Cloning and Functional Expression of a Mammalian Gene for a Peroxisomal Sarcosine Oxidase*

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Sarcosine oxidation in mammals occurs via a mitochondrial dehydrogenase closely linked to the electron transport chain. An additional \text{H}_2\text{O}_2\text{-producing sarcosine oxidase has now been purified from rabbit kidney. A corresponding cDNA was cloned from rabbit liver and the gene designated \text{sox}. This rabbit \text{sox} gene encodes a protein of 390 amino acids and a molecular mass of 44 kDa identical to the molecular mass estimated for the purified enzyme. Sequence analysis revealed an N-terminal ADP-\beta\text{S}-binding fold, a motif highly conserved in tightly bound flavoproteins, and a C-terminal peroxisomal targeting signal 1. Sarcosine oxidase from rabbit liver exhibits high sequence homology (25–28\% identity) to monomeric bacterial sarcosine oxidases. Both purified sarcosine oxidase and a recombinant fusion protein synthesized in \textit{Escherichia coli} contain a covalently bound flavin, metabolize sarcosine, L-pipecolic acid, and l-proline, and cross-react with antibodies raised against L-pipecolic acid oxidase from monkey liver. Subcellular fractionation demonstrated that sarcosine oxidase is a peroxisomal enzyme in rabbit kidney. Transfection of human fibroblast cell lines and CV-1 cells (monkey kidney epithelial cells) with the \text{sox} cDNA resulted in a peroxisomal localization of sarcosine oxidase and revealed that the import into the peroxisomes is mediated by the peroxisomal targeting signal 1 pathway.

In mammals a variety of \text{H}_2\text{O}_2\text{-producing oxidases including }\beta\text{-amino acid oxidase, }\beta\text{-aspartate oxidase, L-hydroxy-acid oxidase, acyl-CoA oxidase, and L-pipecolic acid oxidase are compartmentalized in peroxisomes. The \text{H}_2\text{O}_2} generated from these reactions is then converted to \text{H}_2\text{O} and \text{O}_2 by the peroxisomal matrix enzyme catalase (1). Several disorders have been described in which there is a defect in peroxisomal assembly that results in a partial or total absence of peroxisomal functions (for a review see Ref. 2). Patients with these peroxisomal disorders such as Zellweger syndrome, neonatal adrenoleukodystrophy, infantile Refsum disease, and hyperpipecolic acidemia all have elevated levels of L-pipecolic acid, an imino acid, which in human and monkey liver is oxidized by a peroxisomal L-pipecolic acid oxidase (3). Indeed, L-pipecolic acid oxidase activity was not detected in liver samples from patients with Zellweger syndrome (4). Primates dehydrogenate L-pipecolic acid to δ-piperideine-6-carboxylate which is spontaneously converted to \(\alpha\)-aminoacidipic acid γ-semialdehyde.

The subcellular localization of this pathway seems to differ in other mammalian species. In rabbits, guinea pigs, dogs, and sheep L-pipecolic acid oxidation is primarily mitochondrial (5). However, during our studies examining the subcellular distribution of L-pipecolic acid oxidation in rabbits, a considerable amount of L-pipecolic acid oxidation was detected in the peroxisomes, in addition to the previously reported mitochondrial activity (6). Interestingly, this peroxisomal enzyme showed a high specific activity for sarcosine and also oxidized L-pipecolic acid and L-proline. This finding raised the question whether the purified enzyme is also a sarcosine oxidase.

In mammals, the oxidative removal of the methyl group from sarcosine is catalyzed by sarcosine dehydrogenase (EC 1.5.99.1) and dimethylglycine dehydrogenase (EC 1.5.99.2) in mitochondria. These enzymes were characterized as the two main folate-containing enzymes in rat liver mitochondria. Both enzymes are closely linked to the electron transport chain and form 5,10-methylenetetrahydrofolate. This “active” formaldehyde is predominantly used for the formation of serine from glycine by serine hydroxymethylase (7–11).

The enzyme investigated in this study differs from the classical mammalian sarcosine dehydrogenase described earlier. The reaction takes place in peroxisomes rather than in mitochondria. The reaction mechanism is an \text{H}_2\text{O}_2\text{-generating oxi-}
dation and not an electron transport chain-linked dehydroge-
nation. Although no mammalian sarcosine oxidases are known, several sarcosine oxidases from bacteria have been purified and characterized. They can be classified as monomeric enzymes (e.g. \textit{Arthrobacter} sp. TE 1826 (12), \textit{Bacillus} sp. NS-129 (13), \textit{Bacillus} sp. B-0618 (14), \textit{Streptomyces} sp. KB210–85Y (15), and \textit{Cylindrocarpon didymum} M-1 (16) and as tetrameric enzymes (e.g. \textit{Arthrobacter ureafaciens} (17), from \textit{Corynebacterium} sp. U-96 (18) and \textit{Corynebacterium} sp P-1 (19)). The monomeric enzymes have a molecular mass of 42–45 kDa which is similar to the size of the \(\beta\)-subunit of the tetrameric enzymes. All enzymes contain a covalently attached flavin (\(\beta\)-subunit of the tetrameric enzymes); the tetrameric enzymes also have a noncovalently bound flavin and at least the enzyme from \textit{Corynebacterium} sp. P-1 probably contains an additional tightly bound NAD (20). While the covalent attachment is not unusual for bacterial oxidases, it has been rarely found in mammals. The list of mammalian enzymes with covalently attached flavins includes the mitochondrial sarcosine dehydrogenase and dimethylglycine dehydrogenase from rat and the peroxisomal L-pipecolic acid oxidase from monkey liver (3).

Like the mitochondrial dehydrogenases, the bacterial tet-
rameric sarcosine oxidases (21) can bind tetrahydrofolate and then yield 5,10-methylenetetrahydrofolate instead of formalde-
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hyde. It is not known whether the monomeric enzymes react with tetrahydrofolate.

A mammalian sarcosine oxidase from rabbit kidney has now been purified to investigate the association between the oxidation of the imino acid L-pipecolic acid and the methyl group acceptor sarcosine. Subsequently, the sarcosine oxidase gene was cloned from rabbit liver and expressed in Echerichia coli and in mammalian cells.

EXPERIMENTAL PROCEDURES

Materials—Sarcosine, L-pipecolic acid, 4-hydroxyphenylacetic acid, protease inhibitors, protein molecular weight standards, and protein standards for isoelectric focusing were from Sigma (Deisenhofen, Germany). L-Pipecolic acid was also purchased from Bachem (Heidelberg, Germany). FAD was obtained from Boehringer Mannheim (Mannheim, Germany).

Bacterial Strains, Libraries, and Cells—The E. coli strains K802, DH5α, and TG1 were used for all molecular screening and cloning procedures. To obtain the sox sequence, a rabbit liver cDNA library constructed in Agt10 (kindly provided by Dr. M. Kilimann) and a rabbit liver genomic library cloned into EMBL3 SP6/T7 (Clontech, Palo Alto, CA) were screened.

The human cell lines were obtained and cultured as described by Moser et al. (22). The transformed derivatives were kindly provided by Dr. S. J. Gould. African Green Monkey CV-1 cells were obtained from ATCC (Rockville, MD; ATCC number CCL70).

Sarcosine Oxidase Assay—Sarcosine oxidase activity was determined by measuring the hydrogen peroxide formation in a horseradish peroxidase-coupled fluorimetric assay, as described by Poesch and Yamazaki (23). The reaction mixture contained in a total volume of 550 μl, 55 mM Tris, pH 8.4, 1 mM 4-hydroxyphenylacetic acid, 4 units of horseradish peroxidase (Boehringer Mannheim, types I or II), 9 mM sodium azide, 1 mM FAD, and 9.8 mM sarcosine. The reaction was started with sarcosine, proceeded for various periods (15–60 min) at 37 °C in the dark, and was stopped by the addition of 1.5 ml of 0.2 mM glycine/sodium carbonate buffer, pH 10.5. The fluorescence at 415 nm (excitation 318 nm) was determined with the spectrophotometer JY 3D (Jobin-Yvon, France) or with the spectrophotometer LS 50 (Perkin-Elmer, Weiterstadt, Germany).

Protein concentrations were estimated by the method of Bradford (24) using the Coomassie Protein Plus reagent (Pierce). Bovine serum albumin was used as standard.

Purification of Sarcosine Oxidase—All purification steps were performed at 4 °C under conditions that minimize light exposure of the enzyme. Kidneys from New Zealand White rabbits were cut in half to isolate the kidney cortex. The cortex (10 g) was minced and homogenized in 5 volumes (v/w) of homogenization buffer (250 mM sucrose, 6.8 mM Mops, pH 7.5, 1 mM EDTA, pH 7.5, containing 0.5 mM phenylmethylsulfonyl fluoride, 1 mM pepstatin, and 1 mM leupeptin) with 3 up-and-down passages of a loose-fitting pestle in an Potter-Elvehjem homogenizer at 800 rpm (Braun, Melsungen, Germany). The homogenate was centrifuged at 600 × g for 10 min. The resulting pellet was resuspended in 3.5 volumes (v/w) homogenization buffer and again centrifuged at 600 × g for 10 min. Both supernatants were combined and further centrifuged at 5,100 × g for 15 min to pellet the M fraction which contained most of the mitochondria and peroxisomes. This pellet was gently rehomogenized in 6 volumes of homogenization buffer. To release the enzyme from the organelles, the M fraction was frozen in a thin layer at -70 °C for at least 15 min, quickly thawed at 37 °C, chilled, and centrifuged at 26,000 × g for 15 min. The supernatant containing the solubilized enzyme was saved.

Heat Denaturation—The enzyme solution was brought to 46 °C, stirred continuously for 10 min, and then chilled immediately. The denatured proteins were removed by centrifugation at 26,600 × g for 15 min. The resulting supernatant was used for CM-cellulose chromatography.

CM-52 Cellulose Batch Chromatography—CM-52 cellulose (Whatman), equilibrated in 1 mM potassium phosphate buffer, 1 mM EGTA, pH 6.0, was mixed with 2 volumes of enzyme solution (~70 ml) and shaken at 4 °C for 30 min. Afterward, the gel suspension was transferred onto a glass frit (porosity G3), and a light vacuum was applied to remove the filtrate. The remaining gel was washed 5 times with 20 ml of equilibration buffer, and the enzyme was eluted in 7 fractions of 15 ml each (200 mM potassium phosphate buffer, 1 mM EGTA, pH 8.3). The enzymatically active fractions were combined and concentrated by di-1

1 The abbreviations used are: MOPS, 4-morpholinopropanesulfonic acid; PAGE, polyacrylamide gel electrophoresis; PTS1, peroxisomal targetting signal 1; DIG, digoxigenin; IPTG, isopropylthio-β-D-galactoside; GST, glutathione S-transferase, MBP, maltose binding protein; RCDP, rhizomelic chondrodysplasia punctata; bp, base pair(s); RT-PCR, reverse transcription-polymerase chain reaction.
The yellow pellet was resuspended in 1 ml of 5M guanidine hydrochloride. The protein dissolved in 5M guanidine hydrochloride was concentrated using a Centricon 30 microconcentrator (Amicon). The protein solution was dialyzed against 5 μM guanidine hydrochloride, and the record's spectrum was compared with the spectrum of the corresponding filtrate.

Sequencing and RT-PCR—Nucleotide sequences were determined by primer walking using the Sequenase Version 2.0 and T7 DNA Polymerase (U. S. Biochemical Corp.) following the method of Sanger et al. (33). Alternatively, samples were sequenced on a DNA sequencer 373A (Applied Biosystems, Weiterstadt, Germany) utilizing the Prism Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit.

The complete cDNA for sarcosine oxidase was constructed by RT-PCR. 1 μg of total rabbit liver RNA was reverse transcribed with 2 pmol of T3-1 primer (5'-GGGAGAAGATTGTTGTA-3') and 200 units of SuperScript RT (Life Technologies, Inc., Eugene, Germany). The synthesized cDNA was used as template for a PCR with two specially constructed primers A (5'-TAGAGCTCTAGATGGGCGCTCAGAGAT-3') and B (5'-CCGTCTAGATCTCAGGGACACTCC-5'), which annealed at bp 1-18 and bp 1245-1262, respectively, and contained either a XhoI or a XbaI restriction site. The major part of the PCR product, the fragment between base pairs 189 and 1168, was exchanged against the corresponding fragment from the cDNA clone obtained by library screening to avoid Taq polymerase errors. The rest of the PCR fragment was confirmed by sequencing. This PCR fragment was cloned into the XhoI and XbaI sites of pKS+ (Stratagene, La Jolla, CA) to yield the pBR1 construct.

Plasmid—In addition to pBR1, the constructs pBR2-4 were used to express full-length sarcosine oxidase in E. coli and for transfection experiments in mammalian cell cultures. Cloning of the XhoI/XbaI fragment from pBR1 into the SalI/HindIII sites of pGEX4T-3 (Pharmacia) resulted in the pBR2 construct which encodes a N-terminal fusion with glutathione S-transferase (GST). To obtain the pBR3 construct, coding for an N-terminal fusion with maltose binding protein (MBP), the XhoI/XbaI fragment of pBR1 was cloned into the SalI/HindIII sites of pMALc2 (Invitrogen, NVLeek, The Netherlands). The expression of sox in Mammalian Cells—CV-1 cells and human fibroblast cell lines were transfected with 3 μg of pBR4 plasmid in the presence of 30 μg of lipofectamine (Life Technologies, Inc.) according to the manufacturer's suggestions. After 48 h cells were analyzed by indirect immunofluorescence as described by Slawecki et al. (40). Best results were obtained at a dilution of 1:400 for affinity-purified α-picolinic acid oxidase antibodies and 1:100 for α-catalase antibodies (The Binding Site, Heidelberg Germany). The fluorescein isothiocyanate- and tetramethylrhodamine isothiocyanate-conjugated secondary antibodies (Dianova, Hamburg, Germany) were diluted 1:100. Micrographs were taken with Tmax 400 and Ektachrome 400 films (Kodak).

RESULTS

Purification of Sarcosine Oxidase—Rabbit kidney sarcosine oxidase catalyzes the demethylation of sarcosine to glycine, formaldehyde, and H₂O₂. The H₂O₂ formation can be quantified in a fluorescence coupled assay with 4-hydroxyphenylac-
etic acid as fluorophore. The purification procedure for sarcosine oxidase from rabbit kidney is summarized in Table I. We obtained the M fraction containing peroxisomes, mitochondria, and lysosomes by differential centrifugation. Although preliminary experiments determining the subcellular distribution of sarcosine oxidase suggested a peroxisomal localization for sarcosine oxidase, an additional centrifugation step to purify the peroxisomes resulted in a lower yield of sarcosine oxidase, probably due to breakage of the organelles and loss of the enzyme into the supernatant. Sarcosine oxidase was purified 666-fold with an overall yield of 12.8% (Table I). The purest fraction separated into two closely migrating bands by SDS-PAGE (Fig. 1). These bands appear to have nearly identical pI and molecular mass in two-dimensional gel electrophoresis. The molecular mass of sarcosine oxidase was estimated at 44 kDa under denaturing conditions (Fig. 1). Isoelectric focusing in 6 M urea and 10% Nonidet P40 gave a pI of 7.8. The optimal enzyme activity in Tris buffer at 37°C was found at a pH of 8.6.

Sarcosine Oxidase Oxidizes Sarcosine, L-Pipecolic Acid, and L-Proline—Substrate studies at a fixed concentration of 9.8 mM, which are summarized in Table II, identify sarcosine as the major substrate. Other prominent substrates are L-pipecolic acid and L-proline, and pipecolic acid reacted to a minor extent, but we could not rule out a small contamination of the protein preparation with D-aspartate oxidase. No H₂O₂ was formed with dimethylglycine. This substrate pattern is quite different from that for D-aspartic acid oxidase and D-aspartate oxidase (41) but similar to that of mammalian L-pipecolic acid oxidase isolated from monkey liver (3). L-pipecolic acid oxidase predominantly catalyzes the oxidation of L-pipecolic acid but also reacts with L-proline and sarcosine with rates of 23 and 10%, respectively.

The catalytic efficiency, the ratio of \( k_c/K_m \), is more suitable to determine which substrate is predominantly metabolized when several competing substrates are present (42). As shown in Table III, the \( k_c \) for sarcosine is about 7 times higher than the \( k_c \) for L-pipecolic acid, but the \( K_m \) value for L-pipecolic acid (5.88 mM) is much lower than that for sarcosine (66.7 mM). Thus, the calculated catalytic efficiencies of 0.158 mM⁻¹ min⁻¹ for L-pipecolic acid and 0.093 mM⁻¹ min⁻¹ for sarcosine suggest that L-pipecolic acid is a slightly favored substrate.

The effect of benzoate, a known competitive inhibitor of other peroxisomal oxidases (D-amino-acid oxidase and L-pipecolic acid oxidase) was investigated with L-pipecolic acid and sarcosine as substrates. When L-pipecolic acid was the substrate, a \( K_i \) of 2.04 mM was calculated for benzoate, with competitive inhibition (Fig. 2B). This value is similar to the \( K_i \) of 0.75 mM previously reported for L-pipecolic acid oxidase (3). The \( K_i \) value for benzoate with sarcosine as substrate was estimated at 5.46 mM. However, in this case the inhibition type was noncompetitive (Fig. 2A).

### Table II
Relative activity of sarcosine oxidase toward different substrates

| Substrate       | Relative activity |
|-----------------|-------------------|
| Sarcosine       | 100               |
| L-Pipecolic acid| 30                |
| L-Proline       | 21                |
| L-Alanine       | 2.2               |
| L-Lysine        | 1.6               |
| D-Aspartic acid | 4.3               |
| D-Alanine       | 3.3               |
| D-Proline       | 3.3               |
| D-Pipecolic acid| 1.6               |
| DL-Pipecolic acid| 18                |
| Dimethylglycine | 0                 |

### Table III
Kinetic values for purified sarcosine oxidase

| Substrate       | \( K_m \)  | \( k_c \)  | \( k_c/K_m \) |
|-----------------|-----------|-----------|--------------|
| Sarcosine       | 66.7  M   | 0.20  min⁻¹| 0.093  mM⁻¹ min⁻¹|
| L-Pipecolic acid| 5.88 M    | 0.846  min⁻¹| 0.158  mM⁻¹ min⁻¹|

**Fig. 2. Inhibition of sarcosine oxidase by benzoic acid using sarcosine or L-pipecolic acid as substrate.** H₂O₂ formation by purified sarcosine oxidase was measured with sarcosine at substrate concentrations between 5.9 and 29.8 mM (A) and L-pipecolic acid at different substrate concentrations between 1.4 and 17.7 mM (B). The concentration of the inhibitor benzoic acid varied between 0 and 5 mM. The \( K_i \) using sarcosine as substrate was estimated at 5.46 mM and for L-pipecolic acid as substrate at 2.04 mM.
Sarcosine Oxidase Contains a Covalently Bound Flavin—

Purified sarcosine oxidase had a yellow color, and its sarcosine oxidizing activity was not dependent on FAD addition. Its absorption spectrum with a peak at 450 nm was typical for a flavin (30). Both, sarcosine oxidase and L-pipecolic acid oxidase reacted with the antibodies, suggesting that their flavin was covalently bound, whereas D-amino-acid oxidase, which has a noncovalently bound flavin, did not react with the antibodies (Fig. 4). The same samples were analyzed with antibodies raised against L-pipecolic acid oxidase from monkey liver (3). Sarcosine oxidase from rabbit kidney was recognized by the antibodies, but the antibodies did not cross-react with D-amino-acid oxidase (Fig. 4). Sarcosine oxidase migrated slightly faster than L-pipecolic acid oxidase during SDS-PAGE.

Sarcosine Oxidase Is a Peroxisomal Enzyme—Because the antibodies against L-pipecolic acid oxidase cross-reacted with sarcosine oxidase, they could be used to determine the subcellular localization of sarcosine oxidase. For these studies, a heavy mitochondrial fraction (M fraction) from rabbit kidney cortex was further separated in a Nycodenz gradient. Peak fractions of peroxisomes, mitochondria, and the supernatant fractions, identified by marker enzyme analysis, were separated by SDS-PAGE and blotted onto nitrocellulose. The blots were analyzed with anti-L-pipecolic acid oxidase antibodies, a single protein band with a molecular mass of 44 kDa was detected in the peroxisomal fraction.
identified in the peroxisomal fractions (Fig. 5). All of the faint cross-reacting bands in the mitochondrial fractions and in the supernatant fractions also appeared in control blots incubated with preimmune serum from rabbits.

Molecular Cloning of Rabbit Liver Sarcosine Oxidase—The similarity between L-pipecolic acid oxidase from monkey liver (3) and sarcosine oxidase from rabbit kidney, especially the substrate specificity and the immunological cross-reactivity with L-pipecolic acid oxidase antibodies, encouraged us to investigate the sarcosine oxidase gene from rabbit liver.

When we probed a Northern blot with RNA from rabbit kidney and liver with a partial cDNA clone for human pipecolic acid oxidase, two hybridizing RNAs (approximately 2.3 kilobases (Fig. 6)) were detected in both kidney and liver with a higher expression level in kidney.

This same partial cDNA clone from human pipecolic acid oxidase was chosen to screen a rabbit liver cDNA library. A distinct cDNA clone (bp 157–2083) including the polyadenylation signal but lacking a putative start codon was obtained. Since we were not able to identify the 5'-end of the gene applying the rapid amplification of cDNA ends protocol (44), we probed a genomic rabbit liver library with a cDNA fragment (188 bp) from the very 5’-end of the human liver L-pipecolic acid oxidase gene. Several clones containing the putative translation start site and additional 5’ bp were isolated. A full-length cDNA was compiled by RT-PCR. We designated the rabbit sarcosine oxidase gene as sox. The sequence of the complete cDNA and the deduced amino acid sequence are shown in Fig. 7. The cDNA consists of a 12-bp 5’-untranslated region, an open reading frame of 1170 bp, and 913 bp of 3’-untranslated region and encodes for a protein Sox with 390 amino acids and a molecular mass of 44 kDa. The length of the cDNA is consistent with both the size of the native enzyme and with the size of the detected mRNA (Fig. 6), allowing 150–200 bp for the poly(A) tail. The N terminus of the protein has sequence homology to an ADP-ribosyl-binding fold, typical for proteins that bind FAD, NAD+, or NADP+ (45, 46). The last three amino acids, AHL, represent a peroxisomal targeting signal 1 (PTS1), characteristic for mammalian proteins that are translocated into peroxisomes (47).

Rabbit Sarcosine Oxidase Shows High Homology to Bacterial Sarcosine Oxidases—This mammalian sarcosine oxidase shows high homologies to monomeric bacterial sarcosine oxidases from *Streptomyces* sp. KB210-8SY (15), *Bacillus* sp. NS-129 (13), *Bacillus* sp. B-0618 (14), and *Arthrobacter* sp. TE 1826 (12). A sequence alignment with several different sarcosine oxidases obtained after a BLAST search (38) is shown in Fig. 8. The amino acid identities over the whole protein between
rabbit Sox and the four monomeric bacterial sarcosine oxidases are between 25 and 28%. Apart from the ADP-ββ-binding fold, three other almost identical regions were identified among the proteins. These segments are labeled 1–4 in Fig. 8. While the binding fold is characteristic for many enzymes, the three other homology regions are unique for monomeric sarcosine oxidases. Lower identities (14.9, 14.6, and 12.3%) were found with the β-subunit of the heterotetrameric sarcosine oxidase from *Corynebacterium* sp. P-1 (49), with the N terminus of dimethylglycine dehydrogenase from rat liver (50), and with the amino acid deaminase from *Proteus mirabilis* (51). High identities occurred with an not yet identified gene product (accession number U23529) from *Caenorhabditis elegans*. Interestingly, the encoded protein of *C. elegans* showed an N-terminal duplication of the first 300 amino acids of Sox. The identities to rabbit Sox are 25.1% for the first part and 27.6% for the C-terminal complete part (Fig. 9). As with the rabbit sarcosine oxidase, the *C. elegans* protein contains a PTS1 signal at the C terminus (AHL for rabbit Sox and SKI for the *C. elegans* gene product).

Comparison of Purified Sarcosine Oxidase to the Recombinant Gene Product of the Rabbit Liver sox Gene—To ensure that the right gene had been cloned, three characteristics of the purified sarcosine oxidase, the covalently attached flavin, the unusual substrate specificity, and the peroxisomal localization were investigated with recombinant sarcosine oxidase synthesized in *E. coli*.

**Bacterial Expression of sox Revealed That Sarcosine Oxidase Has a Covalently Attached Flavin**—Several approaches were used to express sox in *E. coli*. After transformation of *E. coli* with the pBR2 plasmid, a GST-fusion protein with a molecular mass of 80 kDa could be purified with a glutathione-Sepharose column. The recombinant protein had a slightly yellowish color. The spectrum of the recombinant protein showed absorption maxima at 380 and 450 nm (Fig. 3A) which were similar to those of the purified enzyme (Fig. 3B) and consistent with a bound flavin (43). When the protein was trichloroacetic acid-precipitated as described for the purified enzyme, no flavin was detected in the supernatant. The spectrum recorded for the recombinant enzyme (Fig. 3A) is similar to the spectra described for sarcosine oxidases (52). Interestingly, the shoulder at 480 nm, which has been described for the monomeric bacterial sarcosine oxidases, was more pronounced with the purified enzyme (52) (Fig. 3B).

Although the fusion protein could be synthesized with the covalently bound flavin, we were not able to isolate the protein in an enzymatically active form. Even after induction of the protein expression at a low IPTG concentration and at a temperature of 30 °C, the formation of inclusion bodies (53) could not be prevented. The GST-fusion protein was only solubilized and purified after treatment with detergents.

**Sarcosine Oxidase Synthesized as Fusion with Maltose-binding Protein Has Enzymatic Activity toward Sarcosine, L-Pipolic Acid, and L-Proline**—A different fusion protein was used to isolate enzymatically active sarcosine oxidase. After transformation of *E. coli* with the pBR3 construct, an MBP-fusion protein with a molecular mass of 86 kDa was partially purified with an amylose resin. After separation by SDS-PAGE the protein levels observed for the sarcosine oxidase fusion were lower than for the fusion protein found when the pMALc2 plasmid alone was used for expression in *E. coli*, even if the induction was carried out for 7 h (Fig. 10). Immunoblotting with the previously used antibodies against l-pipolic acid.
oxidase revealed an 86-kDa fusion protein cross-reacting with the antibodies, as well as a lower migrating protein band, likely due to partial degradation of the MBP-Sox protein (Fig. 10). Fractions purified by affinity chromatography with an amylase resin were investigated for oxidase activity using different substrates. The fusion protein oxidized the substrates sarcosine, L-pipecolic acid, and L-proline with different kinetics as shown by their $K_m$ and $k_c$ values (Table IV). Interestingly, the lowest $K_m$ was determined for L-pipecolic acid as substrate (1.9 mM), but $k_c$ was lower than that for sarcosine and L-proline. Sarcosine and L-proline had $K_m$ values of 6.7 and 8.0 mM, respectively. A marked inhibition was noted with sarcosine at higher substrate concentrations (above 13 mM). Catalytic efficiencies were best for L-pipecolic acid, followed by L-proline and sarcosine. When the pMALc2 vector alone was expressed in E. coli, the similarly processed protein product showed no activity with any of the three substrates.

Rabbit Sox Is A Peroxisomal Protein in Mammalian Cells—To investigate the localization of Sox in mammalian cells, the cDNA was cloned into the mammalian expression vector pcDNA3 creating pBR4. Three different human skin fibroblast cell lines, which were already transformed with SV40 large T antigen and CV-1 cells (monkey kidney epithelial cells), were transfected with pBR4, using Lipofectamine. Two days after transfection, the cells were analyzed by indirect immunofluorescence. All cell lines expressed the gene with transfection rates of 10–20%. Because rabbit sarcosine oxidase has a PTS1 signal at the C terminus, a peroxisomal localization of the enzyme was expected. After normal fibroblasts (GM5756) were transformed with pBR4 and subjected to indirect immunofluorescence using the antibodies against L-pipecolic acid oxidase (3), a punctate staining pattern was obtained (Fig. 11A). Double staining to include antibodies against the peroxisomal matrix protein catalase revealed that Sox colocalized with catalase, suggesting that it is found in or at the peroxisomes (Fig. 11B). The second investigated cell line 005-T is a transformed fibroblast cell line from a patient lacking a PTS1 receptor (complementation group 2 of the peroxisomal biogenesis disorders (40, 54)) that results in a peroxisomal import defect for PTS1 and PTS2 proteins. When pBR4 was transfected into these cells, Sox was synthesized, but the protein was found throughout the cytoplasm of the cells (Fig. 11C). Again, this protein gave the same staining pattern as catalase, which is not imported into the peroxisomes of 005-T cells (Fig. 11D). The third cell line, which was from a patient with classical rhizomelic chondrodysplasia punctata (RCDP), has an isolated peroxisome import defect for the PTS2 protein thiolase but normal PTS1 import (40, 55). Transfection of pBR4 resulted in a peroxisomal localization (Fig. 11E) of sarcosine oxidase (note the same subcellular distribution for catalase in Fig. 11F) in this cell line. These results indicate that sarcosine oxidase is a peroxisomal protein imported into the organelles by the PTS1-dependent pathway. The same results were obtained when different plasmids encoding an N-terminal histidine-tagged sarcosine oxidase (pBR5) or a protein fusion with maltose-binding protein (pBR6) were transfected. Expression of sarcosine oxidase cDNA in CV-1 cells also resulted in a peroxisomal localization of the gene product.

DISCUSSION

During our studies of L-pipecolic acid oxidase, we discovered that rabbits have a similar enzyme, but this enzyme also oxidizes sarcosine. When the gene for this protein was cloned, we found that its amino acid sequence showed the most homology to the monomeric sarcosine oxidases.

Previously, it had been reported that while in human and monkey liver, L-pipecolic acid is oxidized in peroxisomes (3); in rabbit liver and kidney L-pipecolic acid is primarily oxidized in mitochondria (6). However, our studies with rabbit kidneys suggested that there might be L-pipecolic acid oxidation in peroxisomes as well. When we reexamined the oxidation of 14C-radiolabeled L-pipecolic acid in subcellular fractions of a Nycodenz gradient by measuring the formation of aminoacidic acid (6), approximately 80% was attributable to mitochondrial activity, and 20% could be accounted for as peroxisomal activity. When oxidase activity was determined by H2O2 formation from L-pipecolic acid, almost all activity was found in the peroxisomal fraction, confirming two different systems for the oxidation of L-pipecolic acid in rabbit kidney. Like L-pipecolic acid, sarcosine has been reported to be oxidized by mitochondria in certain mammals (7, 9).

Since antibodies raised against pipecolic acid oxidase from

3 S. J. Gould and G. Dodt, unpublished data.

4 G. Dodt, unpublished data.
monkey liver (3) cross-reacted with rabbit sarcosine oxidase, as well as with the recombinant enzyme, we were able to use them in immunoblot analysis of fractionated rabbit kidney to show that rabbit Sox was solely peroxisomal. This peroxisomal localization was further supported by the finding that the rabbit sarcosine oxidase sequence encoded for a C-terminal PTS1 (tripeptide AHL), as well as the sequence for the hypothetical C. elegans protein (tripeptide SKI) (accession number U23529), but the sequences for the bacterial sarcosine oxidases (12–15) did not contain a peroxisomal targeting signal.

Even if skin fibroblasts are not typical cells for the expression of amino-acid oxidases, the availability of cell lines with different peroxisomal import defects made them an excellent tool to study the intracellular localization of Sox in mammalian cells. Expression of cDNA for the rabbit sox gene resulted in an exclusively peroxisomal localization of the corresponding gene product in normal fibroblasts and in kidney CV-1 cells. In contrast, the peroxisomal biogenesis disorder cell line 005-T, which has no detectable PTS1 receptor, synthesized the Sox when transfected with the corresponding plasmid but did not import it into the peroxisomes. Instead, it remained in the cytoplasm like the peroxisomal matrix protein catalase. This finding indicated that the import of sarcosine oxidase into peroxisomes was dependent on an intact PTS1 receptor. In contrast, when sox was expressed in a cell line from a patient with RCDP, with a distinct defect in the import of PTS2 proteins, the protein was targeted to peroxisomes as in normal fibroblasts, further suggesting that rabbit sarcosine oxidase behaves like a typical PTS1 protein.

When the amino acid sequence for rabbit sarcosine oxidase was submitted to a BLAST search (48), the highest homology was seen with an unknown gene product from C. elegans, followed by the monomeric bacterial sarcosine oxidases. The gene for heterotetrameric enzyme from Corynebacterium sp. P-1 is organized as an operon containing genes that encode for all four subunits and the glob gene, which encodes serine hydroxymethyltransferase (14). The a-subunit of this sarcosine oxidase shows less homology to Sox from rabbit than the monomeric enzymes but has a close relationship to the N terminus of mitochondrial dimethylglycine dehydrogenase from rat liver. The relationship to this mitochondrial dehydrogenase is supported by the finding that the a-subunit of the tetrameric...
TABLE V
Comparison of relative catalytic constants of different oxidases

|                     | Recombinant sarcosine oxidasea | Rabbit kidney sarcosine oxidaseb | Monkey liver L-pipecolic acid oxidasec % | % | % |
|---------------------|-------------------------------|---------------------------------|----------------------------------------|---|---|---|
| Sarcosine           | 46                            | 100                             | 10                                      |
| L-Pipecolic acid    | 53                            | 30                              | 100                                     |
| L-Proline           | 100                           | 23                              | 23                                      |

a Recombinant sarcosine oxidase (fusion with maltose-binding protein), this study, substrate concentration 9.8 mM.
b Purified sarcosine oxidase from rabbit kidney, this study, substrate concentration 9.8 mM.
c Purified L-pipecolic acid oxidase from monkey liver, Mikhailik et al. (3), substrate concentration 5 mM.

enzyme has additional homology to the C terminus of dimethylglycine dehydrogenase (49). The tetrameric sarcosine oxidases share more similarities with the mitochondrial dimethylglycine dehydrogenases (the sequence for sarcosine dehydrogenase is not known), whereas the monomeric sarcosine oxidases are closer to the peroxisomal enzyme in mammalian cells.

The close relationship between purified and recombinant rabbit sarcosine oxidases, L-pipecolic acid oxidase from monkey liver and the bacterial sarcosine oxidases is further reflected in their substrate specificity. The tetrameric sarcosine oxidase from Corynebacterium sp. P-1 is able to metabolize L-proline and L-pipecolic acid but at turnover rates 220-fold less than that for sarcosine (56). No information is available as to whether L-proline or L-pipecolic acid are substrates for monomeric sarcosine oxidases.

When the substrate specificities of native rabbit kidney sarcosine were compared with that of the recombinant fusion protein investigated in this study and that of pipecolic acid oxidase from monkey liver (Table V), all three enzymes utilized the same amino acids as substrates but the kinetics were different. The common link between these substrates is the imino moiety (Fig. 12) and the similarity of the reaction mechanisms. Oxidation of these substrates by sarcosine oxidase or pipecolic acid oxidase would yield glycine and formaldehyde for sarcosine, Δ-piperideine-6-carboxylate for L-pipecolic acid, and Δ-pyrroline-5-carboxylate for L-proline. In contrast, the oxidation by N-amino-acid oxidase would result in the formation of Δ-piperideine-2-carboxylate from D-pipecolic acid.

Sarcosine oxidase from rabbit liver exhibits an ADP-ββ′-binding fold satisfying the 11 consensus sequence requirements postulated by Wierenga et al. (57). The aspartate in position 1 does not fit the consensus, but in several well-characterized FAD-binding sites an aspartate has been observed in this position (49, 58). The absorption spectrum of the GST-Sox fusion protein and of the native protein suggests that sarcosine oxidase has a tightly attached flavin as coenzyme. The flavin attachment site has not yet been identified. As in the monomeric sarcosine oxidases, the enzyme contains a histidine (His-49 for rabbit Sox) aligns with the flavin attachment site (His-84) of dimethylglycine dehydrogenase (50, 60). Newer results from Willie and Jorns (20) and Willie et al. (52) indicate that in the case of the enzyme from Corynebacterium sp. P-1 (19) this flavin is FMN, whereas two monomeric sarcosine oxidases contain FAD.

It might be remarkable that the fourth fragment of high homology at the C terminus (Fig. 2) shows high similarity to the N-terminal ADP-binding fold but did not fit the consensus of Wierenga et al. (57) exactly.

Several lines of evidence indicate that the purified rabbit enzyme is identical to the sox gene product. Both the native enzyme from rabbit kidney and the recombinant enzyme were localized in or targeted to peroxisomes. The calculated size from the sox sequence was identical to that estimated by SDS-PAGE of the native enzyme. The same polyclonal antibodies recognized both the purified and the recombinant enzyme. Both enzymes utilized the same substrates with similar catalytic efficiencies for sarcosine and L-pipecolic acid. A covalently attached flavin was found in both enzyme preparations. Some features such as the peroxisomal localization and the involvement of a nucleotide could be predicted from the primary sequence of the sox gene and were confirmed with the native protein.

In summary, L-pipecolic acid, L-proline, and sarcosine can be degraded by the same enzyme via an identical reaction mechanism. This enzyme belongs to a family of sarcosine oxidases which are all characterized by a flavin moiety which is covalently bound. This common type of flavin binding to the protein suggests that it may be required for this reaction mechanism. In contrast, the N-amino-acid oxidases, which have a similar but chirally opposite mechanism, do not contain a covalently bound flavin. Future studies of this reaction mechanism should help in elucidating just how flavin binding to enzymes is associated with particular reaction mechanisms.

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