Figure 2). XL1-Blue cells transformed with pLJC305ΔClaI formed white colonies on indicator plates. Similar results were obtained when XL1-Blue cells were transformed with a series of constructs that contained larger deletions from the 3′ end. The results show that a fragment of at least 1.0 kb but less than 4.0 kb can be deleted from the 3′ end of the 7.3-kb corynebacterial insert in pLJC305, apparently without interfering with sarcosine oxidase expression. The results are consistent with the estimated size, about 5.0 kb, of the sarcosine oxidase operon.

Identification of the Sarcosine Oxidase Activity Expressed in XL1-Blue/pLJC305 Cells. Crude cell lysates from XL1-Blue/pLJC305 cells grown on LB media were prepared and subjected to native gel electrophoresis. A prominent protein band (27% of total protein) was observed in extracts from cells grown in the presence of IPTG (Figure 3A, lane 4). The same band was observed in extracts from cells grown in the absence of inducer (Figure 3A, lane 3) but was reduced in intensity (10% of total protein). This band comigrated with authentic sarcosine oxidase purified from *Corynebacterium* sp. P-1 (Figure 3A, lanes 1 and 6) and a band observed in crude extracts prepared from *Corynebacterium* sp. P-1 grown in the presence of sarcosine (Figure 3A, lane 5). The band was not detected in cell extracts prepared from XL1-Blue/pLJCXXX cells (Figure 3A, lane 2), a random colony isolated along with XL1-Blue/pLJC305 that formed white colonies on indicator plates. Figure 3B shows a duplicate gel, prepared under the same conditions, but stained for sarcosine oxidase activity instead of protein. Except for the extract from XL1-Blue/pLJCXXX (Figure 3B, lane 2), a single activity band was observed for all cell lysates (Figure 3B, lanes 3–5). The activity band comigrated with the prominent protein band present in these extracts and with the activity band observed with sarcosine oxidase purified from *Corynebacterium* sp. P-1 (Figure 3B, lanes 1 and 6). The results suggested that XL1-Blue/pLJC305 cells express a sarcosine oxidase similar to the enzyme isolated from *Corynebacterium* sp. P-1 and prompted further studies to characterize the recombinant enzyme.

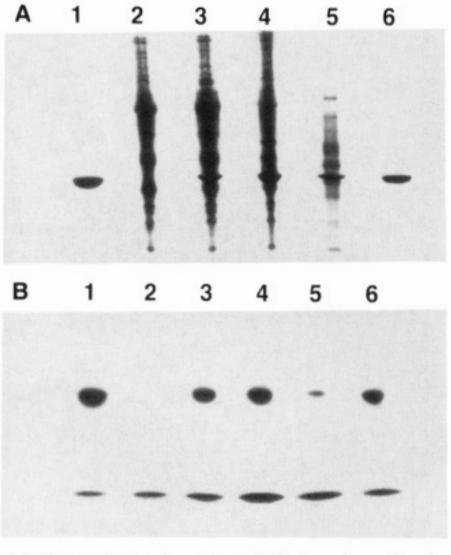


FIGURE 3: Native polyacrylamide gel electrophoresis of crude cell lysates. Identical gels were stained for protein (panel A) or sarcosine oxidase activity (panel B). Sarcosine oxidase purified from *Corynebacterium* sp. P-1 (grown in the presence of sarcosine) was run in lanes 1 (17 μg) and 6 (10 μg). The crude corynebacterial cell extract is shown in lane 5. Lysates from XL1-Blue/pLJC305 cells grown on LB media in the presence and absence of IPTG were run in lanes 4 and 3, respectively. A lysate from XL1-Blue/pLJCXXX cells (a random colony isolated along with pLJC305) grown on LB media in the presence of IPTG was run in lane 2. The lower band in each of the lanes in panel B is the tracking dye.

Purification of Recombinant Sarcosine Oxidase. The optimal time for harvesting XL1-Blue/pLJC305 cells was estimated by measuring cell density (A_{650}) and sarcosine oxidase activity in cell lysates at various times after induction with IPTG. Both parameters began to plateau at 8 h, with small increases up to 18 h, and then slowly declined at longer times after induction (data not shown). On the basis of this information, cells for enzyme isolation were routinely harvested at 12–16 h after induction. XL1-Blue/pLJC305 cells are readily disrupted by sonication in the cold, unlike *Corynebacterium* sp. P-1, where cell lysis requires treatment with lysozyme at 25 °C. The cell lysate from XL1-Blue/pLJC305 is bright yellow, whereas a similar extract from XL1-Blue/pBluescript II KS (+) is nearly colorless. The cell lysate from XL1-Blue/pLJC305 cells was subjected to ammonium sulfate fractionation, and the fraction containing sarcosine oxidase was further purified by gel filtration on Ultrogel ACA 34. These two steps parallel a procedure previously described for the purification of sarcosine from oxidase from *Corynebacterium* sp. P-1 (Kvalnes-Krick & Jorns, 1986). Similar behavior was observed, except that the recombinant enzyme was consistently recovered in the 20–70% ammonium sulfate precipitate, whereas a significant amount of enzyme in extracts from *Corynebacterium* sp. P-1 will sometimes precipitate in the 0–20% range. After gel filtration, the recombinant enzyme was chromatographed on Phenyl–Sepharose. This step replaces the DEAE-cellulose and hydroxyapatite columns in a previous procedure (Kvalnes-Krick & Jorns, 1986) and is also currently used for the purification of enzyme from *Corynebacterium* sp. P-1. The last step in the purification, chromatography on DEAE-Sephacel, removes catalase. This impurity is present in much smaller amounts at this stage of the purification, as compared with enzyme from *Corynebacterium* sp. P-1.

The recombinant enzyme is purified 14.4-fold by this procedure and recovered in greater than 50% yield (Table I). More than 0.5 g of purified enzyme is obtained starting from about 40 g of cells, a value 5-fold greater than the amount of sarcosine oxidase isolated from a similar mass of sarcosine-induced *Corynebacterium* sp. P-1 cells. The purified recombinant enzyme exhibited a single band in native gel electrophoresis when the gels were stained either for protein or activity and comigrated with sarcosine oxidase isolated from *Corynebacterium* sp. P-1 (data not shown). Since a 14.4-fold

Table I: Purification of Recombinant Corynebacterial Sarcosine Oxidase Expressed in *E. coli*

step	total activity (units)[a]	protein (mg)	specific activity (units/mg)	yield (%)	x-fold purified
cell lysate[b]	12 600	14 800	0.85	100	1.0
$(NH_4)_2SO_4$ precipitate	9200	11 150	1.08	73.0	1.3
Ultrogel AcA 34	7110	1340	5.32	56.4	6.3
Phenyl-Sepharose	7050	788	8.95	56.0	10.6
DEAE-Sephacel	6670	545	12.2	52.9	14.4

[a] Units = μmol formaldehyde/min. [b] The cell lysate was prepared from 39.1 g (wet weight) of *E. coli* (XL1-Blue/pLJC305).

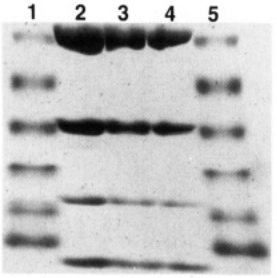


FIGURE 4: Subunit composition of recombinant sarcosine oxidase. Samples were denatured (2 min at 100 °C in 1% SDS) and subjected to SDS polyacrylamide gradient electrophoresis. A mixture of protein markers [rabbit muscle phosphorylase b (M_r 97 400), bovine serum albumin (M_r 66 200), hen egg white ovalbumin (M_r 45 000), bovine carbonic anhydrase (M_r 31 000), soybean trypsin inhibitor (M_r 21 500), and hen egg white lysozyme (M_r 14 400)] was run in lanes 1 and 5. Sarcosine oxidase (30 μg) from *Corynebacterium* sp. P-1 was run in lane 2, and two different preparations of recombinant enzyme were run in lanes 3 and 4.

purification is required to yield pure enzyme, the results suggest that the recombinant enzyme consitutes about 7% of the protein in the crude cell extract. A considerably higher value (27%) is estimated from native gel electrophoretic studies (*vide supra*). The basis for this discrepancy is unclear.

Subunit Composition of Recombinant Sarcosine Oxidase. Four bands with molecular weights of 100 000, 42 000, 20 000, and 6000 were observed when the purified recombinant enzyme was subjected to SDS gradient gel electrophoresis (Figure 4, lanes 3 and 4), as judged by their comigration with the subunits observed for enzyme isolated from *Corynebacterium* sp. P-1 (Figure 4, lane 2). In a separate experiment, a crude cell extract of XL1-Blue/pLJC305 was subjected to native gel electrophoresis, and the sarcosine oxidase band was located by staining for activity. This band was sliced from the gel, electroeluted into a dialysis bag, and subjected to SDS gradient gel electrophoresis. The sample exhibited the same four bands observed for enzyme purified by the standard procedure (data not shown). The results indicate that the unusual subunit composition of recombinant enzyme is not an artifact of the isolation procedure, similar to that observed for enzyme produced in *Corynebacterium* sp. P-1 (Kvalnes-Krick & Jorns, 1986).

Activity of Recombinant Enzyme. Enzyme activity was measured using a discontinuous assay where formaldehyde is determined by the Nash procedure (Nash, 1953; Jorns, 1985) after a 3-min incubation (37 °C, pH 8.3) or by a continuous method under somewhat different conditions (25 °C, pH 7.0), where H_2O_2 formation is monitored in a NADH peroxidase-coupled assay (Kvalnes-Krick & Jorns, 1987). Using either assay, the specific activity determined for recombinant enzyme was nearly identical to values obtained for enzyme isolated from *Corynebacterium* sp. P-1 (Table II). As expected for a coupled assay, a small initial lag is observed in the NADH

Table II: Comparison of Recombinant Sarcosine Oxidase with Enzyme Isolated from *Cornyebacterium* sp. P-1

property	Enzyme Preparation: recombinant	Enzyme Preparation: *Corynebacterium* sp. P-1
specific activity[a]		
μmol HCHO/min mg	12.2	12.5
μmol H_2O_2/min mg	4.77	4.69
A_{280}/A_{450}	16.9	12.2
A_{368}/A_{450}	1.0	0.83
ϵ_{450} (mM^{-1} cm^{-1})	9.7	12.7[b]
noncovalent FAD/covalent FAD	1.0	1.0[b]

[a] Enzyme activity was estimated by measuring HCHO production (pH 8.3, 37 °C) or H_2O_2 formation (pH 7.0, 25 °C). [b] Kvalnes-Krick & Jorns, 1986.

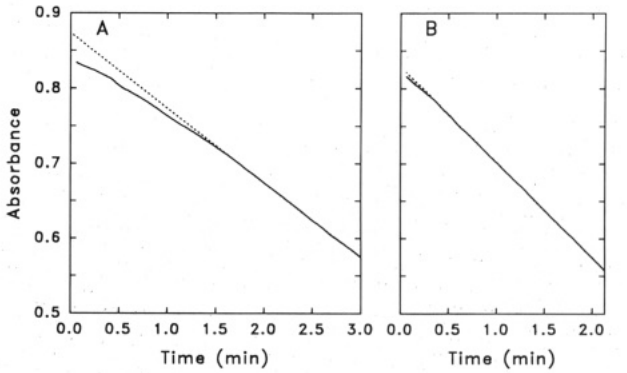


FIGURE 5: Catalytic assays using an NADH peroxidase coupled assay. The solid traces show the time course of absorbance changes at 340 nm in assays initiated by addition of recombinant sarcosine oxidase (panel A) or enzyme from *Corynebacterium* sp. P-1 (panel B). Straight lines drawn through the linear portion of the assay traces are shown by dashes.

peroxidase assay with enzyme from *Corynebacterium* sp. P-1. Surprisingly, a rather pronounced lag was observed in assays using a similar amount of recombinant enzyme (Figure 5).

Flavin Composition and Spectral Properties of Recombinant Enzyme. The noncovalent flavin in recombinant enzyme was estimated on the basis of the absorbance at 450 nm released into solution upon denaturation with 5% TCA. The covalent flavin was determined on the basis of the absorbance at 450 nm observed when the TCA pellet was redissolved in buffer containing 4 M guanidine hydrochloride. Values obtained for the molar ratio of noncovalent to covalent FAD with two different preparations (0.95, 1.03) indicate that the recombinant enzyme contains equimolar amounts of the two flavins, similar to enzyme from *Corynebacterium* sp. P-1 (Kvalnes-Krick & Jorns, 1986). As judged by the single yellow fluorescent band observed when SDS gels were viewed under ultraviolet light prior to protein staining, the covalent flavin is attached to same subunit in recombinant enzyme (M_r 42 000), as observed with enzyme from *Corynebacterium* sp. P-1 (Kvalnes-Krick & Jorns, 1986).

The recombinant enzyme exhibits visible absorption maxima (λ_{max} = 450, 368 nm) similar to those observed for enzyme from *Corynebacterium* sp. P-1 (λ_{max} = 450, 366 nm) (Figure 6). However, the enzyme preparations exhibit a 20% difference in the relative intensity of the visible absorption bands (Table II). An even larger difference is observed for the relative intensity of protein *versus* flavin absorbance, as judged by the ratio A_{280}/A_{450}, which is nearly 40% larger with recombinant enzyme (A_{280}/A_{450} = 16.9) than with enzyme from *Corynebacterium* sp. P-1 (A_{280}/A_{450} = 12.2).

The extinction coefficient of the intact recombinant enzyme was estimated on the basis of the total amount of flavin

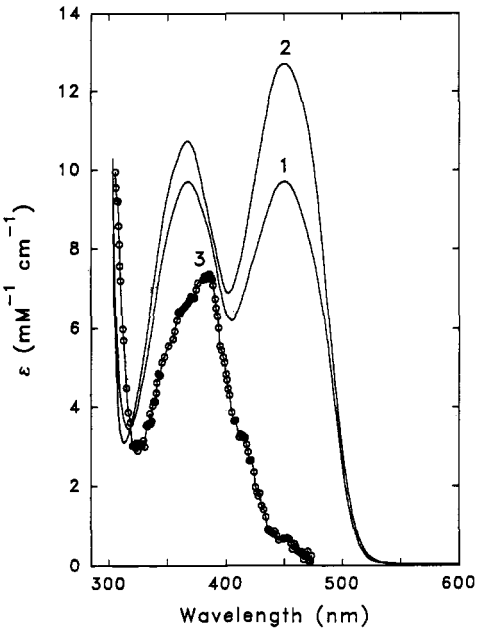


FIGURE 6: Comparison of the spectral properties of recombinant sarcosine oxidase (curve 1) with enzyme from *Corynebacterium* sp. P-1 (curve 2). Spectra were recorded in 10 mM potassium phosphate buffer, pH 8.0, at 20 °C. Curve 3 is the absorption spectrum of the modified covalent flavin in recombinant enzyme, calculated as described in the text.

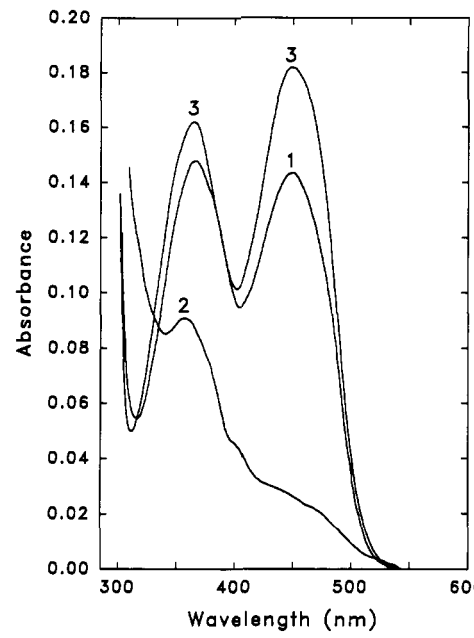


FIGURE 7: Reduction of recombinant enzyme with sarcosine under aerobic conditions. Curve 1 is the initial absorption spectrum of recombinant enzyme in 10 mM potassium phosphate buffer, pH 8.0, at 25 °C. Curve 2 was recorded immediately after addition of 2.0 mM sarcosine. Curve 3 was recorded after 145 min.

recovered after denaturation with TCA ($\epsilon_{450} = 9.7 \times 10^3$ M^{-1} cm^{-1}). A similar value was estimated from the absorbance change observed after heat denaturation under conditions where protein precipitation does not occur ($\epsilon_{450} = 9.6 \times 10^3$ M^{-1} cm^{-1}). These values are about 25% less than those observed for enzyme from *Corynebacterium* sp. P-1 ($\epsilon_{450} = 12.7 \times 10^3$ M^{-1} cm^{-1}). When the ratio A_{280}/A_{450} is corrected for this difference in flavin extinction, the value obtained for recombinant enzyme [A_{280}/A_{450}(calc) = 12.9] is similar to that for enzyme from *Corynebacterium* sp. P-1. The results indicate that *E. coli* is able to efficiency flavinylate a foreign polypeptide and produce recombinant sarcosine oxidase with a flavin content similar to that of enzyme from *Corynebacterium* sp. P-1. However, the large decrease in flavin extinction at 450 nm suggests that a significant difference in the environment of the prosthetic group(s) must occur when the enzyme is produced in *E. coli*.

Reaction of Recombinant Enzyme with Sarcosine and Its Oxidation Products. Enzyme from *Corynebacterium* sp. P-1 undergoes multiple turnover events prior to cell harvesting because expression is induced by using sarcosine as the source of carbon and energy. In contrast, the recombinant enzyme may experience minimal or no turnover prior to isolation because sarcosine is absent from the growth medium and XL1-Blue/pLJC305 cells have a limited endogenous supply of sarcosine, as judged by the fact that only white colonies are formed when these cells are grown on sarcosine-free indicator plates. It is conceivable that turnover with substrate may modify an amino acid residue at the active site. If recombinant enzyme represents a "virgin" form of sarcosine oxidase, then this residue would be unmodified and may alter the enzyme's spectral properties by interacting with the flavin.

Evidence to evaluate this hypothesis was sought by monitoring the effect of sarcosine on the spectral properties of recombinant enzyme. The recombinant enzyme is readily reduced by 2.0 mM sarcosine under aerobic conditions, as judged by the spectrum observed immediately after substrate addition (Figure 7, curve 2). Complete reoxidation was observed after 145 min. However, the spectrum of the reoxidized enzyme (Figure 7, curve 3) did not coincide with the initial spectrum (Figure 7, curve 1) but instead more closely resembled the spectrum observed for enzyme from *Corynebacterium* sp. P-1, as judged by the increase in absorbance at 450 nm (27%) accompanied by a decrease in the relative intensity of the visible absorption bands ($A_{368}/A_{450} = 0.89$). No spectral changes were observed in a control experiment with recombinant enzyme when sarcosine was omitted from the incubation mixture. The spectral properties of enzyme from *Corynebacterium* sp. P-1 are unaffected by turnover with sarcosine (Kvalnes-Krick & Jorns, 1986; Jorns, 1985).

Further information regarding the effect of turnover on recombinant enzyme was sought by determining whether any of the products of sarcosine oxidation might affect the spectral properties of the enzyme. No spectral changes were observed after a 2-h incubation with glycine or formaldehyde (2.0 mM). However, incubation of recombinant enzyme with hydrogen peroxide (2.0 mM) resulted in a 28% increase in absorbance at 450 nm and a decrease in the ratio A_{368}/A_{450} to a value (0.84) similar to that observed for enzyme from *Corynebacterium* sp. P-1. The reaction was complete within 180 min, with most of the spectral change (95%) occurring within 80 min (Figure 8). Hydrogen peroxide does not effect the absorption spectrum of enzyme from *Corynebacterium* sp. P-1, as judged by results obtained during a 180-min incubation with 2.0 mM hydrogen peroxide.

The effect of hydrogen peroxide on recombinant enzyme is not due to the formation of a Michaelis-type complex, as judged by the fact that the spectral change observed with 2.0 mM hydrogen peroxide was not reversed upon incubation (2 h at 25 °C, pH 8.0) with excess catalase (26.2 units/mL). This amount of catalase will rapidly decompose 2.0 mM hydrogen peroxide, as judged by a control experiment where the order of addition of catalase and hydrogen peroxide was reversed. Similar spectral changes were observed in studies at various hydrogen peroxide concentrations in the range from 1.0 to 10 mM. In each case, the increase in absorbance at

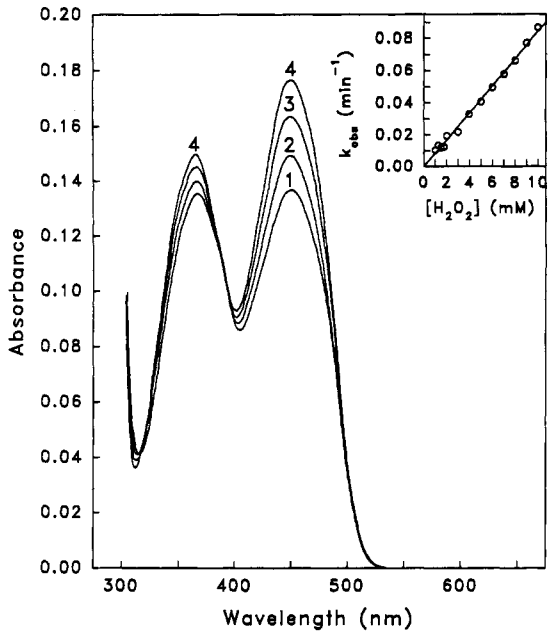


FIGURE 8: Reaction of recombinant enzyme with hydrogen peroxide. Curve 1 is the initial absorption spectrum of recombinant enzyme in 10 mM potassium phosphate buffer, pH 8.0, 25 °C. Curves 2–4 were recorded 25, 60, and 180 min, respectively, after addition of 2.0 mM hydrogen peroxide. Inset: Pseudo-first-order rate constants observed for the reaction of hydrogen peroxide with recombinant enzyme are plotted *versus* the hydrogen peroxide concentration.

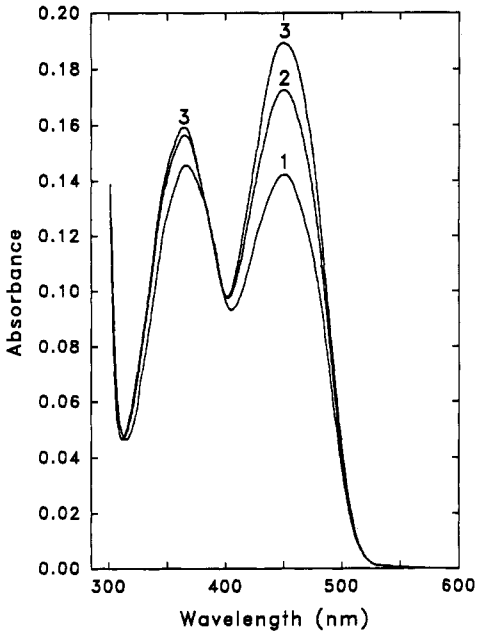


FIGURE 9: Reaction of recombinant enzyme with methyl methanethiosulfonate. Curve 1 is the initial absorption spectrum of the enzyme in 10 mM potassium phosphate buffer, pH 8.0 at 20 °C. Curves 2 and 3 were recorded 0 and 60 min, respectively, after addition of 1.0 mM MMTS.

450 nm exhibited pseudo-first-order kinetics, and the observed rate constant was directly proportional to the reagent concentration (Figure 8, inset).

Reaction with hydrogen peroxide eliminates the pronounced lag in the NADH peroxidase-coupled assay but does not otherwise affect the activity of recombinant enzyme, as judged by measurements conducted after reaction of the enzyme with 0.5 mM hydrogen peroxide (7.5 h, 25 °C). The lag was not eliminated in a control sample incubated in the absence of hydrogen peroxide.

The preceding results suggested that the spectral change observed after turnover of the recombinant enzyme with sarcosine might be due to the reaction of the enzyme with free hydrogen peroxide. Prior addition of excess catalase (26.2 units/mL) interfered but did not completely prevent the spectral change observed with recombinant enzyme and 2.0 mM sarcosine; the increase in absorbance at 450 nm after a 160-min incubation in the presence of catalase was 25% that observed for a control sample incubated with sarcosine alone (data not shown). The data suggest that reaction with hydrogen peroxide (or a precursor) prior to its dissociation from the active site may partially account for the spectral change caused by turnover with sarcosine.

The Modified Flavin Spectrum in Recombinant Enzyme is Due to Interaction with a Thiol. As described above, reaction of hydrogen peroxide with recombinant sarcosine oxidase yields enzyme with spectral properties similar to those of enzyme from *Corynebacterium* sp. P-1. In proteins, hydrogen peroxide reacts principally by oxidizing cysteine and methionine residues (Means & Feeney, 1971). Evidence to distinguish between these possibilities was sought in studies with methyl methanethiosulfonate (MMTS), a reagent that specifically reacts with cysteine residues (Smith et al., 1975). Reaction of recombinant enzyme with 1.0 mM MMTS results in an increase in absorbance at 450 nm (33.5%) accompanied by a decrease in the ratio A_{368}/A_{450} to a value (0.84) similar to that observed for the reaction with hydrogen peroxide. The reaction with 1.0 mM MMTS is quite rapid; 65% of the spectral change is observed immediately after mixing at 20 °C (Figure 9). MMTS has no effect on the absorption spectrum of enzyme from *Corynebacterium* sp. P-1. In a separate experiment, the recombinant enzyme was incubated with 0.6 mM MMTS, an aliquot was withdrawn when the spectral change was complete (20 min at room temperature), and enzyme activity was measured using the NADH peroxidase-coupled assay. Reaction with MMTS eliminated the pronounced lag in the assay but did not otherwise affect activity. A loss of activity was observed only after prolonged incubation with MMTS (45% decrease after 120 min).

The reaction of MMTS with cysteine residues is readily reversed upon addition of excess thiol (Smith et al., 1975). To determine whether excess thiol might reverse the spectral change observed with recombinant enzyme and MMTS, a sample was incubated with 1.0 mM MMTS until the absorbance change was complete (10 min at room temperature), and then 20 mM DTT was added. The sample was incubated for an additional 2 h at the same temperature and then dialyzed against thiol-free buffer at 4 °C. [The dialysis step is necessary because the flavin in recombinant enzyme is readily reduced by DTT, similar to the reaction observed with enzyme from *Corynebacterium* sp. P-1 (Zeller et al., 1989; Kvalnes-Krick & Jorns, 1987)]. The spectral properties of the dialyzed sample were identical to those observed for untreated enzyme (data not shown).

The results indicate that the modified flavin absorption spectrum observed with recombinant enzyme is due to the reversible interaction of the prosthetic group(s) with a cysteine residue. The interaction is disrupted when the thiol is oxidized or alkylated, as judged by results obtained with hydrogen peroxide or MMTS, respectively. The thiol residue may also be susceptible to air oxidation in a slow reaction, as judged by the absorbance change observed upon prolonged incubation (5–8 days) of the enzyme at 25 °C (data not shown). This spectral change was not reversed upon subsequent storage at 4 °C. The spectrum of the isolated recombinant enzyme is

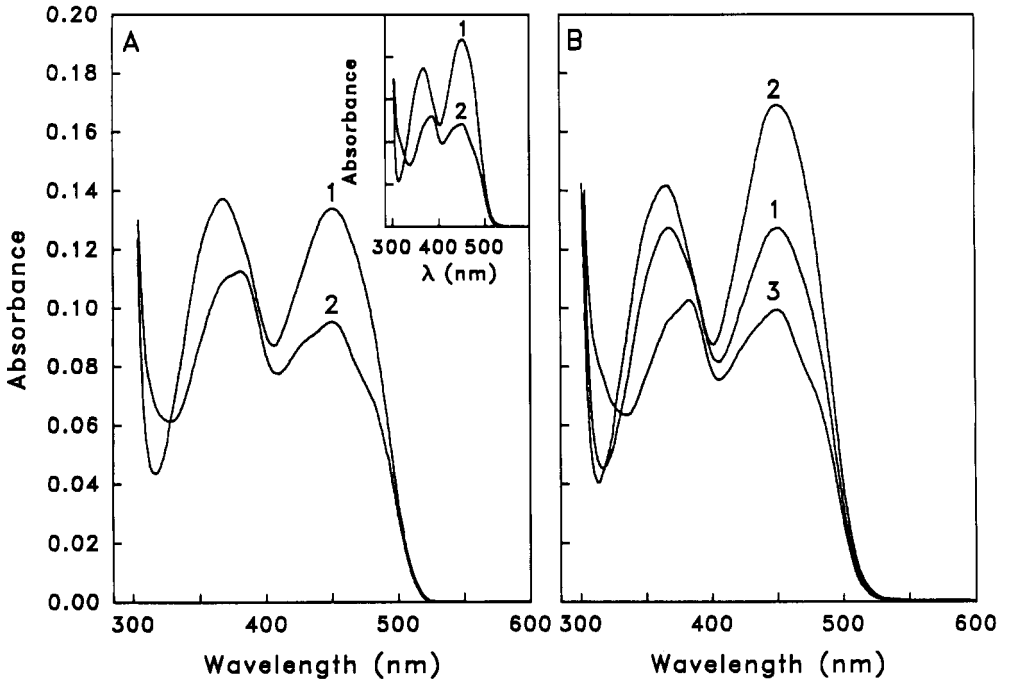


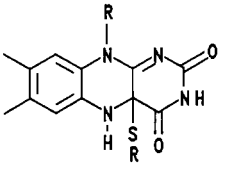
FIGURE 10: Reaction of recombinant enzyme with sulfite. Reactions were conducted in 10 mM potassium phosphate buffer, pH 8.0, at 20 °C. Panel A: Absorption spectra of recombinant enzyme observed before and after addition of 16 mM sulfite are shown in curves 1 and 2, respectively. The inset shows spectra of enzyme from *Corynebacterium* sp. P-1 recorded before (curve 1) and after addition of 16 mM sulfite (curve 2). Panel B: Spectra of recombinant enzyme were recorded before (curve 1) and after (curve 2) reaction with 1 mM MMTS. Curve 3 was obtained after addition of 16 mM sulfite to MMTS-treated enzyme.

stable for at least 2 days at 4 °C or during long-term storage at −70 °C.

Identification of the Flavin That Interacts with the Thiol. The rate of increase in absorbance at 450 nm during long-term storage of recombinant enzyme at 25 °C or upon reaction with 1.0 mM MMTS at 8 °C was unaffected by addition of 6.0 mM 2-furoic acid. Since 2-furoic acid is a competitive inhibitor (K_d = 0.34 mM) that selectively binds near the noncovalent (dehydrogenase) flavin (Zeller et al., 1989), the results suggested that the thiol might selectively interact with the covalent (oxidase) flavin.

Further evidence was sought by studying the reaction of the recombinant enzyme with sulfite. Previous studies with enzyme from *Corynebacterium* sp. P-1 have shown that sulfite reacts reversibly and selectively with the covalent flavin to form a flavin–sulfite adduct (K_d = 0.12 mM at pH 8.0, 5 °C). This results in a loss of half of the flavin absorbance at 450 nm since the flavin–sulfite adduct exhibits negligible absorbance at this wavelength (Kvalnes-Krick & Jorns, 1986; Jorns, 1985). Results in agreement with previous studies were obtained in a control experiment with enzyme from *Corynebacterium* sp. P-1, where nearly half (45%) of the flavin absorbance at 450 nm was lost upon reaction with excess sulfite (Figure 10A, inset). In contrast, reaction of recombinant enzyme with excess sulfite caused a much smaller decrease in absorbance at 450 nm (28.8%) (Figure 10A, curve 2). In a separate experiment, recombinant enzyme was first treated with MMTS to block interaction of the thiol with the flavin and then reacted with excess sulfite. The enzyme–sulfite complex (Figure 10B, curve 3) exhibited a 42% loss of absorbance when compared with MMTS-treated enzyme (Figure 10B, curve 2) but only a 22% decrease when compared with untreated enzyme (Figure 10B, curve 1). The results indicate that the covalent flavin is the flavin that interacts with a thiol residue. This interaction affects about half of the covalent flavin in the isolated recombinant enzyme used in these studies.

Nature of the Flavin–Thiol Interaction in Recombinant Enzyme. The preceding studies indicate that about 75% of total flavin in recombinant enzyme is unmodified. The absorption spectrum of the modified covalent flavin was calculated by subtracting the contribution from unmodified flavin, estimated on the basis of the spectral properties observed for enzyme isolated from *Corynebacterium* sp. P-1. The spectrum calculated for the modified flavin (Figure 6, curve 3) exhibits a single absorption maximum at 383 nm (ϵ_{383} = 7.3 mM^{-1} cm^{-1}). It is identified as an adduct formed between the sulfur of a cysteine residue and the C(4a) position of the flavin, based on the similarity of its spectral properties with covalent thiolate adducts observed with dihydrolipoamide dehydrogenase (λ_{max} = 380, ϵ_{380} = 7 mM^{-1} cm^{-1}) and mercuric reductase (λ_{max} = 382, ϵ_{382} = 7.5 mM^{-1} cm^{-1}) (Williams, 1992).



4a-thiolate adduct

DISCUSSION

A clone, XL1-Blue/pLJC305, that overexpressed corynebacterial sarcosine oxidase was isolated in a single step by using indicator plates to screen a genomic library for colonies that generated hydrogen peroxide in a sarcosine-dependent reaction. The genomic library was constructed by using an expression vector, pBluescript II SK (+), that could accommodate DNA fragments large enough (5.0–9.4 kb) to code for the putative 5.0-kb sarcosine oxidase operon. A number of genes from *C. glutamicum* have been expressed in *E. coli* under the control of corynebacterial promoters, at least at levels sufficient to yield heterologous complementation of

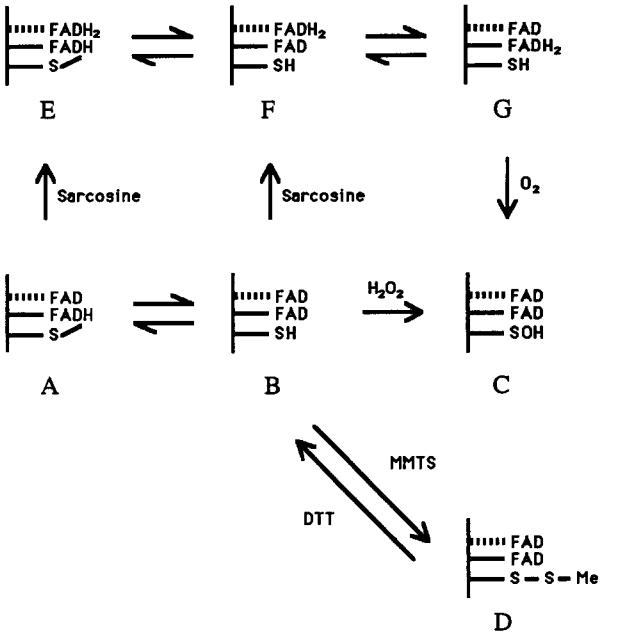
several *E. coli* auxotrophic mutants (Follettie & Sinskey, 1986). However, expression of sarcosine oxidase in XL1-Blue/pLJC305 cells is entirely under the influence of the vector-encoded *lac* promoter, as judged by the absence of expression in a construct which reversed the orientation of the corynebacterial insert with respect to the *lac* promoter. Genes encoding monomeric sarcosine oxidase from *Bacillus* sp. 0618 or *Bacillus* sp. NS-129 have been isolated by screening genomic libraries for hydrogen peroxide (Sagai et al., 1989) or formaldehyde production (Koyama et al., 1991), respectively. However, in these studies, the initial clones contained inserts that were 5–10-fold larger than the target genes; overexpression was achieved when smaller fragments were subcloned into pBR322.

Recombinant corynebacterial sarcosine oxidase is isolated as a heterotetramer containing equimolar amounts of covalent and noncovalent flavin, identical to that observed for enzyme isolated from *Corynebacterium* sp. P-1. Since a separate gene is generally required for each polypeptide produced in procaryotic systems, the results suggest that the corynebacterial DNA insert in pLJC305 (7.3 kb) contains at least four genes to code for the enzyme's subunits. At least 1.0 kb, but less than 4.0 kb, can be deleted from the 3′ end of the 7.3 kb insert without affecting sarcosine oxidase expression, consistent with the estimated operon size. An open reading frame coding for the β subunit and a region corresponding to the amino terminus of the α subunit have been identified in sequencing studies currently in progress (Chlumsky and Jorns, unpublished results). The ability of *E. coli* to efficiently flavinylate the β subunit and then assemble four foreign polypeptides into a functional enzyme is impressive, particularly since extensive *in vitro* refolding experiments with this enzyme have thus far been totally unsuccessful (Zhang and Jorns, unpublished data). This suggests that folding errors during *in vivo* biosynthesis may be avoided by the participation of molecular chaperones.

Despite its similar flavin content, recombinant sarcosine oxidase exhibits spectral properties significantly different from those of enzyme from *Corynebacterium* sp. P-1 (Table II, Figure 6). This difference is due to the fact that about half of the covalent flavin in recombinant enzyme forms a reversible covalent 4a-adduct with a cysteine residue. As indicated in Scheme I, the equilibrium can be shifted in favor of adduct dissociation by oxidizing the cysteine residue with hydrogen peroxide (B → C) or by alkylation with MMTS (B → D). The reaction with MMTS is fully reversible upon addition of excess DTT. The reaction with hydrogen peroxide is first order with respect to hydrogen peroxide. This is consistent with the mechanism shown in Scheme I, provided that equilibration of A and B is fast compared with the reaction of B with hydrogen peroxide and that the equilibrium between A and B lies strongly in favor of A. The kinetics are also compatible with peroxide oxidation of the 4a-adduct to the corresponding *S*-oxide, followed by adduct dissociation (not shown). Cysteinesulfenic acid is tentatively proposed as the oxidation product formed with hydrogen peroxide, assuming that the active site does not contain a pair of vicinal thiols.

Although the same specific activity is observed for recombinant enzyme and enzyme from *Corynebacterium* sp. P-1, the recombinant enzyme exhibits a pronounced lag in the NADH peroxidase coupled assay. The lag is eliminated by prior disruption of the 4a-thiolate adduct via reaction with hydrogen peroxide or MMTS. As suggested in Scheme I, electron transfer from sarcosine to the noncovalent flavin may occur prior to (A → E) or after (B → F) dissociation of the 4a-thiolate adduct. This proposal is consistent with previous

Scheme I: Proposed Mechanism for the Effect of Sarcosine, Hydrogen Peroxide, or MMTS on the Stability of the 4a-Thiolate Adduct Formed between a Cysteine Residue and the Covalent Flavin in Recombinant Sarcosine Oxidase[a]



[a] The noncovalent and covalent flavins are indicated by dashed and solid lines, respectively.

studies which show that electron transfer from sarcosine to the noncovalent flavin is unaffected by sulfite; sulfite adds reversibly to the N(5) position of the covalent flavin to form a N(5)-adduct (Zeller et al., 1989; Ali et al., 1991). On the other hand, the 4a-thiolate adduct will clearly interfere with interflavin electron transfer (F ⇄ G). The lag in the catalytic assay is attributed to adduct dissociation (E ⇄ F or A ⇄ B). Reformation of the adduct is blocked by oxidation of the cysteine residue by hydrogen peroxide (or a precursor) formed during the reoxidation of the reduced covalent flavin (G → C). This is consistent with spectral studies which show that the adduct is disrupted by turnover with sarcosine. The effect of catalase on this reaction suggests that reaction of hydrogen peroxide (or a precursor) with the cysteine residue partially competes with the release of hydrogen peroxide into solution. The observed *in situ* activation of recombinant enzyme in the NADH peroxidase-coupled assay is likely to involve the former pathway(s) since assay conditions are specifically designed to scavenge free hydrogen peroxide. [The consumption of hydrogen peroxide (or precursor) in the activation process itself is insignificant and would not affect the rate of NADH oxidation since assays are conducted using only 10–30 nM sarcosine oxidase.] Although no intermediate has yet been detected in the oxidative half-reaction with flavoprotein oxidases, a 4a-hydroperoxyflavin intermediate has been observed with flavin monooxygenases (Entsch et al., 1976) and bacterial luciferase (Hastings et al., 1973; Vervoort et al., 1986). Model studies show that oxidation of an alkyl sulfide to the corresponding *S*-oxide is nearly 4 orders of magnitude faster with 4a-hydroperoxyflavin than with hydrogen peroxide (Bruice, 1983; Bruice et al., 1983). A redox-active cysteinesulfenic acid has been identified in NADH oxidase and NADH peroxidase. This residue is reduced to cysteine upon reaction with NADH and then reoxidized by reaction with a 4a-hydroperoxyflavin or hydrogen peroxide. Only the latter oxidant is feasible in the NADH peroxidase reaction (Stehle et al., 1993; Ahmed & Claiborne, 1992).

4a-Thiolate adducts have been proposed as intermediates in the reduction of free flavin by dithiols (Loechler & Hollocher, 1975). Pyridine nucleotide disulfide oxidoreductases, including dihydrolipoamide dehydrogenase and mercuric reductase, constitute a family of flavoenzymes that contain a redox-active disulfide which is reduced by dithiol substrates. A 4a-thiolate adduct is probably formed transiently as an intermediate during electron transfer between the flavin and the active center disulfide and can also be stabilized under certain conditions (Williams, 1992). In contrast, the 4a-thiolate adduct formed with sarcosine oxidase represents an inactive form of the enzyme which can be activated by reaction with sarcosine in what appears to be the first example of a posttranslational modification associated with turnover. Complete activation occurs *in vivo* when sarcosine oxidase is produced in *Corynebacterium* sp. P-1, where enzyme synthesis is induced by growth of the organism with sarcosine as the source of carbon and energy. The opportunity for *in vivo* activation is greatly diminished when the recombinant enzyme is produced in *E. coli* under the control of the *lac* promoter and half of the covalent flavin in the isolated enzyme is present as a 4a-thiolate adduct. At present, the possible physiological significance of the 4a-thiolate adduct in sarcosine oxidase remains elusive.

REFERENCES

Ahmed, S. A., & Claiborne, A. (1992) *J. Biol. Chem. 36*, 25822–25829.

Ali, S. N., Zeller, H.-D., Calisto, M. K., & Jorns, M. S. (1991) *Biochemistry 30*, 10980–10986.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidmen, J. G., Smith, J. A., & Struhl, K. (1899) in *Current Protocols in Molecular Biology*, John Wiley & Sons, New York.

Bergmeyer, H. U. (1974) in *Methods of Enzymatic Analysis*, Academic Press, New York.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–255.

Bredt, D. S., Hwang, P. M., Glatt, C. E., Lowenstein, C., Reed, R. R., & Snyder, S. H. (1991) *Nature 351*, 714–718.

Bruice, T. C. (1983) *J. Chem. Soc., Chem. Commun.* 14–15.

Bruice, T. C., Noar, J. B., Ball, S. S., & Venkataram, U. V. (1983) *J. Am. Chem. Soc. 105*, 2452–2463.

Cole, S. T., Condon, C., Lemire, B. D., & Weiner, J. H. (1985) *Biochim. Biophys. Acta 811*, 381–403.

Davis, B. J. (1964) *Ann. N.Y. Acad. Sci. 121*, 404–427.

Entsch, B., Ballou, D. P., & Massey, V. (1976) *J. Biol. Chem. 251*, 2550–2563.

Follettie, M. T., & Sinskey, A. J. (1986) *Food Tech.* 88–94.

Hanahan, D. (1983) *J. Mol. Biol. 166*, 557–580.

Hastings, J. W., Balny, C., LePeuch, C., & Douzou, P. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3468–3472.

Hevel, J. M., White, K. A., & Marletta, M. A. (1991) *J. Biol. Chem. 266*, 22789–22791.

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

Koyama, Y., Yamamoto-Otake, H., Suzuki, M., & Nakano, E. (1991) *Agric. Biol. Chem. 55*, 1259–1293.

Kvalnes-Krick, K., & Jorns, M. S. (1986) *Biochemistry 25*, 6061–6069.

Kvalnes-Krick, K., & Jorns, M. S. (1987) *Biochemistry 26*, 7391–7395.

Kvalnes-Krick, K., & Jorns, M. S. (1991) in *Chemistry and biochemistry of flavoenzymes, Volume 2* (Muller, F., ed.), pp 425–435, CRC Press, Boca Raton, FL.

Loechler, E. L., & Hollocher, T. C. (1975) *J. Am. Chem. Soc. 97*, 3235–3237.

Mayer, B., John, M., Heinzel, B., Werner, E. R., Wachter, H., Schultz, G., & Bohme, E. (1991) *FEBS Lett. 288*, 187–191.

Means, G. E., & Feeney, R. E. (1971) in *Chemical modification of proteins*, Holden-Day, Inc., San Francisco, CA.

Nash, T. (1953) *Biochem. J. 55*, 416–421.

Oprian, D. D., & Coon, M. J. (1982) *J. Biol. Chem. 257*, 8935–8944.

Ostrowski, J., Barber, M. J., Rueger, D. C., Miller, B. E., Siegel, L. M., & Kredich, N. M. (1989) *J. Biol. Chem. 264*, 15796–15808.

Porter, D. H., Cook, R. J., & Wagner, C. (1985) *Arch. Biochem. Biophys. 243*, 396–407.

Sagai, H., Masujima, H., Ikuta, S., Suzuki, K., & Toyo Jozo Co., Ltd. (1989) French Patent 2619395, Feb 17, 1989, 43 p.; Int. Cl[4] C12N15/00, Aug 10, 1988.

Siegel, L. M., Kamin, H., Rueger, D. C., Presswood, R. P., & Gibson, Q. H. (1971) in *Flavins and flavoproteins* (Kamin, H., ed.), pp 523–553, University Park Press, Baltimore, MD.

Skoog, D. A., & West, D. M. (1976) *Fundamentals of analytical chemistry*, Saunders Golden Sunburst Series, Philadelphia, PA.

Smith, D. J., Maggio, E. T., & Kenyon, G. L. (1975) *Biochemistry 14*, 766–771.

Steenkamp, D. J., & Husain, M. (1982) *Biochem. J. 203*, 707–715.

Stehle, T., Claiborne, A., & Schulz, G. E. (1993) *Eur. J. Biochem. 211*, 221–226.

Stewart, V. (1988) *Microbiol Rev 52*, 190–232.

Stuehr, D. J., Cho, H. J., Kwon, N. S., Weise, M. F., & Nathan, C. F. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 7773–7777.

Suzuki, M. (1981) *J. Biochem. (Tokyo) 89*, 599–607.

Swaney, L. B., & Kuehl, K. S. (1976) *Biochim. Biophys. Acta 446*, 561–565.

Vermillion, J. L., Ballou, D. P., Massey, V., & Coon, M. J. (1981) *J. Biol. Chem. 256*, 266–277.

Vervoort, J., Muller, F., Lee, J., van den Berg, W. A. M., & Moonen, C. T. W. (1986) *Biochemistry 25*, 8062–8067.

Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406–4412.

Williams, C. H. (1992) in *Chemistry and biochemistry of flavoenzymes, Volume III* (Muller, F., ed.), pp 121–211, CRC Press, Boca Raton, FL.

Wittwer, A. J., & Wagner, C. (1981) *J. Biol. Chem. 256*, 4102–4108.

Wood, D., Darlison, M. G., Wilde, R. J., & Guest, J. R. (1984) *Biochem. J. 222*, 519–534.

Zeller, H.-D., Hille, R., & Jorns, M. S. (1989) *Biochemistry 28*, 5145–5154.