Minami, Y., Wakabayashi, S., Wada, K., Matsubara, H., Kerscher, L., & Oesterhelt, D. (1985) *J. Biochem. (Tokyo) 97*, 745–753.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007–4021.
Rabinowitz, J. C. (1972) *Methods Enzymol. 24B*, 431–446.
Robson, R. L. (1979) *Biochem. J. 181*, 569–575.
Sands, R. H., & Dunham, W. R. (1975) *Q. Rev. Biophys. 7*, 443–504.
Shethna, Y. I., Der Vartanian, D. V., & Beinert, H. (1968) *Biochem. Biophys. Res. Commun. 31*, 862–868.
Smithies, O., Gibson, D., Fanning, E. M., Goodfliesh, R. M., Gilman, J. G., & Ballantyne, D. L. (1971) *Biochemistry 10*, 4912–4921.
Spackman, D. H., Stein, W. H., & Moore, S. (1958) *Anal. Chem. 30*, 1190–1206.
Stout, C. D. (1982) in *Iron-Sulfur Proteins* (Spiro, T. G., Ed.) pp 97–146, Wiley-Interscience, New York.
Subramanian, V., Liu, T. N., Yeh, W. K., Serdar, C. M., Wackett, L. P., & Gibson, D. T. (1985) *J. Biol. Chem. 260*, 2355–2363.
Tanaka, M., Nakashima, T., Benson, A., Mower, H., & Yasunobu, K. T. (1966) *Biochemistry 5*, 1666–1681.
Tanaka, M., Haniu, M., Yasunobu, K. T., & Kimura, T. (1973) *J. Biol. Chem. 248*, 1141–1157.
Tanaka, M., Haniu, M., Yasunobu, K. T., Dus, K., & Gunsalus, I. C. (1974) *J. Biol. Chem. 249*, 3689–3701.
Tarr, G. E., Beecher, J. F., Bell, M., & McKean, D. J. (1978) *Anal. Biochem. 84*, 622–627.
Tsö, M. Y. W., Ljones, T., & Burris, R. H. (1972) *Biochim. Biophys. Acta 267*, 600–604.
Tsukihara, T., Fukuyama, K., Nakamura, M., Katsube, Y., Tanaka, N., Kakudo, M., Wada, K., Hase, T., & Matsubara, H. (1981) *J. Biochem. (Tokyo) 90*, 1763–1773.
Tsukihara, T., Katsube, Y., Hase, T., Wada, K., & Matsubara, H. (1982) in *Molecular Evolution, Protein Polymorphism and the Neutral Theory* (Kimura, M., Ed.) pp 299–312, Japan Scientific Societies Press, Tokyo.
Tuckey, R. C., & Stevenson, P. M. (1984) *Int. J. Biochem. 16*, 489–495.
Wakabayashi, S., Fujimoto, N., Wada, K., Matsubara, H., Kerscher, L., & Oesterhelt, D. (1983) *FEBS Lett. 162*, 21–24.
Woese, C. R., Debrunner-Vossbrinck, B. A., Oyaizu, H., Stackebrandt, E., & Ludwig, W. (1985) *Science (Washington, D.C.) 229*, 762–765.
Yachandra, V. K., Hare, J., Gewirth, A., Czernuszewicz, R. S., Kimura, T., Holm, R. H., & Spiro, T. G. (1983) *J. Am. Chem. Soc. 105*, 6462–6468.
Yamanaka, T., & Kamen, M. D. (1967) *Biochim. Biophys. Acta 131*, 317–329.
Yoon, P. S., & DeLuca, H. F. (1980) *Biochemistry 19*, 2165–2171.

Bacterial Sarcosine Oxidase: Comparison of Two Multisubunit Enzymes Containing both Covalent and Noncovalent Flavin†

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ABSTRACT: Sarcosine oxidase was purified to homogeneity from *Corynebacterium* sp. P-1, a soil organism isolated by a serial enrichment technique. The enzyme contains 1 mol of noncovalently bound flavin [flavin adenine dinucleotide (FAD)] plus 1 mol of covalently bound flavin [8α-(N^3-histidyl)-FAD] per mole of enzyme (M_r 168 000). The two flavins appear to have different roles in catalysis. The enzyme has an unusual subunit composition, containing four dissimilar subunits (M_r 100 000, 42 000, 20 000, and 6000). The same subunits are detected in Western blot analysis of cell extracts prepared in the presence of trichloroacetic acid, indicating that the subunits are a genuine property of the enzyme as it exists in vivo. The presence of both covalent and noncovalent flavin in a single enzyme is extremely unusual and has previously been observed only with a sarcosine oxidase from a soil *Corynebacterium* isolated in Japan. The enzymes exhibit many similarities but are distinguishable in electrophoretic studies. Immunologically, the enzymes are cross-reactive but not identical. The results indicate that the synthesis of a sarcosine oxidase containing both covalent and noncovalent flavin is not a particularly unusual event in corynebacteria.

Sarcosine oxidase from *Corynebacterium* sp. U-96, a soil organism isolated in Japan, is the first example of a flavoprotein containing both covalently bound flavin [1 mol of 8α-(N^3-histidyl)-FAD[1]/mol of enzyme] and noncovalently bound flavin (1 mol of FAD/mol of enzyme) (Hayashi et al., 1980, 1982; Suzuki, 1981). The enzyme catalyzes the oxidative demethylation of sarcosine (eq 1). Recent studies by

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

Jorns (1985) suggest that the two flavins in this enzyme have different roles in catalysis. The noncovalent flavin appears to function as a dehydrogenase flavin, accepting electrons from sarcosine and then transferring them to the covalent flavin. Oxygen is reduced to hydrogen peroxide by the covalent flavin

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[1] Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; EDTA, ethylenediaminetetraacetic acid; sarcosine oxidase P, sarcosine oxidase from *Corynebacterium* sp. P-1; sarcosine oxidase J, sarcosine oxidase from *Corynebacterium* sp. U-96; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; Tween 20, poly(oxyethylene) sorbitan monolaurate; TCA, trichloroacetic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)-aminomethane.

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which acts as an oxidase flavin.

To determine whether the sarcosine oxidase from the Japanese *Corynebacterium* was unique, we decided to purify and characterize sarcosine oxidase from a soil *Corynebacterium* isolated in Philadelphia (*Corynebacterium* sp. P-1). To avoid confusion, enzyme from *Corynebacterium* sp. P-1 and *Corynebacterium* sp. U-96 will be referred to as sarcosine oxidase P and sarcosine oxidase J, respectively. In this paper, we show that the two enzymes exhibit many similarities but are distinguishable in electrophoretic studies. Immunologically, the enzymes are cross-reactive but not identical. 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.

Experimental Procedures

Materials. Sarcosine oxidase J was a gift from Dr. Masaru Suzuki. The properties of the enzyme are similar to those previously reported (Jorns, 1985). BALB/c mice, from Jackson Laboratories, were a gift from Dr. Carole Long. Deoxyribonuclease I, ribonuclease A, lysozyme, iodonitrotetrazolium violet, anti-mouse IgG alkaline phosphatase conjugate, all protease inhibitors, all molecular weight protein standards, alkaline phosphatase, and *Naja naja kaouthia* snake venom (used as a source of phosphodiesterase) were purchased from Sigma. Nitrocellulose (0.45-μm pore size) was from Schleicher & Schuell. Freund's complete and incomplete adjuvants were obtained from Difco. Avidin conjugated to alkaline phosphatase was from Cooper Diagnostics. Biotinylated protein A was purchased from Zymed. DEAE-cellulose (DE-52) was from Whatman. Ultrogel AcA 34 was obtained from LKB. Hydroxyapatite was from Bio-Rad. DEAE-Sephacel was purchased from Pharmacia. Leucine aminopeptidase was purchased from P-L Biochemicals Inc. Silica gel 60 F_{254} TLC plates were from Merck. Cellulose (Cel 300-10 UV_{254}) TLC plates were from Brinkmann Instruments. Flavodoxin from *Megasphaera elsdenii* was a gift from Dr. Steven Mayhew. 8α-(N^1-Histidyl)riboflavin was a gift from Dr. William McIntire. It was purified before use as described by McIntire et al. (1985). 8α-(N^3-Histidyl)riboflavin was a gift from Dr. Dale Edmondson.

Isolation of Corynebacterium sp. P-1. Isolation was initiated by mixing 10-g samples of soil from various locations in the Philadelphia area with 50 mL of growth media at pH 6.5 containing 0.5 g of sarcosine, 0.11 g of yeast extract, 25 mg of KH_2PO_4, 100 mg of K_2HPO_4, 5 mg of $MgSO_4 \cdot 7H_2O$, 2.5 mg of $FeCl_3$, and 100 mg of NH_4Cl. After incubation at 30 °C for 4 days with shaking, a sample was withdrawn and used to inoculate a second 50-mL aliquot of fresh media. After 4 days, the process was repeated using a third aliquot of fresh media. The latter was incubated for 4 days, and then bacterial colonies were isolated on plates containing growth media minus NH_4Cl plus 2% agar. Gram stains and growth on potassium tellurite (0.038%) were performed according to the procedures described by Gerhardt (1981) with a slight modification. The methylene blue stain was performed as described by Branson (1972).

Activity and Protein Assays. Sarcosine oxidase activity was measured in 0.06 M glycylglycine buffer, pH 8.3, containing 0.12 M sarcosine at 37 °C by monitoring oxygen consumption or formaldehyde production as previously described (Jorns, 1985). A unit of activity is defined as the amount of enzyme needed to convert 1 μmol of substrate to product in 1 min. Protein concentration was determined from the absorbance of the purified enzymes at 280 nm by using the extinction coefficient ($E^{1\%}$ = 13.1) reported by Suzuki (1981). During purification of sarcosine oxidase P, protein was estimated by the method of Lowry et al. (1951) using bovine serum albumin as standard. With purified sarcosine oxidase P, the same protein concentration was obtained when either method was used.

Purification of Sarcosine Oxidase P. Unless specifically noted, all isolation and handling of sarcosine oxidase J and sarcosine oxidase P were conducted at 0–4 °C under yellow light. During purification of sarcosine oxidase P, each dialysis step was conducted over a period of 20 h with three changes of a 60-fold excess of buffer. *Corynebacterium* sp. P-1 was grown on a reciprocal shaker in 2-L flasks containing 400 mL of growth media minus NH_4Cl. After 24 h, the cells were harvested by centrifugation and washed twice with 0.01 M potassium phosphate buffer, pH 8.0. The yield is about 6 g (wet weight) of cells per liter of medium. If necessary, washed cells can be stored frozen. For lysis, the cells are suspended in 0.02 M potassium phosphate buffer, pH 7, containing 1.0 M sucrose, (9.4 mL of buffer/g of cells). Lysozyme (1 mg/mL) is added along with a mixture of protease inhibitors (phenylmethanesulfonyl fluoride, 25 mg/L; aprotinin, 1.25 mL/L; soybean trypsin inhibitor, 12.5 mg/L; N^{α}-*p*-tosyl-L-lysine chloromethyl ketone, 3 mg/L), nucleases (deoxyribonuclease, 20 mg/L; ribonuclease, 20 mg/L), and $MgSO_4$ (2.5×10^{-4} M). The mixture is stirred slowly for 1 h at 25 °C, a second aliquot of protease inhibitors is added, and the sample is centrifuged. The supernatant contains cell lysate and is stored at 4 °C. The precipitate contains cell debris plus unbroken cells. It is suspended in 0.01 M potassium phosphate buffer, pH 8.0 (4.7 mL of buffer/g of cells), mixed with a third aliquot of protease inhibitors, and incubated at room temperature. After 15 min, the material is centrifuged, and the supernatant is combined with the first supernatant. The cell extract is brought to 20% saturation with ammonium sulfate (107 g/L) and centrifuged. (Sarcosine oxidase generally does not precipitate in this range, but it is advisable to test the pellet for activity since, on one occasion, a significant amount of activity did appear in the 0–20% pellet.) The supernatant is brought to 70% saturation with ammonium sulfate (315 g/L). The precipitate is collected by centrifugation and dissolved in a minimal volume of 0.01 M potassium phosphate buffer, pH 8.0. The enzyme is dialyzed against the same buffer, centrifuged, and then applied to an Ultrogel AcA 34 column equilibrated with the dialysis buffer. Fractions with a specific activity greater than 2 units/mg are pooled and dialyzed vs. 5 mM potassium phosphate buffer, pH 7.5, containing 0.3 mM EDTA. In a typical run, 2500 units are applied to a column measuring 2.8 × 95 cm and developed at a flow rate of 7.8 mL/h. A preparation starting with 108 g of cells was processed in three successive batches and then pooled for all subsequent chromatography steps where column sizes refer to those used for this size preparation. The dialyzed enzyme was applied to a DEAE-cellulose column (4.5 × 60 cm) equilibrated with 5 mM potassium phosphate, pH 7.5, containing 0.3 mM EDTA. The column was washed with 2 column volumes of the column equilibration buffer and then with 5 mM potassium phosphate, pH 7.5, containing 0.3 mM EDTA plus 0.25 M KCl until the A_{280} of the eluate fell below 0.01. The enzyme was eluted with a linear gradient formed with 2 L each of 5 mM potassium phosphate, pH 7.5, containing 0.3 mM EDTA plus 0.25 or 0.60 M KCl. Fractions containing enzyme with a specific activity greater than 7.0 units/mg are pooled and dialyzed vs. the column equilibration buffer. The dialyzed enzyme was applied to a hydroxyapatite column (3.6 × 52 cm) equilibrated with the same buffer. The

column was washed with 2 column volumes of the starting buffer and then eluted with 0.1 M potassium phosphate, pH 7.5, containing 0.3 mM EDTA. Fractions with a specific activity greater than 9.5 units/mg were pooled and dialyzed vs. 5 mM potassium phosphate, pH 7.5, containing 0.3 mM EDTA. The dialyzed enzyme was applied to a DEAE-Sephacel column (1.6 × 49 cm) equilibrated with the same buffer. The column was washed with 5 mM potassium phosphate, pH 7.5, containing 0.3 mM EDTA plus 0.25 M KCl until the A_{280} of the eluate was less than 0.01. The enzyme was eluted with 5 mM potassium phosphate, pH 7.5, containing 0.3 mM EDTA plus 0.30 M KCl. Fractions were assayed for both sarcosine oxidase and catalase activity (Bergmeyer, 1974). Catalase-free fractions with a specific activity greater than 11 units/mg were pooled and concentrated by addition of ammonium sulfate to 70% saturation. The precipitate was dissolved and dialyzed vs. 0.01 M potassium phosphate, pH 8.0. The dialyzed enzyme is stored in aliquots at −70 °C.

Flavin Composition of Sarcosine Oxidase P. A sample of enzyme in 0.1 M potassium phosphate buffer, pH 7.6, was precipitated with 5% trichloroacetic acid and centrifuged. The supernatant was neutralized with 1 M K_2HPO_4. The precipitate was resuspended in starting buffer containing 5% trichloroacetic acid and centrifuged. The supernatant was neutralized and combined with the first supernatant. The precipitate was resuspended in 0.3 M potassium phosphate buffer, pH 8.5, containing chymotrypsin and trypsin (0.1 mg of each protease/mg of sarcosine oxidase) and digested for 20 h at 37 °C. Absorption spectra were recorded (Perkin-Elmer Lambda 3 spectrophotometer) and corrected for dilution.

In other experiments, the TCA supernatant was desalted by extraction with phenol (Jorns & Hersh, 1975) and used for thin-layer chromatography (TLC) studies with the noncovalent flavin.

In studies to identify the covalent flavin, protease-digested TCA precipitate (6.6×10^{-8} mol with respect to covalent flavin) was chromatographed on a Bio-Gel P-2 column (0.7 × 34 cm) equilibrated with water. Fractions absorbing at 447 nm were combined and lyophilized. The FMN peptide (5.5×10^{-8} mol) was dissolved in water and treated with alkaline phosphatase (185 units) for 2 h at 37 °C. The resulting riboflavin peptide (4.8×10^{-8} mol) was lyophilized, dissolved in 0.14 M Tris, pH 8.0, and incubated with aminopeptidase (18.4 μg) at 38 °C. After 24 h, an additional 11.6 μg of aminopeptidase was added, and the sample was incubated for an additional 12 h. The aminopeptidase-treated peptide was desalted by phenol extraction, as previously described (Jorns & Hersh, 1975), except ether was replaced by chloroform. The peptide was mixed with an equal volume of 12 N HCl and hydrolyzed under argon in a sealed tube for 16 h at 95 °C. The aminoacylriboflavin preparation was neutralized with NaOH in an ice–salt bath and then desalted by phenol extraction, again using chloroform in place of ether. Apoflavodoxin was prepared, standardized, and reacted with FMN derivatives or FAD derivatives treated with phosphodiesterase as described by Wassink and Mayhew (1975). AMP was determined by the method of Bergmeyer (1974). Fluorescence measurements were made with an Amino-Bowman spectrophotofluorometer. FMN and riboflavin peptides were estimated on the basis of flavin content by using an extinction coefficient of 12.5×10^3 M^{-1} cm^{-1} at 445 nm.

Gel Filtration. The determination of the molecular weight of sarcosine oxidase by gel filtration was performed on an Ultrogel AcA 34 column (2.4 × 46.5 cm) equilibrated with 10 mM potassium phosphate, pH 8.0. The column was calibrated by measuring the elution volume for the following proteins: carbonic anhydrase (M_r 29 000), bovine serum albumin (M_r 66 000), alcohol dehydrogenase (M_r 150 000), and β-amylase (M_r 200 000). The void volume was determined with blue dextran.

Electrophoresis. Studies with native enzyme were conducted by using polyacrylamide slab gels (14% resolving gel, 2.5% stacking gel) at pH 8.3 according to the method of Davis (1964). Protein was visualized by staining with Coomassie blue (Weber & Osborn, 1969). To stain for sarcosine oxidase activity, the gel was placed in 0.06 M glycylglycine buffer, pH 8.3, containing 0.12 M sarcosine plus 0.32 M iodonitrotetrazolium violet.

Subunit composition studies were conducted by using polyacrylamide gradient (3–27% acrylamide) slab gels in the presence of SDS (1%) according to the method of Swaney and Kuehl (1976). Protein bands were stained with Coomassie blue (Swaney & Kuehl, 1976), and electrophoretic mobilities were calculated from scans recorded with an E-C scanning densitometer. Electrophoretic transfer to nitrocellulose (Western blot) of the unstained SDS–polyacrylamide gradient gel was performed according to a modification of the method of Towbin (1979). A homemade device, similar to the Trans-Blot cell from Bio-Rad, was used. Crude cell extracts for Western blot analysis were prepared by suspending *Corynebacterium* sp. P-1 (4 g) in 10 mM potassium phosphate, pH 8.0, containing 10% trichloroacetic acid (19 mL). The suspension was sonicated in an ice–salt bath for 45 s (Branson sonifier 350, 130-W power output) and then centrifuged. The pellet was mixed with 19 mL of 130 mM Tris buffer, pH 9.2, containing 50 mM DTT, 10 mM sodium phosphate, 1% SDS, and 10% glycerol. The mixture was incubated at 100 °C for 10 min and then centrifuged to remove a small amount of insoluble material. Except for sonication, a similar procedure was used to prepare samples of denatured purified enzyme for Western blot analysis and also for immunoblot experiments.

Antibody Preparation. A sample of purified sarcosine oxidase P or sarcosine oxidase J in 10 mM potassium phosphate, pH 8.0, was mixed with an equal volume of complete Freund's adjuvant using a Tekmar tissumizer. Aliquots, containing 0.1 mg of enzyme, were injected subcutaneously into BALB/c mice (three mice per enzyme). After 3 weeks, the mice received a second, similar injection. Three weeks later, each mouse received a final intraperitoneal injection containing 0.1 mg of enzyme in incomplete Freund's adjuvant. A group of two control mice received a similar series of injections except that enzyme was omitted. One week after the final injection, the mice were anesthetized and bled from the retroorbital sinus. The serum was collected after the blood had coagulated. It was screened for antibody titer by using Ouchterlony double-diffusion agar plates (Ouchterlony, 1949) and then stored in aliquots at −70 °C.

Immunochemical Studies. For immunoblot experiments, a sheet of nitrocellulose was wet with phosphate-buffered saline (PBS) (6.7 mM potassium phosphate buffer, pH 7.0, containing 0.14 M sodium chloride plus 3.1 mM sodium azide) and then assembled into a Bio-Dot apparatus (Bio-Rad). Aliquots (5 μg) of native or denatured sarcosine oxidase P were applied to each well. After 1 h, the membrane was dried, and the wells were washed with PBS blocking buffer (0.5% Tween 20 in PBS). After 30 min, the wells were washed with additional PBS blocking buffer, dried, treated with varying amounts of antibody against sarcosine oxidase P or with serum

from control mice, and dried after 1 h. The nitrocellulose sheet was then removed from the Bio-Dot apparatus, and the entire sheet was washed with PBS blocking buffer. It was then gently shaken at room temperature with a solution containing anti-mouse IgG alkaline phosphatase conjugate in PBS blocking buffer. After 30 min, the sheet was removed and washed with PBS blocking buffer and then with 0.15 M veronal acetate buffer, pH 9.6. For color development, the sheet was incubated for 15 min at room temperature with alkaline phosphatase histochemical stain (Blake et al., 1984) and then washed with water. Except for certain modifications, a similar ELISA assay was used to detect antigen–antibody complexes in Western blot analysis.

A modification of a procedure described by Majarian (1985) was used for ELISA assays on microtiter plates. Sarcosine oxidase J or sarcosine oxidase P (1 μg) was added to the assay wells. After an overnight incubation at 4 °C, the plate was washed with Tris-buffered saline (TBS) (25 mM Tris, pH 8.0, containing 150 mM NaCl) and then with 1% TBS blocking buffer (TBS containing 1.0% Tween 20). The wells were treated with an aliquot (50 μL) containing antibody against sarcosine oxidase P or sarcosine oxidase J and incubated overnight at 4 °C. (For competition assays, an additional amount of sarcosine oxidase J or sarcosine oxidase P was added along with antibody.) The wells were washed with 0.1% TBS blocking buffer (TBS containing 0.1% Tween 20), and then biotinylated protein A was added. After a 1-h incubation at room temperature, the plate was washed with 0.1% TBS blocking buffer. Avidin conjugated to alkaline phosphatase was added, and the plate was washed with 0.1% TBS blocking buffer. Alkaline phosphatase assay mixture (200 μL) was then added. After 30 min at 37 °C, the reaction was stopped by adding 5 N NaOH (25 μL). Color development at 405 nm was measured by using a Dynatech Multiscan automated microplate reader.

Results

Isolation of Corynebacterium sp. P-1. Bacteria able to grow with sarcosine as a source of carbon and energy were isolated from Philadelphia soil samples by a serial enrichment technique. Out of a total of 26 colonies, 3 were identified as corynebacteria on the basis of criteria described by Davis et al. (1973). In particular, all three organisms were Gram-positive rods, exhibited a palisade cellular arrangement, grew on potassium tellurite, forming gray or black colonies, and exhibited polar metachromatic granules when stained with methylene blue.

The *Corynebacterium* (*Corynebacterium* sp. P-1) that exhibited the highest sarcosine oxidase activity (measured with whole cells in an oxygen electrode assay) was chosen for further study. No sarcosine oxidase activity was observed with *Corynebacterium* sp. P-1 when sarcosine in the growth medium was replaced by glucose, suggesting that sarcosine oxidase P is an inducible enzyme.

Purification of Sarcosine Oxidase P. Corynebacterium sp. P-1 is readily disrupted by treatment with lysozyme. Sarcosine oxidase P is present in the soluble cell lysate. After ammonium sulfate fractionation, an opalescent impurity that interferes with most columns is removed by gel filtration. In the next step, sarcosine oxidase elutes from DEAE-cellulose when the KCl concentration in the gradient buffer reaches 0.35 M. The eluate exhibits a typical flavoprotein absorption spectrum except for a small peak at 405 nm. The latter is eliminated by chromatography on hydroxyapatite, yielding enzyme that shows no spectral evidence for a hemoprotein contaminant. However, a catalase impurity was detected by activity assays and removed by chromatography on DEAE-Sephacel. The overall purification, summarized in Table I, proceeds with a 32% recovery of activity and yields enzyme that exhibits a single band in polyacrylamide slab gel electrophoresis when the gel is stained either for protein (Figure 1) or for activity. Sarcosine oxidase P constitutes about 3% of the protein in the crude cell extract, as estimated by the fact that a 37-fold purification yields homogeneous enzyme.

Table I: Purification of Sarcosine Oxidase P

step	total act. (units)	total protein (mg)	sp act. (units/mg)	% yield	x-fold purified
cell lysate[a]	11100	33800	0.33	100	1.0
$(NH_4)_2SO_4$ precipitate	7920	5560	1.42	71.4	4.3
Ultrogel AcA 34	7530	2180	3.45	67.9	10.5
DEAE-cellulose	5660	707	8.00	51.0	24.2
hydroxyapatite	5450	472	11.6	49.2	35.0
DEAE-Sephacel	3510	287	12.2	31.7	37.1

[a] The cell lysate was prepared from 108 g of *Corynebacterium* sp. P-1.

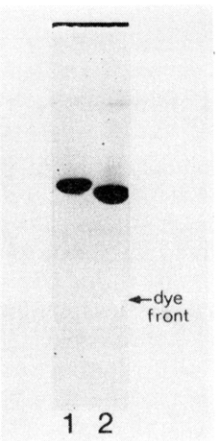


FIGURE 1: Polyacrylamide gel electrophoresis of native sarcosine oxidase. Sarcosine oxidase P (25 μg) and sarcosine oxidase J (25 μg) were run in lanes 1 and 2, respectively. The gels were stained for protein with Coomassie blue.

Purified enzyme in 10 mM potassium phosphate, pH 8.0, is stable for at least 1 year at −70 °C, 2 weeks at 0 °C, or 24 h at 25 °C. No activity loss was detected after 3 cycles of freezing and thawing within 1 week.

Flavin Composition of Sarcosine Oxidase P. The enzyme exhibits visible absorption maxima at 450 and 366 nm and a value of 11.8 for the ratio A_{280}/A_{450}. Nearly half (48%) of the absorbance of the enzyme at 450 nm is released into solution upon denaturation with trichloroacetic acid. The remainder (49%) is associated with a yellow protein precipitate that can be solubilized by treating with a mixture of chymotrypsin and trypsin. The results show that the enzyme contains both covalently bound and noncovalently bound flavin. A heat denaturation procedure (Jorns, 1985) was used to determine an extinction coefficient for the enzyme at 450 nm ($\epsilon = 12.7 \times 10^3\ M^{-1}\ cm^{-1}$) that represents an average of the contributions from both flavins.

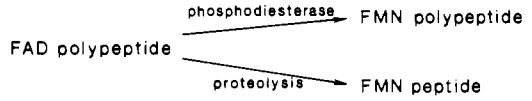
The noncovalent flavin was identified as FAD on the basis of TLC studies (Table II). For identification of the covalent flavin, the solubilized TCA precipitate was chromatographed on Bio-Gel P-2. Several observations indicate that the flavin in this peptide preparation is present at the FMN level. First, the flavin fluorescence of the peptide at 525 nm (excitation at 445 nm) is quenched upon addition of apoflavodoxin, a protein which is specific for FMN derivatives. On the other

Table II: Identification of the Noncovalent Flavin in Sarcosine Oxidase P

solvent[a]	matrix	R_f FAD	R_f TCA extract	R_f FMN
A	silica	0.09	0.09	0.14
B	silica	0.45	0.45	0.31
C	cellulose	0.13	0.12	0.20

[a] The following solvent systems were used: (A) butanol–acetic acid–water (12:3:5); (B) butanol–ethanol–0.01 M sodium acetate, pH 5.5 (1:1:1); (C) butanol–acetic acid–water (4:1:5).

hand, phosphodiesterase did not cause an increase in fluorescence which would be expected for an FAD derivative. That the covalent flavin in sarcosine oxidase P might be an FMN derivative was surprising in view of results obtained with other sarcosine oxidases (Hayashi et al., 1982) and dehydrogenases (Patek & Frisell, 1972; Sato et al., 1981; Pinto & Frisell, 1975) where the covalent flavin is present at the FAD level. Further analysis of the FMN peptide preparation showed that it contained AMP in amounts stoichiometric with flavin (1.0 mol of AMP/mol of flavin). Separate control studies showed that AMP would cochromatograph with the flavin peptide on Bio-Gel P-2. The results suggested that the covalent flavin in sarcosine oxidase P might be at the FAD level but had undergone hydrolysis to the FMN level during the preparation of the flavin peptide, releasing AMP. Consistent with this hypothesis, no AMP was detected after the enzyme was heated for 5 min at 100 °C in 10 mM potassium phosphate, pH 8.0. Enzyme denatured under these conditions remains soluble and was separated from the released noncovalent flavin on a Sephadex G-25 column. The fluorescence observed for the flavin polypeptide preparation was not quenched by apoflavodoxin. An increase (2-fold) in flavin fluorescence was observed when the flavin polypeptide was treated with phosphodiesterase. Quenching with apoflavodoxin (1.2-fold excess) was observed after phosphodiesterase treatment. Proteolytic digestion of the flavin polypeptide, under the same conditions used to solubilize the TCA precipitate, was accompanied by the release of stoichiometric amounts of AMP (0.85 mol/mol of flavin). After proteolysis, treatment with phosphodiesterase was no longer required for the quenching of flavin fluorescence with apoflavodoxin. The results show that the covalent flavin in sarcosine oxidase P is at the FAD level. Hydrolysis to the FMN level occurs during protein digestion (16 h at 37 °C) or treatment with phosphodiesterase (eq 2).



(2)

The FMN peptide, obtained after digestion of the TCA precipitate, was converted to the corresponding riboflavin peptide by treating with alkaline phosphatase, a reaction which prevents quenching of flavin fluorescence with apoflavodoxin. The riboflavin peptide was then treated with aminopeptidase in the hope of generating the corresponding aminoacylriboflavin derivative with a single amino acid directly attached to the flavin ring. Aminopeptidase-treated material exhibits absorption maxima at 445 and 350 nm.

There are five known types of covalently bound flavin. The type involving a link between position 6 of the flavin and a cysteinyl residue can be excluded as a possibility for sarcosine oxidase P since it exhibits very different spectral properties (Table III). The remaining four types all involve 8α-substituents (N^3-histidyl, N^1-histidyl, *O*-tyrosyl, and *S*-cysteinyl).

Table III: Properties of Various Riboflavin Derivatives

compound	λ_{max} (nm)	pK_a	$NaBH_4$ reduction[h]	R_f solvent D[i]	R_f solvent E[k]
riboflavin peptide from sarcosine oxidase P[a]	445, 350	4.75	no	0.82[j]	
aminoacylriboflavin from sarcosine oxidase P[b]			no	0.52	0.42
8α-(N^3-histidyl)riboflavin[c]	445, 355	4.70	no	0.52	0.42
8α-(N^1-histidyl)riboflavin[d]	445, 355	5.75	yes		0.36
8α-(*S*-cysteinyl)riboflavin[e]	448, 367				
8α-(*O*-tyrosyl)riboflavin[f]	446, 359				
6-(*S*-cysteinyl)riboflavin[g]	437				
riboflavin	445, 373				

[a] The TCA precipitate was solubilized with chymotrypsin and trypsin and then treated with aminopeptidase. [b] The aminopeptidase-treated riboflavin peptide was hydrolyzed with 6 N HCl. [c] Spectral data are from Walker et al. (1972), who also obtained a pK_a value of 4.7. [d] Spectral data are from Edmondson et al. (1976), who reported a pK_a value of 5.25. [e] Data are from Walker et al. (1971). [f] Data are from McIntire et al. (1981). [g] Data are from Steenkamp et al. (1978). [h] Reactions were conducted in 100 mM citrate/phosphate buffer, pH 3.5. Solid $NaBH_4$ was added, and the reaction was followed fluorometrically. [i] Butanol–acetic acid–water (4:2:4) on cellulose plates. [j] The observed R_f value was unaffected by further incubation with aminopeptidase. An R_f value of 0.03 was observed before aminopeptidase treatment. [k] Pyridine–butanol–acetic acid–water (15:10:3:12) on cellulose plates.

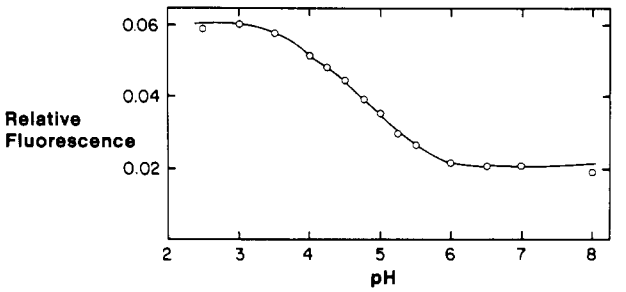


FIGURE 2: Fluorometric titration of the covalent flavin from sarcosine oxidase P. Titration of the aminopeptidase-treated riboflavin peptide was conducted at 25 °C in 100 mM citrate/phosphate buffer. Emission and excitation wavelengths were 525 and 445 nm, respectively.

A hypsochromic shift of the near-UV band, as compared with riboflavin, is a characteristic feature of 8α-substituted flavins. This feature is also observed for the covalent flavin from sarcosine oxidase P (Table III). The spectral properties of the latter most closely resemble those observed for 8α-(histidyl)flavin. Flavins with a histidyl moiety at the 8α position exhibit a characteristic increase in fluorescence below pH 7 where pK_a values of 4.70 and 5.25 have been reported for 8α-(N^3-histidyl)riboflavin and 8α-(N^1-histidyl)riboflavin, respectively (Walker et al., 1972; Edmondson et al., 1976). The other known 8α-substituted flavins exhibit a weak pH-independent fluorescence (McIntire et al., 1981; Walker et al., 1971). An increase in fluorescence is observed with the aminopeptidase-treated riboflavin peptide from sarcosine oxidase (Figure 2). The observed pK_a (pK_a = 4.75) is in excellent agreement with what we obtained in titrations with 8α-(N^3-histidyl)riboflavin (pK_a = 4.70) whereas a higher value (pK_a = 5.75) was observed with 8α-(N^1-histidyl)riboflavin. [The reason for the difference between the latter pK_a and the value reported by Edmondson et al. (1976) is unclear.] In

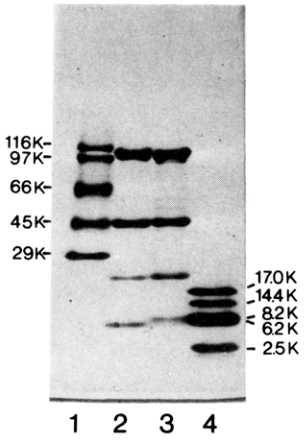


FIGURE 3: Subunit composition of sarcosine oxidase. Samples were denatured (2 min at 100 °C in 1% SDS) and subjected to SDS–polyacrylamide gradient electrophoresis. A mixture of protein markers [carbonic anhydrase (M_r 29000), ovalbumin (M_r 45000), bovine serum albumin (M_r 66000), phosphorylase *b* (M_r 97400), and β-galactosidase (M_r 116000)] was run in lane 1. Sarcosine oxidase P (22 μg) and sarcosine oxidase J (21 μg) were run in lanes 2 and 3, respectively. A mixture of myoglobin polypeptides (M_r 16950, 14400, 8160, 6210, and 2510) was run in lane 4.

addition to pK_a differences, 8α-(N^3-histidyl)riboflavin and 8α-(N^1-histidyl)riboflavin differ in reactivity with borohydride. As reported by Edmondson et al. (1976) and repeated in our laboratory, the N^1-isomer is reduced by borohydride, accompanied by a decrease in fluorescence, while the N^3-isomer is unreactive. No reaction was observed with borohydride and the aminopeptidase-treated riboflavin peptide from sarcosine oxidase P, providing further evidence in favor of the N^3-isomer. In TLC studies, the aminopeptidase-treated riboflavin peptide did not comigrate with 8α-(N^3-histidyl)riboflavin. This difference is attributed to incomplete hydrolysis by aminopeptidase since subsequent acid hydrolysis yielded material with an R_f value identical with that observed for the N^3-isomer (Table III). The peptide moiety that is resistant to hydrolysis by aminopeptidase causes quenching (58%) of flavin fluorescence at acid pH but not at neutral pH. When corrected for quenching, the ratio of fluorescence at neutral vs. acid pH observed with the aminopeptidase-treated riboflavin peptide (Figure 2) decreases from a value of 0.35 to 0.14, similar to the ratio (0.12) obtained with authentic 8α-(N^3-histidyl)riboflavin.

Subunits of Sarcosine Oxidase P. A value of 168000 was obtained for the molecular weight by gel filtration. Four subunits with molecular weights of 100000, 42000, 20000, and 6000 are detected when the enzyme is denatured and subjected to polyacrylamide gradient electrophoresis in the presence of SDS (Figure 3). When unstained gels are viewed under ultraviolet light, a single yellow fluorescent band is observed with an electrophoretic mobility corresponding to a molecular weight of 42000. The results indicate that the covalent flavin is attached to the next to the largest subunit.

The presence of four nonidentical subunits, including ones that are fairly small, raises the possibility that the enzyme may have suffered proteolytic damage during isolation. Initial evidence against this possibility is provided by the fact that the four subunits are observed after purification by a procedure where the crude extract is repeatedly treated with a mixture of protease inhibitors (phenylmethanesulfonyl fluoride, aprotinin, soybean trypsin inhibitor, and N^{α}-*p*-tosyl-L-lysine chloromethyl ketone). Further evidence was sought in experiments to determine whether the same four subunits are present in crude extracts prepared under conditions where all

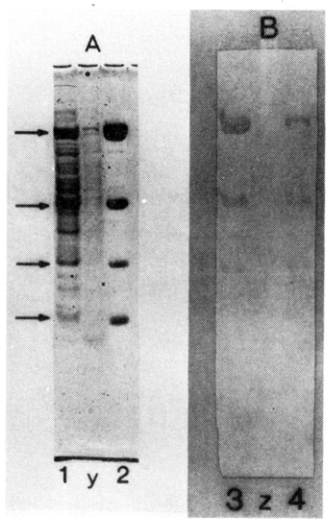


FIGURE 4: Western blot analysis of sarcosine oxidase P subunits in crude extracts of *Corynebacterium* sp. P-1. Samples were subjected to SDS–polyacrylamide gradient electrophoresis. The gel was stained for protein (panel A) or subjected to Western blot transfer to nitrocellulose where subunits were detected with an ELISA assay (panel B). Crude cell extract (130 μg) was applied in lanes 1 and 4. Purified sarcosine oxidase P (25 μg) was applied in lanes 2 and 3. Arrows at lane 1 mark the positions of bands in the crude extract that appear to correspond to sarcosine oxidase subunits. (An unrelated experiment appears in lanes y and z.)

proteins, including proteases, are denatured immediately upon release from the cell. With *Corynebacterium* sp. P-1, the latter is easily accomplished by sonicating a cell suspension in 10% trichloroacetic acid.

An immunochemical method was sought to detect sarcosine oxidase subunits in crude extracts. Antibody against sarcosine oxidase P was available from mice immunized with purified, native enzyme. Since subunit analysis is conducted with denatured enzyme, immunoblot experiments were conducted to determine whether treatment with SDS might interfere with the antibody–antigen reaction. In these experiments, native or denatured, purified enzyme is applied to nitrocellulose in a series of separate spots. The sheet is washed and treated to block any unoccupied protein binding sites. The antigen spots are then treated with varying amounts of antibody against sarcosine oxidase P, and antigen–antibody complexes are visualized as pink spots using an enzyme-linked immunosorbent assay (ELISA). Antibody–antigen complexes were observed with both native and denatured enzyme. The intensity of the pink spots was unaffected by SDS treatment but did vary depending on the amount of antibody used. (A 1:100 dilution of antisera gave a good reaction and was used in further experiments.) No reaction was observed with serum from control mice. The results show that the antibody–antigen reaction is unaffected by denaturation of sarcosine oxidase.

Multiple protein bands are observed after SDS–polyacrylamide gradient electrophoresis of the crude extract prepared by sonicating *Corynebacterium* sp. P-1 in trichloroacetic acid. As indicated by the arrows in Figure 4, there appear to be four bands in the crude extract that migrate at positions corresponding to the four subunits seen with purified enzyme. That the sarcosine oxidase subunits might be prominent in the crude extract is not surprising since the enzyme is a major protein when *Corynebacterium* sp. P-1 is grown in the presence of sarcosine. To obtain direct evidence for sarcosine oxidase subunits, the gel also included several sections that were not stained for protein. Each section included a lane for pure enzyme, crude extract, and a mixture of molecular weight markers. The separated polypeptide chains in these sections

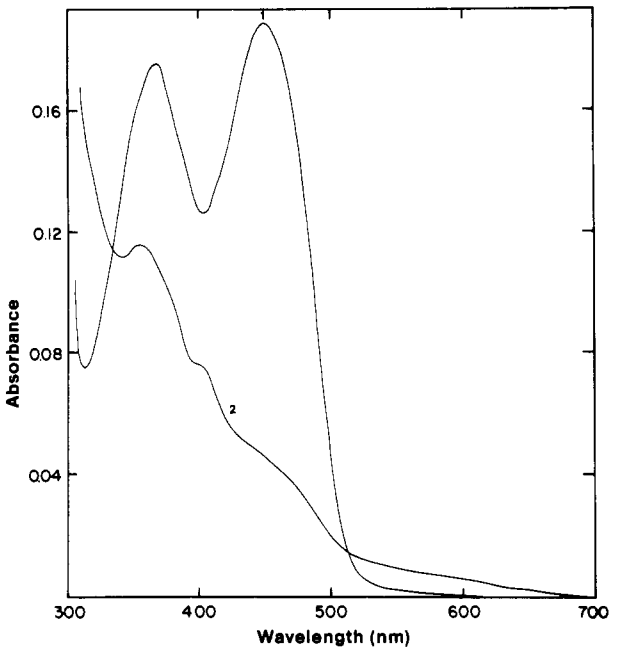


FIGURE 5: Reaction of sarcosine oxidase P with sarcosine. Curve 1 is the spectrum of the enzyme in 10 mM potassium phosphate, pH 8.0, at 5 °C. Curve 2 was recorded immediately after the aerobic addition of 4.0 mM sarcosine.

were electrophoretically transferred to nitrocellulose (Western blot). One section was stained for protein with amido black. The results (data not shown) were similar to that obtained when a section of the original gel was stained with Coomassie blue, showing that protein transfer did occur. Another section of the nitrocellulose was mixed with antibody against sarcosine oxidase P. Antigen–antibody complexes were visualized by an ELISA assay similar to that used for immunoblot experiments. With purified enzyme, an antigen–antibody complex is observed with the three largest subunits. This suggests that the smallest subunit lacks an antigenic determinant. A very faint band was observed for the largest subunit in control assays where sarcosine oxidase P antibody was omitted or replaced with serum from nonimmunized mice (data not shown). This blank reaction was negligible as compared with the intense band seen in the presence of sarcosine oxidase P antibody. Three bands are observed with the crude cell extract, appearing at positions that coincide with those observed with purified enzyme (Figure 4). (A very faint blank reaction for the largest subunit was seen.) There is no evidence for reaction with a larger polypeptide in the crude extract that might represent a precursor of the subunits seen with purified enzyme. The results provide strong evidence against proteolytic degradation and suggest that sarcosine oxidase is present in intact *Corynebacterium* sp. P-1 as a multisubunit enzyme.

Reaction of Sarcosine Oxidase P with Sarcosine and Sulfite. Both flavins in sarcosine oxidase P are reducible (either directly or indirectly) by substrate, as evidenced by the decrease in absorbance at 450 nm observed immediately after the aerobic addition of 4.0 mM sarcosine (Figure 5). Complete reoxidation of the enzyme was observed after 7 h. The spectrum of reduced sarcosine oxidase P is very similar to that observed for sarcosine oxidase J (Jorns, 1985). In both cases, reduction is accompanied by an increase in absorbance at $\lambda > 510$ nm.

Flavoprotein oxidases characteristically react with sulfite to form a reversible, covalent complex that exhibits negligible absorbance at 450 nm (Massey et al., 1969; Jorns & Hersh, 1976). As expected for sulfite complex formation, titration of sarcosine oxidase P with sulfite results in a progressive loss of absorbance at 450 nm ($K_d = 1.2 \times 10^{-4}$ M at 5 °C in 10 mM potassium phosphate, pH 8.0). However, only half of the flavin in the enzyme reacts with sulfite, as evidenced by the residual absorbance at 450 nm in the presence of excess sulfite. [The spectral course of the titration (data not shown) is very similar to that reported for sarcosine oxidase J (Jorns, 1985).] The enzyme–sulfite complex is catalytically inactive, as shown by the nearly complete inhibition (97%) observed when activity (O_2 consumption) is measured in the presence of excess sulfite. The activity loss is reversible since quantitative return of activity (97%) is observed when enzyme is mixed with excess sulfite but then diluted in order to dissociate the complex under assay conditions. Addition of sarcosine (4.0 mM) in the presence of excess sulfite (3.3×10^{-2} M) results in a rapid reduction of the sulfite-unreactive flavin. However, no reoxidation was observed after 20 h whereas complete reoxidation is observed in 7 h for a control reaction in the absence of sulfite. The results suggest that the two flavins in sarcosine oxidase P have different roles in catalysis. Sulfite inactivates the enzyme by reacting with only one of the flavins. Sulfite does not appear to block electron input from sarcosine but does inhibit the reoxidation of reduced enzyme, suggesting that sulfite reacts with the flavin that transfers electrons to oxygen.

Comparison of Sarcosine Oxidase P with Sarcosine Oxidase J. Similar values are obtained for specific activity and for the ratio A_{280}/A_{450} with sarcosine oxidase P (12.2 units/mg, 11.8) and sarcosine oxidase J (10.8 units/mg, 12.7). Both enzymes contain covalent and noncovalent flavin. In each enzyme, the flavins appear to have different roles in catalysis. The covalent flavin in sarcosine oxidase P is identified as 8α-(N^3-histidyl)-FAD while the noncovalent flavin is shown to be FAD. The same flavin composition is found in sarcosine oxidase J (Hayashi et al., 1982). A molecular weight of 168 000 is observed for both enzymes in gel filtration experiments. A similar value for the molecular weight of sarcosine oxidase J (174 000) was obtained by Suzuki (1981) in sedimentation experiments. That the enzymes are not identical is suggested by a small, but reproducible, difference in mobility observed with the native enzymes in polyacrylamide gel electrophoresis (Figure 1). Both enzymes contain four nonidentical subunits. The molecular weights of the two larger subunits (100 000 and 42 000) are the same for both enzymes, as judged by SDS–polyacrylamide gradient electrophoresis (Figure 3). In each case, the covalent flavin is attached to the subunit with a molecular weight of 42 000. Differences are observed for the molecular weights of the other subunits (sarcosine oxidase P, M_r 20 000 and 6000, vs. sarcosine oxidase J, M_r 21 000 and 7000) (Figure 3). Values of 110 000, 44 000, 21 000, and 10 000 were reported by Suzuki (1981) for the subunits of sarcosine oxidase J.

That sarcosine oxidase P and sarcosine oxidase J are similar but not identical is confirmed by various immunochemical studies. In one type of experiment, a fixed amount of enzyme is bound to a microtiter plate and then mixed with varying amounts of polyclonal antibody. Formation of antibody–antigen complexes is quantitated by an ELISA assay. Antibody–antigen complexes are formed with both enzymes by using antibody formed against sarcosine oxidase P. However, for a given, nonsaturating amount of antibody, a greater number of complexes are observed with sarcosine oxidase P (Figure 6). This suggests that a fraction of the polyclonal antibody preparation had been formed against antigenic determinants present in sarcosine oxidase P but not in sarcosine oxidase J. Similar results were obtained with antibody against

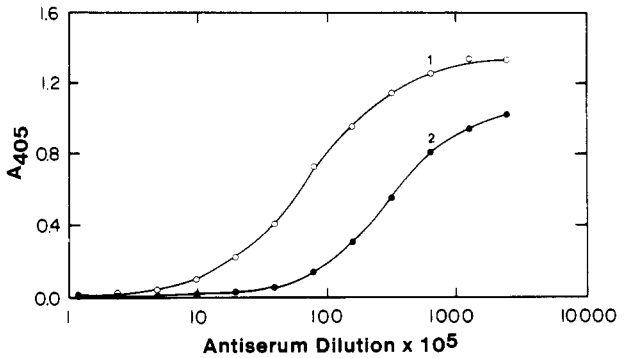


FIGURE 6: Reaction of sarcosine oxidase P and sarcosine oxidase J with antibody against sarcosine oxidase P. Antibody–antigen complex formation, as measured at 405 nm in an ELISA assay, is plotted vs. the amount of antibody (50 μL of various antiserum dilutions) added to a fixed amount of antigen (1 μg). The data with sarcosine oxidase P and sarcosine oxidase J are plotted in curves 1 and 2, respectively.

sarcosine oxidase J where a weaker reaction was observed with sarcosine oxidase P.

Further evidence was sought in competition experiments where a fixed amount of sarcosine oxidase J (1 μg) was bound to a microtiter plate and then reacted with antibody (50 μl of a 1:1000 dilution of antiserum) against sarcosine oxidase J in the presence of variable amounts (0.1–22.5 μg) of unbound sarcosine oxidase J or sarcosine oxidase P. In this experiment, the free antigen competes with the bound antigen for a limited amount of antibody. If sufficient amounts of the competing antigen (2.5 μg) are added, reaction of the antibody with bound antigen is completely blocked in the case where the free and bound antigens are both sarcosine oxidase J. On the other hand, when the competing antigen is sarcosine oxidase P, the amount of complex formed with bound sarcosine oxidase J and antibody against sarcosine oxidase J is diminished (a plateau is reached with 2.5 μg of competing antigen) but not eliminated (data not shown). The results strongly indicate that sarcosine oxidase P can react only with a fraction of the polyclonal antibodies against sarcosine oxidase J. This means that the enzymes do not contain the same set of antigenic determinants. Similar results are obtained in competition experiments using antibody against sarcosine oxidase P and sarcosine oxidase P as the bound antigen.

Discussion

In this paper, we have shown that sarcosine oxidase P contains both covalent [8α-(N^3-histidyl)-FAD] and noncovalent flavin (FAD). Hydrolysis of the covalent flavin from the FAD to the FMN level occurred during initial protein digestion under conditions that have been successfully used in many other covalent flavin studies [e.g., see McIntire et al. (1980, 1981), Fukuyama & Miyaka (1979), Kenney et al. (1979), Steenkamp et al. (1978), and Weiner & Dickie (1979)]. In view of these results, it may be prudent to test for AMP release in cases where the data suggest an FMN analogue. Our data also suggest that incubation of a flavin peptide with aminopeptidase until the R_f value in TLC studies changes to a constant value may not always be a reliable indicator of complete hydrolysis, which should be checked by HCl hydrolysis. That apoflavodoxin is able to bind the FMN polypeptide from sarcosine oxidase P (M_r 42000) indicates that it can accommodate a remarkably large substituent at the 8α-position. This is consistent with other studies which show that the aromatic ring of FMN bound to flavodoxin sticks out into the solvent and that apoflavodoxin can bind FMN derivatives bearing bulky substituents in the aromatic ring (Mayhew, 1971; Oestreicher et al., 1976). The results suggest that an apoflavodoxin affinity column (Mayhew & Strating, 1975) could be useful in purifying flavin peptides for amino acid sequence analysis.

The flavin composition found for sarcosine oxidase P is the same as that reported for sarcosine oxidase J (Hayashi et al., 1982). The flavins in sarcosine oxidase P appear to have different roles in catalysis, similar to sarcosine oxidase J (Jorns, 1985). The two enzymes exhibit many other similarities but are not identical, as judged by electrophoretic and immunochemical studies. The results suggest that the synthesis of a sarcosine oxidase containing both covalent and noncovalent flavin is not a very unusual event in corynebacteria. Studies are currently in progress to determine whether other flavoproteins synthesized by *Corynebacterium* sp. P-1 exhibit the same feature. The presence of both covalent and noncovalent flavin does not appear to be a general property of sarcosine oxidizing enzymes, as evidenced by the fact that only covalently bound flavin is found in sarcosine oxidase from *Cylindrocarpon didymum* (Mori et al., 1980) and in sarcosine dehydrogenase from rat liver (Patek & Frisell, 1972; Sato et al., 1981) or from *Pseudomonas* sp. (Pinto & Frisell, 1975). The covalently bound flavin has been identified only in the latter two enzymes where it was shown to be 8α-(N^3-histidyl)-FAD. Among other flavoenzymes, only NADPH–cytochrome P-450 reductase (Vermilion et al., 1981), NADPH–sulfite reductase (Siegel et al., 1972), and dihydroorotate dehydrogenase (Aleman et al., 1966) contain two nonequivalent flavins. The flavins (FAD and FMN) in these enzymes also have different catalytic functions, but both flavins are noncovalently bound.

Sarcosine oxidase from *Cylindrocarpon didymus* (Mori et al., 1980) and sarcosine dehydrogenase from rat liver (Satu et al., 1981) are monomeric proteins (M_r 45000 and 94000, respectively). Sarcosine dehydrogenase from *Pseudomonus putida* (M_r 170000, flavin coenzyme not identified) contains four identical subunits (Oka et al., 1979). Sarcosine oxidase P and sarcosine oxidase J are isolated as multisubunit proteins, containing four dissimilar subunits. That this is an unusual subunit composition is evidenced by the fact that a list of 480 proteins (M_r range 11466–1000000) containing 2 or more subunits included only 6 examples of proteins containing 4 or more dissimilar subunits (Darnall & Klotz, 1976). As compared with sarcosine oxidation, the six examples comprise a group of proteins with highly complex functions (three RNA polymerases from different sources; rabbit muscle myosin, rat ATPase, *Escherichia coli* Qβ-replicase), suggesting that the subunit composition observed for the two corynebacteria sarcosine oxidases might be an artifact caused by proteolytic damage during enzyme purification. Proteolytic artifacts should be eliminated in extracts prepared by sonicating *Corynebacterium* sp. P-1 in the presence of trichloroacetic acid. Western blot analysis of these extracts shows that the unusual subunit composition is a genuine property of the enzyme as it exists in vivo. In contrast, similar studies with glycinamide ribonucleotide transformylase indicate that the enzyme exists in chicken liver as a monomer (M_r 117000) and not as the dimer (subunit M_r 55000) originally isolated (Young et al., 1984). Studies are in progress to characterize the function of each of the subunits in sarcosine oxidase P and to evaluate the metabolic role of the enzyme.

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Registry No. FAD, 146-14-5; 8α-(N^3-histidyl)-FAD, 30017-36-8; sarcosine oxidase, 9029-22-5.

REFERENCES

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

Bergmeyer, H. U. (1974) *Methods of Enzymatic Analysis*, pp 673–680, 2127–2131, Academic Press, New York.

Blake, M. S., Johnston, K. A., Russell-Jones, G. J., & Gotschlich, E. C. (1984) *Anal. Biochem. 136*, 175–179.

Branson, D. (1972) *Methods in Clinical Bacteriology*, p 178, Charles C. Thomas, Springfield, IL.

Darnall, D. W., & Klotz, I. M. (1976) in *Handbook of Biochemistry and Molecular Biology, 3rd Ed.* (Fasman, G. D., Ed.) *Proteins*, Vol. II, pp 325–371, CRC Press, Cleveland, OH.

Davis, B. D., Dulbecco, R., Eisen, H. N., Ginsberg, H. S., & Wood, W. B. (1973) *Microbiology*, pp 682–692, Harper & Row, Hagerstown, MD.

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

Edmondson, D. E., Kenney, W. C., & Singer, T. P. (1976) *Biochemistry 15*, 2937–2945.

Fukuyama, M., & Miyaka, Y. (1979) *J. Biochem. (Tokyo) 85*, 1183–1193.

Gerhardt, P. (1981) *Manual of Methods for General Bacteriology*, pp 26, 120, American Society for Microbiology, Washington, D.C.

Hayashi, S., Nakamura, S., & Suzuki, M. (1980) *Biochem. Biophys. Res. Commun. 96*, 924–930.

Hayashi, S., Suzuki, M., & Nakamura, S. (1982) *Biochem. Int. 4*, 617–620.

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

Jorns, M. S., & Hersh, L. B. (1975) *J. Biol. Chem. 250*, 3620–3628.

Jorns, M. S., & Hersh, L. B. (1976) *J. Biol. Chem. 251*, 4872–4881.

Kenney, W., Edmondson, D. E., Singer, T. P., Nishikimi, M., Noguchi, E., & Yagi, K. (1979) *FEBS Lett. 97*, 40–42.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Majarian, W. R. (1985) Thesis, Hahnemann University, Philadelphia, PA.

Massey, V., Müller, F., Feldberg, R., Schuman, M., Sullivan, P. A., Howell, L. G., Mayhew, S. G., Matthews, R. G., & Foust, G. P. (1969) *J. Biol. Chem. 244*, 3999–4006.

Mayhew, S. G. (1971) *Biochim. Biophys. Acta 235*, 289–302.

McIntire, W., Edmondson, D. E., & Singer, T. P. (1980) *J. Biol. Chem. 255*, 6553–6555.

McIntire, W., Edmondson, D. E., Hopper, D. J., & Singer, T. P. (1981) *Biochemistry 20*, 3068–3075.

McIntire, W., Singer, T. P., Ameyama, M., & Adachi, O. (1985) *Biochem. J. 231*, 651–654.

Mori, N., Sano, M., Tani, Y., & Yamada, H. (1980) *Agric. Biol. Chem. 44*, 1391–1397.

Oestreicher, G., Edmondson, D. E., & Singer, T. P. (1976) in *Flavins and Flavoproteins* (Singer, T. P., Ed.) pp 447–452, Elsevier Scientific Publishing Co., Amsterdam.

Oka, I., Yoshimoto, T., Rikitake, K., Ogushi, S., & Tsura, D. (1978) *Agric. Biol. Chem. 43*, 1197–1203.

Ouchterlony, O. (1949) *Acta Pathol. Microbiol. Scand. 26*, 516–524.

Patek, D. R., & Frisell, W. R. (1972) *Arch. Biochem. Biophys. 150*, 347–354.

Pinto, J. T., & Frisell, W. R. (1975) *Arch. Biochem. Biophys. 169*, 483–491.

Sato, M., Ohnishi, N., & Yagi, K. (1981) *Biochem. Int. 3*, 89–92.

Siegel, L. M., Faeder, E. G., & Kamin, H. (1972) *Z. Naturforsch., B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol. 27B*, 1087–1089.

Steenkamp, D. J., Kenney, W. C., & Singer, T. P. (1978) *J. Biol. Chem. 253*, 2812–2817.

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

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

Towbin, H., Staehelin, T., & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4350–4354.

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

Walker, W. J., Kearney, E. B., Seng, R. L., & Singer, T. P. (1971) *Eur. J. Biochem. 24*, 328–331.

Walker, W. H., Singer, T. P., Ghisla, S., & Hemmerich, P. (1972) *Eur J. Biochem. 26*, 279–289.

Wassink, J. H., & Mayhew, S. G. (1975) *Anal. Biochem. 68*, 609–616.

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

Weiner, J. H., & Dickie, P. (1979) *J. Biol. Chem. 254*, 8590–8593.

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

Young, M., Sammons, D., Mueller, W. T., & Benkovic, S. J. (1984) *Biochemistry 23*, 3979–3986.