Articles

A Novel Structural Basis for Membrane Association of a Protein: Construction of a Chimeric Soluble Mutant of (*S*)-Mandelate Dehydrogenase from *Pseudomonas putida*†

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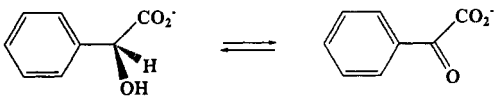
ABSTRACT: The (*S*)-mandelate dehydrogenase (MDH) from *Pseudomonas putida* (ATCC 12633) is the only membrane-associated member of a homologous family of FMN-dependent, α-hydroxy acid dehydrogenases/oxidases that includes the structurally characterized glycolate oxidase from spinach (GOX). We have correlated the membrane association of MDH to a polypeptide segment in the *interior* of the primary sequence. This has been accomplished by construction of a chimeric enzyme in which the putative membrane-binding segment in MDH has been deleted and replaced with the corresponding segment from the soluble GOX. The resulting chimera, MDH-GOX, is soluble and retains partial catalytic activity (~1%) using (*S*)-mandelate as substrate. In contrast, the activities of both the membrane-associated wild-type MDH and the soluble MDH-GOX are nearly the same when (*S*)-phenyllactate is used as substrate. To the best of our knowledge, this is the first example of a membrane-associated protein in which an internal polypeptide segment anchors the protein to the membrane.

(*S*)-Mandelate dehydrogenase (MDH)[1] is an enzyme in the mandelate pathway that occurs in several strains of Pseudomonads, including *Pseudomonas putida* (ATCC 12633). The mandelate pathway converts (*R*)-mandelate to benzoate. MDH, a membrane-associated, FMN-dependent enzyme, oxidizes (*S*)-mandelate to phenylglyoxalate (benzoylformate):



The gene for MDH has been cloned and sequenced (Tsou et al., 1990). The predicted amino acid sequence for MDH shows significant identity to sequences of a family of FMN-dependent, α-hydroxy acid dehydrogenases/oxidases, including glycolate oxidase (GOX) from spinach, lactate oxidase (monooxygenase) from *Mycobacterium*, flavocytochrome b_2 (a lactate dehydrogenase) from yeast, and a long-chain, α-hydroxy acid oxidase from rat kidney (Tsou et al., 1990; Diep Le & Lederer, 1991). While all members of this family catalyze the FMN-dependent oxidation of an α-hydroxy to an α-keto acid, the ultimate fate of the electron pair depends upon the particular enzyme. For example, GOX transfers the electron pair to molecular oxygen to form hydrogen peroxide, flavocytochrome b_2 transfers the electron pair first to a heme domain and then to cytochrome *c*, and MDH transfers the electron pair to an electron transport chain in the membrane.

In contrast to the other known members of this family, MDH is membrane-associated. Examination of the aligned sequences of the members of this enzyme family reveals global sequence similarity except that there is no detectable homology in one large internal region of the sequence. In the membrane-associated MDH, this internal sequence is longer than those observed in the members of the family known to be soluble. High-resolution X-ray structures are available for both GOX (Lindqvist, 1989) and flavocytochrome b_2 (Xia & Mathews, 1990). In these latter structures the internal nonhomologous sequence is associated with a large surface-exposed segment that is adjacent to the active (FMN-binding) site. Therefore, from the sequence alignment and structural data, we hypothesize that this segment is responsible for the membrane

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[1] Abbreviations: BSA, bovine serum albumin; DCPIP, 2,6-dichlorophenolindophenol, sodium salt; DEAE, diethylaminoethyl; EDTA, ethylenediaminetetraacetic acid; FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; GOX, glycolate oxidase from spinach; IPTG, isopropyl β-thiogalactopyranoside; LB medium, Luria–Bertani medium; MDH, (*S*)-mandelate dehydrogenase; MDH(ΔC), MDH with 17 residues deleted at the carboxy terminus; MDH(ΔN), MDH with residues 2–4 deleted; MDH-GOX, a chimeric mutant of MDH with the membrane anchoring loop replaced by a portion of GOX; PCR, polymerase chain reaction; PMS, phenazine methosulfate; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

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association of MDH.

From the studies reported in this paper, we conclude that this segment is, in fact, responsible for the membrane association of MDH. Using PCR techniques, we have created a *soluble* chimeric enzyme in which the putative membrane-binding segment of MDH has been replaced by the analogous segment of the soluble GOX. The purified soluble chimeric enzyme MDH-GOX oxidizes (*S*)-mandelate at ~1% the rate of the membrane-bound, wild-type MDH. Preliminary kinetic data suggest that the rate-determining steps in the reactions catalyzed by the two enzymes may be different.

MATERIALS AND METHODS

Materials. The expression vector pKK223-3 was obtained from Pharmacia Biochemicals. Restriction endonucleases, T4 DNA ligase, and calf intestine phosphatase were from Boehringer Mannheim Biochemicals. Deoxynucleotides were from U.S. Biochemicals. Vent DNA polymerase was from New England Biolabs. Oligonucleotides were purchased from Oligos Etc., Guilford, CT. Geneclean was from Bio101. pMV2, a plasmid carrying the cDNA clone of GOX from spinach, was the generous gift of Dr. Chris Somerville (Michigan State University). Horseradish peroxidase and bovine serum albumin were from Sigma. Chromatographic materials for protein purification were from Pharmacia. All other chemicals were of the highest commercial grade and were obtained from Aldrich or Sigma.

Homology Analysis and Sequence Alignment of MDH. Peptide and nucleotide sequence databases were searched for sequences similar to MDH using the algorithms FASTA (Pearson & Lipman, 1988) and BLAST (Altschul et al., 1990) as implemented by the Computer Graphics Laboratory at the University of California, San Francisco. Additional searches were performed by the National Center for Biotechnology Information (NCBI) using the FASTA and BLAST network services. The highest scoring sequences from these searches were subjected to further analysis to determine the statistical significance of their relationships to MDH. This was accomplished by pairwise comparisons with MDH using the algorithms Lawrence (Lawrence & Goldman, 1988) and Monte (Altschul & Erickson, 1986). On the basis of the results of these analyses, the sequences considered to be the most like MDH were multiply aligned using the PIMA algorithm (Smith & Smith, 1990). Percent identity and percent similarity calculations were performed by pairwise comparisons of sequences as aligned by PIMA using the algorithm sim_calc (Lisa Kim, personal communication).

Modeling Methods. An atomic model of MDH was constructed from the refined coordinates of GOX by a minimum perturbation approach. The high degree of sequence similarity between the two enzymes allowed us to use the backbone dihedral angles of GOX for most of the polypeptide chain of MDH. The exceptions were segments in MDH corresponding to insertions and deletions. Portions of GOX that were deleted in MDH were easy to treat, because they tended to be surface Ω-loops so the ends of the deleted segment were close together in space. Thus, the gap could be resealed by energy minimization with very little distortion of the coordinates of the neighboring polypeptide. Insertions were more difficult to handle, although they also were almost entirely in surface loop segments. Those differing only modestly in length were modeled by selecting homologous fragments from a library of well-refined protein structures. Long insertions were deliberately not modeled to avoid the false impression that we know anything about their structures.

Side-chain replacements were performed conservatively: the conformation of the substituted side chain was selected so that the atoms common between it and the residue it replaced had the same positions in the modeled structure. Additional side-chain components were built from a library of common rotamers. The FMN cofactor was positioned in the active site of MDH to match its orientation in GOX. When all residues had been built, the structure was subjected to energy minimization until convergence. During minimization, the atomic coordinates of the backbone were restrained to remain close to the values set from the structure of GOX. All model building and energy minimization were performed with the program package CHARMM/QUANTA from Molecular Simulations, Inc., Burlington, MA.

Genetic Engineering Methods. The gene for MDH has been cloned from *P. putida* (ATCC 12633) and sequenced as part of a 10.5-kb fragment of genomic DNA that encodes the five enzymes of the mandelate pathway (Tsou et al., 1990). MDH was expressed in *Escherichia coli* JM105 after the gene was subcloned from this fragment into the expression vector pKK223-3 (Pharmacia) using PCR methods. An *Eco*RI site just upstream of the initiation codon and a *Pst*I site immediately following the termination codon were generated by PCR using primers N1 and N2:

N1: 5′-GCG-GAATTC-ATG-AGC-CAG-3′

N2: 5′-GCG-CTGCAG-TCA-TGC-GTG-T-3′

The vector pKK223-3 and the PCR product were digested separately with *Eco*RI and *Pst*I; the two fragments were purified using Geneclean and ligated to give the plasmid pBM. In this plasmid the gene for MDH is under control of the *tac* promoter and, therefore, is inducible by IPTG. pBM was transformed into *E. coli* JM105. The membrane-associated, wild-type MDH was expressed by growing the cells in LB medium supplemented with 50–100 μg/mL ampicillin until the absorbance at 590 nm was 0.8 followed by induction with 0.1 mM IPTG.

An N-terminal deletion mutant of MDH with residues 2–4 deleted, MDH(ΔN), was generated using primers N2 and N3:

N3: 5′-GCG-GAATTC-ATG-CTC-TTT-AAC-G-3′

The PCR was performed using pBM as template, and the mutant gene was recloned into pKK223-3 to give plasmid pBM10.

The C-terminal deletion mutant with the last 17 residues deleted, MDH(ΔC), was generated using primers N1 and N4:

N4: 5′-CC-CTGCAG-TCA-TCC-CTC-GTT-TTG-3′

The PCR was performed using pBM as template, and the mutant gene was recloned into pKK223-3 to give plasmid pBM7.

A chimeric mutant of MDH and GOX was generated by deleting residues 177–229 from the sequence of MDH and inserting residues 176–209 from the sequence of GOX in the same position. The chimera was created by overlap extension PCR as follows (Yon & Fried, 1989). A DNA fragment encoding an N-terminal portion of MDH (residues 1–176) was generated using primers N1 and N5

N5: 5′-TGG-TAT-CTT-GAA-TCG-3′

and pBM. Similarly, a DNA fragment encoding a C-terminal

portion of MDH (residues 230–373) was generated using primers N2 and N6

N6: 5′-AGT-TTC-AAC-TGG-GAG-GC-3′

and the same template. Finally, a short DNA fragment encoding the internal segment of GOX (residues 176–209) that is analogous in sequence position to the putative membrane-binding segment of MDH was generated using primers N7 and N8

N7: 5′-GAT-TCA-AGA-TAC-CAC-CTT-TTC-T-3′

N8: 5′-TGC-CTC-CCA-GTT-GAA-ACT-TCG-ATC-AAT-CTG-3′

and the plasmid pMV2 containing the cDNA clone for GOX (Volokita & Somerville, 1987). The three DNA fragments, each of which had short regions of overlap, were purified and mixed together with primers N1 and N2 for a final step of PCR. The resulting chimeric gene was cloned into pKK223-3 to yield plasmid pBM11.

All PCR amplifications were performed using Vent polymerase. The sequences of the three mutant genes so constructed [MDH(ΔN), MDH(ΔC), and MDH-GOX] were verified by DNA sequence analysis. All other procedures were carried out according to Sambrook et al. (1989).

Purification of Enzymes. Cells transformed with the plasmids encoding wild-type MDH and its three mutants were grown at 37 °C in LB medium supplemented with 50–100 μg/mL of ampicillin. When the absorbance at 590 nm reached 0.8, the gene for MDH (wild-type or mutant) was induced by the addition of 0.1 mM IPTG. The cells were harvested after another 16 h of growth and stored at −20 °C. All purification steps were carried out at 4 °C.

(A) Wild-Type MDH and Membrane-Associated Mutants MDH(ΔN) and MDH(ΔC). The purification protocol for the membrane-bound proteins was modified from that described by Hoey et al. (1987) for the purification of (*S*)-mandelate dehydrogenase from *Acinetobacter calcoaceticus*. Typically, 10 g of frozen cells was thawed and resuspended in 200 mL of 50 mM potassium phosphate buffer, pH 7.5. The cells were broken by sonication, and the lysate was centrifuged at 5000*g* for 20 min. The pellet contained unbroken cells, cell debris, and any inclusion bodies which may have been produced due to overexpression of proteins. The supernatant was subjected to ultracentrifugation at 165000*g* for 90 min. The two fractions from this centrifugation, designated the supernatant and the pellet, were considered to represent the soluble and membrane components of the extract, respectively. The supernatant was discarded, and the pellet was washed twice with 100 mL of phosphate buffer by resuspension and centrifugation. Finally, the pellet was resuspended in approximately 50 mL of 20 mM phosphate buffer, pH 7.5, to a final concentration of 2–4 mg of protein/mL. Triton X-100 was added dropwise to a final concentration of 1%. The suspension was stirred for 30 min and then centrifuged at 165000*g* for 90 min. The supernatant, the Triton X-100 extract, was made 10% (v/v) in ethanediol.

The Triton X-100 extract was loaded on a 12 × 5 cm column of DEAE-Sephacel equilibrated with buffer A (20 mM potassium phosphate, pH 7.5, 10% ethanediol, and 0.05% Triton X-100). The column was washed with 100 mL of buffer A, and the protein was eluted with a linear 1-L gradient of 0.1–0.4 M NaCl in buffer A. Fractions containing greater than 50% of the activity of the fraction containing the highest activity were pooled. The solubilized MDH was >95% pure after this step and was used for most experiments. When necessary, the protein was further purified using a second 5 × 2.5 cm column of DEAE-Sephacel. The protein was eluted from this column with a 200-mL linear gradient of 0.15–0.3 M NaCl in buffer A. Fractions with MDH activity were pooled, made 20% in ethanediol, and stored in aliquots at −70 °C.

(B) Soluble Chimeric Mutant MDH-GOX. Frozen cells (10 g) were thawed and resuspended in 200 mL of 50 mM phosphate buffer, pH 7.5, containing 0.2 μg/mL aprotinin, sonicated, and centrifuged at 165000*g* for 90 min. The supernatant was considered to represent the soluble components of the extract. FMN was added to the supernatant to a final concentration of 0.1 mM. Solid $(NH_4)_2SO_4$ (0.164 g/mL) was added to 30% saturation. The suspension was stirred for 60 min and centrifuged at 12000*g* for 20 min. The supernatant was made 55% saturated in $(NH_4)_2SO_4$ (0.148 g/mL), stirred for 90 min, and centrifuged at 12000*g* for 20 min.

The $(NH_4)_2SO_4$ pellet was taken up in a minimum volume (10–20 mL) of 20 mM potassium phosphate, pH 7.5, containing 0.2 μg/mL aprotinin (buffer B) and desalted by percolation through a 45 × 2.5 cm column of Sephadex G-25 equilibrated in buffer B. The protein was then applied to a 12 × 5 cm column of DEAE-Sephacel equilibrated in buffer B, washed with 100 mL of buffer B, and eluted with a 1.5-L linear gradient of 0–0.4 M NaCl in buffer B. Fractions containing greater than 50% of the activity of the fraction containing the highest activity were pooled, FMN was added to a final concentration of 0.1 mM, and the protein was precipitated by the addition of $(NH_4)_2SO_4$ to 55% saturation. The precipitated protein was taken up in buffer C (20 mM phosphate, pH 7.5, 5% ethanediol, and 0.1 mM FMN) containing 30% $(NH_4)_2SO_4$ and applied to a 0.5 × 5 cm prepacked column of Phenyl Superose (Pharmacia) equilibrated in the same buffer. The MDH-GOX chimera was eluted with a 60-mL linear gradient of 30–0% $(NH_4)_2SO_4$ in buffer C. Fractions with greater than 25% the activity of the peak fraction were pooled. The protein was stored as an $(NH_4)_2SO_4$ precipitate.

The purity of the proteins was assessed by SDS–PAGE (Laemmli, 1970).

Enzyme Assays. (A) Reduction of DCPIP. The rate of oxidation of the $FMNH_2$ intermediate in the reactions catalyzed by the wild-type and mutant versions of MDH was measured by observing the reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm (Hill & Fewson, 1983). The assay mixtures (1 mL) for membrane-associated enzymes [wild-type and the MDH(ΔN) and the MDH(ΔC) mutants] contained 0.1 mM phosphate buffer, pH 7.5, 50 μM DCPIP, 1 mM PMS, and 1.0 mg/mL BSA. For the soluble MDH-GOX, PMS was omitted from the assay buffer. Assays (1 mL) were initiated by adding (*S*)-mandelate to a final concentration of 10 mM. An extinction coefficient of 21.6 mM^{-1} cm^{-1} at 600 nm at pH 7.5 was assumed for DCPIP (Armstrong, 1960). For inhibition studies, the enzyme was incubated in the assay buffer with the inhibitor for 3 min followed by the addition of (*S*)-mandelate to initiate the assay.

(B) Formation of Phenylglyoxalate. The rate of formation of the product phenylglyoxalate was directly assayed by chemical derivatization with *o*-phenylenediamine (R. Li and G. L. Kenyon, unpublished results). At fixed time points, 0.2-mL aliquots were removed from the same assay mixture described in the previous paragraph and quenched by addition to 0.8 mL of 2.5 mM *o*-phenylenediamine solution in 80%

acetic acid. These were heated at 50 °C for 10 min, and the absorbance at 334 nm due to the formation of 3-phenylquinoxaline-2-ol was recorded. A calibration curve was generated by using known concentrations of phenylglyoxalate for the same reaction. This assay is not sensitive at very low phenylglyoxalate concentrations but is linear above 0.1 mM.

(C) Formation of H_2O_2. The formation of H_2O_2 was measured in a coupled-enzyme assay using horseradish peroxidase and *o*-dianisidine. The assay mixture contained 0.1 mM phosphate buffer, pH 7.5, 0.01 mg/mL horseradish peroxidase, 0.5 mM *o*-dianisidine, and 5–10 mM (*S*)-mandelate. The increase in absorbance at 440 nm was recorded.

Analysis of Flavin. All dissociated and free flavin was removed from protein solutions by passing them consecutively through two columns of Sephadex G-25. Noncovalently bound flavin was removed from wild-type MDH and MDH-GOX either by heating the proteins at 100 °C for 5 min or by incubating them with 10% (w/v) trichloroacetic acid for 10 min at 4 °C. In either case, the protein solutions were centrifuged, and the supernatants containing dissociated flavin were removed. UV/vis absorbance spectra of the supernatants were recorded. The identity of the flavin was checked by thin-layer chromatography on Kieselgel F60 plates using a solvent system of 5% Na_2HPO_4 in water and commercially available FMN and FAD as standards.

Protein Determination. Protein concentrations were determined with the bicinchoninic acid reagent from Pierce (Smith et al., 1985) using bovine serum albumin as standard. This assay could be used to measure protein concentrations in the presence of Triton X-100.

RESULTS

Expression of the Gene for MDH in E. coli and the Nature of Its Membrane Association. The gene encoding wild-type MDH from *P. putida* (ATCC 12633) was subcloned into the expression vector pKK223-3 where it is under the control of the *tac* promoter. The MDH was expressed in *E. coli* strain JM105 and was found to be associated with membranes, like the protein in *P. putida* and the MDH in *A. calcoaceticus* (Hoey et al., 1987). The effectiveness of various reagents on solubilizing the protein from the membrane fraction was tested. EDTA at 10 or 20 mM solubilized only ~6% of the activity as did 0.5 or 1.0 M NaCl. Triton X-100 proved to be the most effective detergent; 1% Triton X-100 was effective in solubilizing almost 100% of the activity. Surface association of the protein with the membrane is unlikely because only very small amounts of protein were solubilized by high concentrations of either EDTA or salt.

Identification of a Potential Membrane Binding Segment by Sequence Alignment. An alignment of the sequence of the membrane-associated MDH with the soluble members of the family of FMN-dependent, α-hydroxy acid dehydrogenases/oxidases is shown in Figure 1. An additional sequence, designated Ml.urf, from *Mycobacterium leprae*, the causative agent of leprosy, is also included in the alignment. Since the protein product from this unidentified reading frame has not yet been isolated and characterized, neither its enzymatic identity nor its solubility is known. However, we believe that its sequence similarity with the characterized members of this family suggests that it is also an FMN-dependent, α-hydroxy acid dehydrogenase/oxidase.

The overall sequence identities between MDH and the remaining sequences aligned in Figure 1 range from 27% to 38%. The sequence similarity between MDH and all of the other sequences is ~50%. As deduced by our searches of the available databases, no other sequences appear to be members of this family, including other known membrane-binding proteins. The alignment shows that all of the soluble enzymes and Ml.urf share significant sequence similarity with MDH over the entire sequence, with regions of especially high similarity being enclosed by boxes in Figure 1. Moreover, the residues that have been identified as important to structure–function relationships in the active site of GOX (Lindqvist et al., 1991) are conserved in all of the proteins in the family. The global nature of the sequence similarity suggests that the overall tertiary structures of all members of the family are also similar.

On the basis of these observations, identification of a membrane binding segment of MDH might simply involve locating a segment in MDH that is unlike its counterparts in the soluble enzymes. As shown in Figure 1, such a segment can be readily located roughly in the central regions of the primary sequences. These regions deviate from the global pattern of sequence similarity both in sequence and in length. Thus, this internal segment of the primary sequence may represent a structural element that is less important for overall attainment of tertiary structure. Interestingly, while this region of sequence in MDH is somewhat more hydrophobic than the analogous sequences in the other enzymes in the family, its hydrophobicity is not striking. In addition, construction of a helical wheel for this region reveals no evidence for an amphipathic helix. Nevertheless, we concluded that this internal sequence of MDH is a likely candidate for the membrane-binding segment of MDH.

It is perhaps noteworthy that, in this internal sequence representing the putative membrane-binding region, the unidentified reading frame Ml.urf is more closely related to MDH in both sequence similarity and length than are the members of the family known to be soluble. Thus, it is interesting to speculate that Ml.urf encodes an FMN-dependent, α-hydroxy acid dehydrogenase/oxidase that is also membrane-associated.

Modeling the Sequence of MDH on the Sequence and Structure of Glycolate Oxidase. In order to test whether our predicted candidate for the membrane-binding segment is structurally reasonable, we modeled the structure of MDH on the basis of the available high-resolution structure of glycolate oxidase (GOX; Lindqvist, 1989). The superpositioning of the model of MDH so generated (blue) and the actual structure of GOX (white) is shown in Figure 2.

The overall fold of GOX is that of an αβ barrel. The resulting modeled structure of MDH is also that of an αβ barrel. In the modeled structure for MDH the regions of sequence identity shown in Figure 1 are superimposed, and the conserved active site residues in MDH are in structurally and functionally similar positions to those observed for the analogous residues in GOX. The internal sequences of MDH and GOX which deviate from global sequence similarity are predicted to be in a large loop that is inserted between an α-helix and a β-sheet of the αβ barrel architecture. In GOX, this segment is located on the solvent-exposed surface; in MDH, we predict that this segment is also on the surface of the protein. In the structure of GOX, residues 189–197 are disordered, and their positions in the structure were not specified. In the modeled structure of MDH, an arbitrary backbone conformation has been assigned to the polypeptide segment in this region. This region of MDH is likely to be included in the segment of polypeptide that is responsible for the membrane association of MDH.

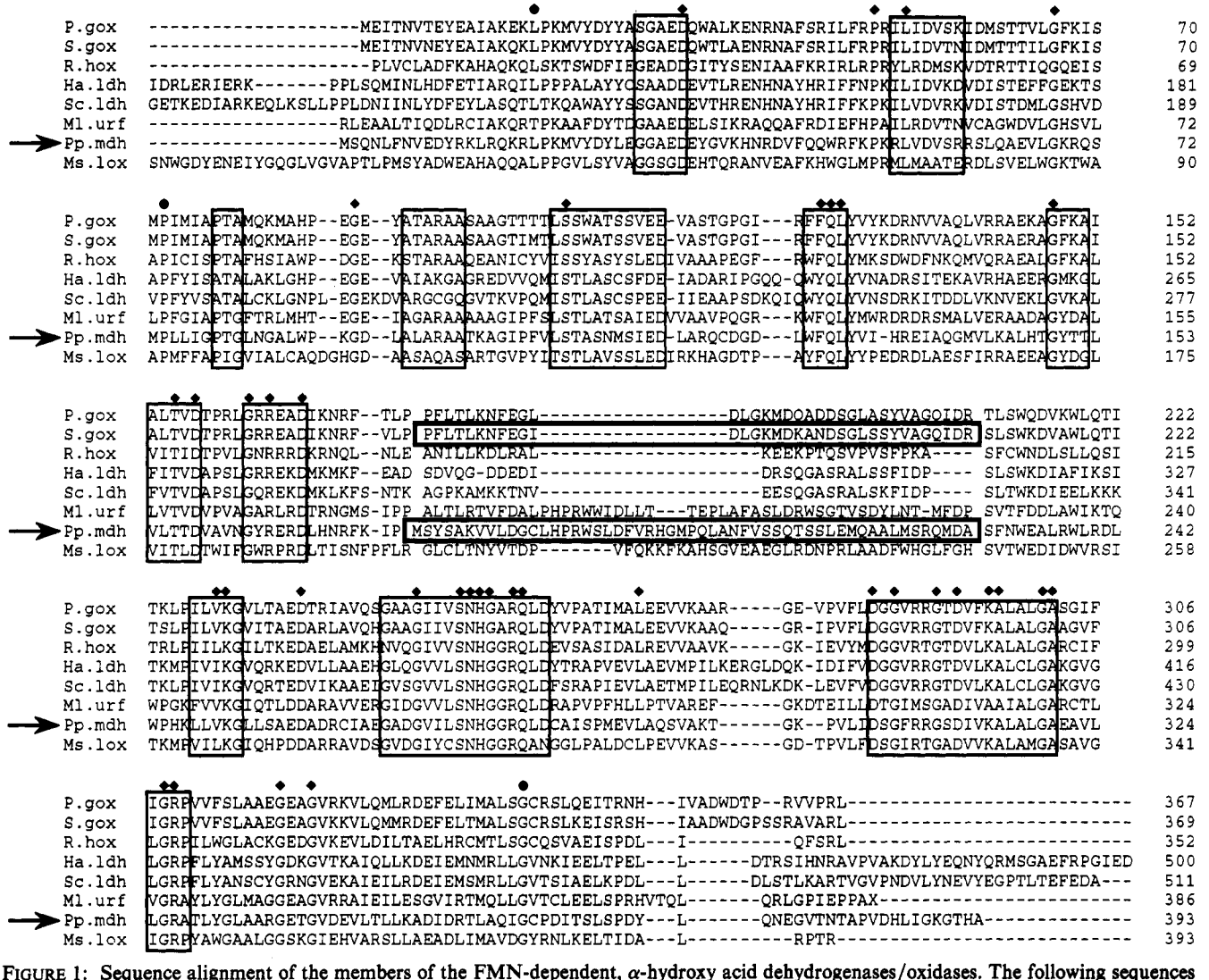

FIGURE 1: Sequence alignment of the members of the FMN-dependent, α-hydroxy acid dehydrogenases/oxidases. The following sequences are shown: P.gox, glycolate oxidase from pumpkin (Tsugeki et al., 1993); S.gox, glycolate oxidase from spinach (Volokita & Somerville, 1987); R.hox, (*S*)-2-hydroxy acid oxidase from rat (Diep Le & Lederer, 1991); Ha.ldh, L-lactate dehydrogenase from *Hansenula anomala* (Risler et al., 1989); Sc.ldh, L-lactate dehydrogenase from *Saccharomyces cerevisae* (Guiard, 1985); Ml.urf, unknown reading frame from *M. leprae* (D. R. Smith, GenBank Database Accession No. LO1095, 1992); Pp.mdh, MDH from *P. putida* (Tsou et al., 1990); Ms.lox, lactate 2-monooxygenase from *Mycobacterium smegmatis* (Giegel et al., 1990). The residues marked by diamonds show positions at which all eight sequences are identical. The sequences enclosed in boxes represent regions of high sequence identity/similarity. The two regions enclosed in boxes in the center of the figure represent the internal sequences associated with membrane binding and are those that were swapped in the MDH-GOX chimera.

The segments of the GOX and MDH that contain the sequences that deviate from global sequence similarity are shown in red and green, respectively. These colored regions [residues 177–229 of MDH (green) and residues 176–209 of GOX (red)] are those interchanged in the construction of the chimera of MDH and GOX (*vide infra*).

Importance of the N- and C-Terminal Sequences in Membrane Association. The N- and C-terminal regions of some membrane-associated proteins are known to be responsible for membrane binding. Therefore, we first determined whether mutations in these regions would cause solubilization of MDH.

The N-terminal region of MDH has a high degree of sequence identity to the soluble members of this family of flavoproteins; hence, a direct insertion of the N-terminus into the membrane is unlikely. However, posttranslational modification of the N-terminal residues is, in principle, possible. We chose to delete residues 2–4 (Ser-Gln-Asn) from the N-terminus to determine whether any unexpected posttranslational modification of these residues might be involved in membrane association. The resulting mutant MDH(ΔN) is membrane-associated as judged by both SDS–PAGE (Figure 3) and the measured distribution of activity between the soluble and membrane fractions (Table I). The kinetic parameters of purified MDH(ΔN) as described in the Materials and Methods section are listed in Table II.

We next examined the C-terminus of MDH. In the family of homologous proteins, the C-terminal regions are quite dissimilar; in addition, the C-terminus of MDH is somewhat hydrophobic on a hydropathy plot. A mutant, MDH(ΔC), was generated by deleting the last 17 residues of MDH. MDH(ΔC) is membrane-associated as judged by both SDS–PAGE (Figure 3) and the measured distribution of activity between the soluble and membrane fractions (Table I). The kinetic parameters of purified MDH(ΔC) as described in the Materials and Methods section are also listed in Table II.

Construction and Solubility of the MDH-GOX Chimeric Enzyme. On the basis of the modeling described in an earlier section, we next constructed a chimeric protein in which the putative membrane binding segment of the membrane-

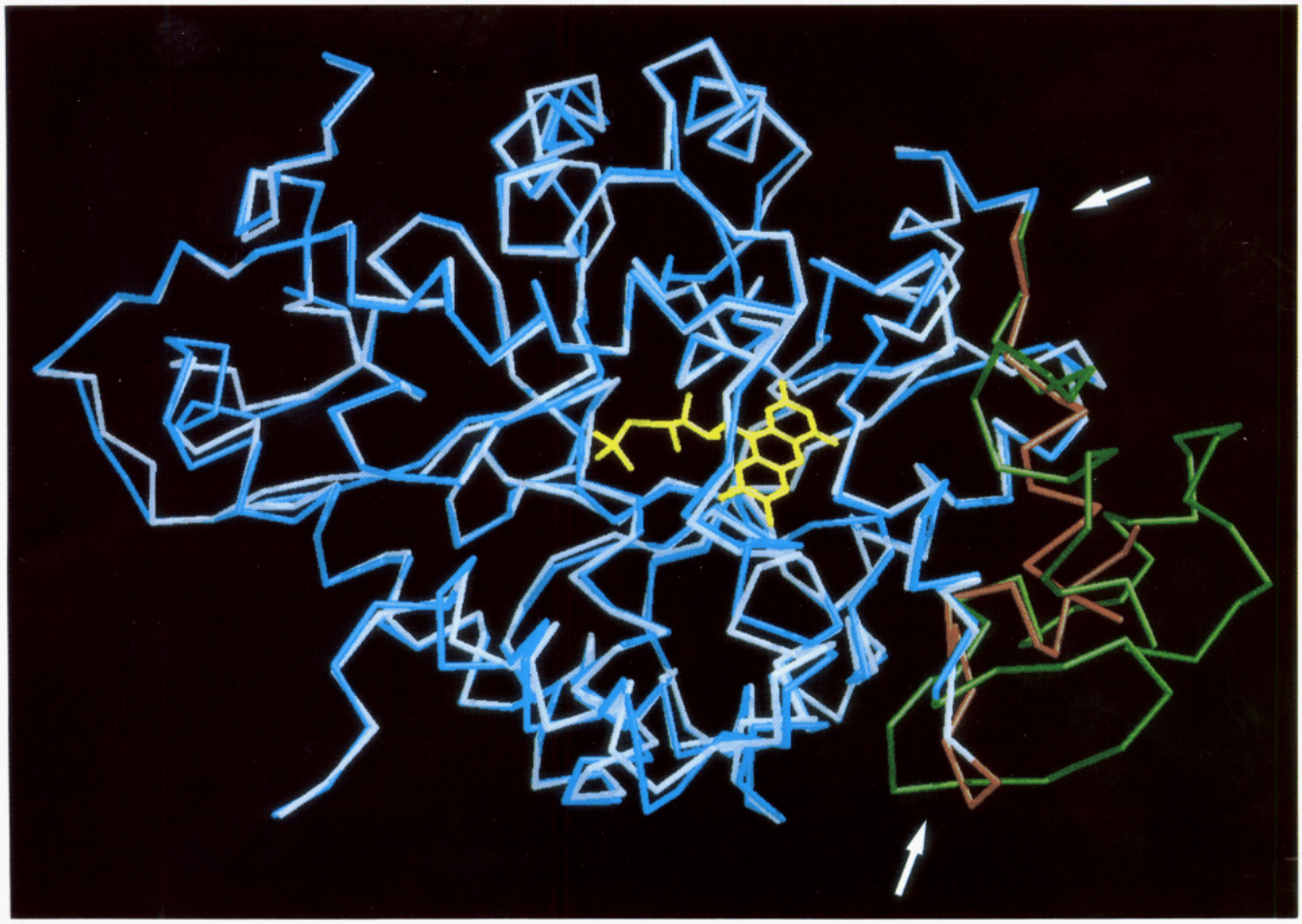

FIGURE 2: Superpositioning of the α-carbon backbones of GOX (white and red) and MDH (blue and green). The white and blue segments are those of the homologous $\alpha\beta$ barrels; the red and green segments are those of the internal sequences that were swapped in the construction of the MDH-GOX chimeric enzyme. Note both that a stretch of 12 amino acids is missing from the red segment of GOX and that the structure of the green segment of MDH has been assigned an arbitrary conformation in modeling.

associated MDH is replaced with the analogous segment from the soluble GOX. Residues 177–229 were deleted from MDH (green segment in Figure 2) and replaced by residues 176–209 of GOX (red segment in Figure 2). This mutant, designated MDH-GOX, is largely soluble as judged by both SDS–PAGE (Figure 3) and the measured distribution of activity between the soluble and membrane fractions (Table I). The kinetic parameters of purified MDH-GOX are also listed in Table II; this chimeric enzyme has 1% of the specific activity of wild-type MDH. Therefore, we conclude that the interior segment of MDH does, in fact, play a major role in its membrane association.

Purification and Storage of the Enzymes. Wild-type MDH was purified using a modification of the procedure of Hoey et al. (1987). The protein was solubilized from the membranes with Triton X-100. Following solubilization, the total activity was consistently somewhat higher than in the membranes; this apparent increase in activity may be due to the presence of an inhibitor associated with the membrane fraction. After two anion-exchange chromatographic steps, wild-type MDH was >95% pure. Wild-type MDH could be stored frozen at −70 °C in 20% ethanediol for weeks without loss of activity; repeated freeze–thaw cycles caused significant losses of activity.

The soluble chimeric mutant MDH-GOX proved to be difficult to purify. It is easily degraded and loses activity in buffers having low ionic strengths. An anion-exchange step followed by hydrophobic interaction chromatography resulted in protein that was >99% pure. This mutant could be stored at 4 °C without loss in activity as a 55% $(NH_4)_2SO_4$ precipitate in the presence of 0.1 mM FMN; precipitation in the absence

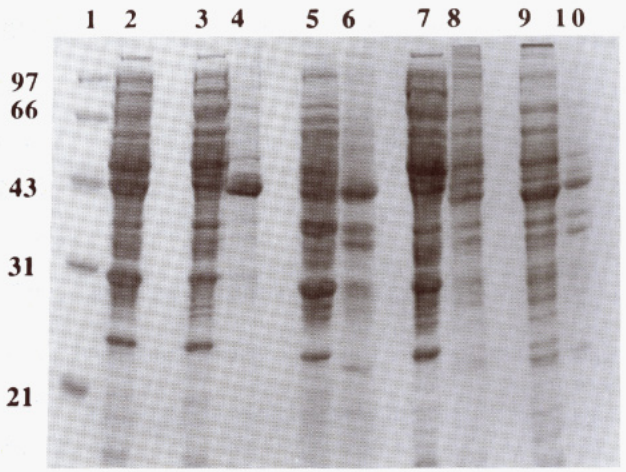

FIGURE 3: Subcellular location of MDH and its mutants analyzed by 12.5% SDS–PAGE stained with Coomassie Blue. Lanes: 1, molecular weight markers; 2, uninduced cells; 3 and 4, soluble and membrane fractions, respectively, of induced cells containing wild-type MDH; 5 and 6, soluble and membrane fractions, respectively, of induced cells containing MDH(ΔN); 7 and 8, soluble and membrane fractions, respectively, of induced cells containing MDH(ΔC); 9 and 10, soluble and membrane fractions, respectively, of induced cells containing MDH-GOX.

Table I: Distribution of MDH and Its Mutants in the Soluble and Membrane Fractions[a]

	% act. in the supernatant	% act. in the pellet
wild-type MDH	13	87
MDH(ΔN)	10	90
MDH(ΔC)	11	89
MDH-GOX	69	31

[a] Sonicated cells were centrifuged at 5000*g* for 15 min. The pellet was discarded, and the supernatant was centrifuged at 165000*g* for 90 min. The supernatant and the membrane pllet were separated. The pellet was resuspended in the same volume as the supernatant, and the activities of the two fractions were determined.

Table II: Kinetic Parameters for Purified Preparations of MDH and Its Mutants[a]

	(*S*)-mandelate		(*S*)-phenyllactate	
	k_{cat} (s^{-1})	K_m (μM)	k_{cat} (s^{-1})	K_m (mM)
wild-type MDH	174 ± 1	206 ± 4	0.27 ± 0.01	2.6 ± 0.2
MDH-GOX	1.6 ± 0.01	229 ± 8	0.77 ± 0.01	0.78 ± 0.05
MDH(ΔN)	9.0 ± 0.4	225 ± 15		
MDH(ΔC)	24 ± 0.5	158 ± 10		

[a] Using the DCPIP assay.

Table III: Purification of Recombinant Wild-Type MDH and Recombinant MDH-GOX from 10 g of *E. coli* JM105

	volume (mL)	[protein] (mg/mL)	total act. (units)	sp act. (units/mg)
(a) Recombinant Wild-Type MDH				
cells	200	5.1	22 800	22.4
membrane fraction	102	2.1	17 540	81.9
Triton X-100 extract	100	1.8	18 200	95.6
first DEAE-Sephacel	308	0.23	14 700	207.0
second DEAE-Sephacel	144	0.28	8 900	221.1
(b) Recombinant MDH-GOX				
cells	200	5.4	198	0.18
$(NH_4)_2SO_4$ precipitate	46	8.4	108	0.28
DEAE-Sephacel	110	0.52	55	0.97
Phenyl Superose	35	0.52	46	2.54

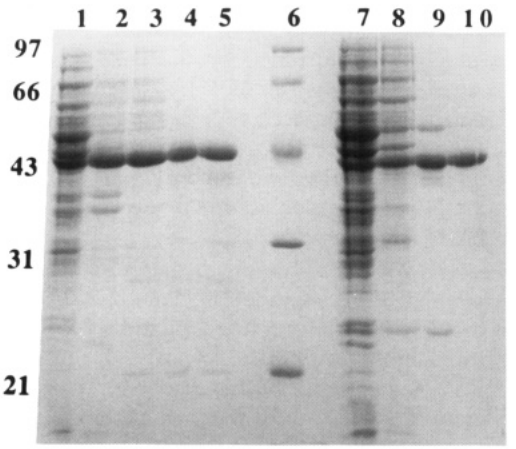

FIGURE 4: SDS–PAGE (12.5%) showing purification of wild-type MDH (lanes 1–5) and MDH-GOX (lanes 7–10). Lanes: 1, broken cells containing wild-type MDH; 2, membrane fraction; 3, after Triton X-100 extraction; 4 and 5, after first and second DEAE-Sephacel columns, respectively; 6, molecular weight markers; 7, broken cells containing MDH-GOX; 8, 30–55% $(NH_4)_2SO_4$ precipitate; 9, after DEAE-Sephacel; 10, after Phenyl Superose.

of added FMN resulted in loss of intrinsic flavin and irreversible inactivation.

The purification of both wild-type MDH and MDH-GOX is summarized in Table III. An SDS-PAGE gel of the purified wild-type MDH and MDH-GOX is shown in Figure 4. The soluble MDH-GOX is 19 residues shorter than wild-type

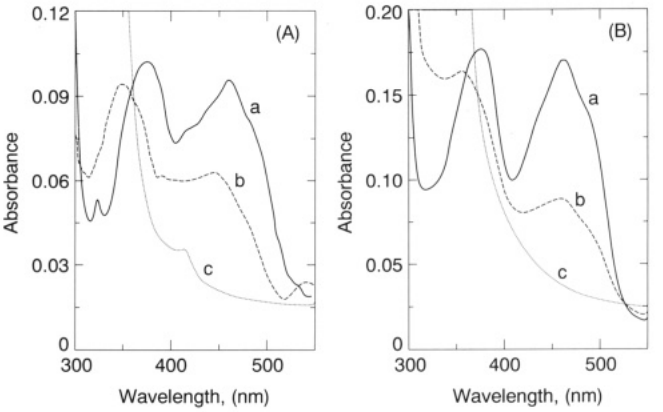

FIGURE 5: Absorbance spectra of wild-type MDH (panel A) and MDH-GOX (panel B) in the oxidized and reduced states. Spectra in each panel: a, oxidized enzyme; b, enzyme after 5 mM (*S*)-mandelate was added; c, enzyme after 1 mM dithionite was added. Conditions: wild-type MDH, ~0.40 mg/mL in 0.1 M phosphate buffer, pH 7.5; MDH-GOX, ~0.60 mg/mL in 0.1 M phosphate buffer containing 0.5 mg/mL BSA.

MDH (373 residues) and has a calculated molecular weight of 41 129 (as compared to 43 352 for wild-type MDH).

Determination of Flavin. The absorbance spectrum of both wild-type MDH (Figure 5A) and MDH-GOX (Figure 5B) showed the absorption maxima characteristic of flavin (spectra a). On addition of (*S*)-mandelate, both wild-type MDH and MDH-GOX showed a decrease in absorbance of the peak at 458 nm and the broad shoulder at 378 nm (spectra b). Addition of dithionite produced the same bleaching effect (spectra c). The reduced states of both the FMN in both enzymes could be maintained for at least 30 min in the presence of oxygen. The spectrum of wild-type MDH, but not that of MDH-GOX, reveals the presence of trace cytochrome/heme impurities as indicated by the absorption peak at 414 nm.

The flavin cofactor could be dissociated from both enzymes by denaturation; hence, it is noncovalently bound. Thin-layer chromatography of the dissociated flavins in comparison to samples of authentic FAD and FMN established that FMN is the cofactor for both proteins.

Enzyme Activity. The rate of oxidation of $FMNH_2$ was measured by the phenazine methosulfate-dependent reduction of DCPIP at pH 7.5. The effects of various substances on the activity were assessed. For wild-type MDH, both BSA at 0.25–1 mg/mL and PMS at 0.5–1 mM together gave the maximal activity. For MDH-GOX, BSA at 1 mg/mL, PMS at 1 mM and Triton X-100 at 0.5% all increased activity to the same extent. However, unlike wild-type MDH, BSA and PMS together did not have an additive effect on the activity of the soluble MDH-GOX.

No hydrogen peroxide formation was observed during the course of the reactions catalyzed by either wild-type MDH or MDH-GOX, indicating that they are dehydrogenases and not oxidases.

The rate of formation of the reaction product phenylglyoxalate was measured by coupling its formation to reaction with *o*-phenylenediamine. For both wild-type MDH and MDH-GOX, the activity measured by reduction of DCPIP was equal to that measured by phenylglyoxalate formation, within experimental error. The equivalence of these rates indicates that neither enzyme catalyzes the decarboxylation of (*S*)-mandelate to form benzoate and carbon dioxide (in analogy to the reaction catalyzed by lactate oxidase/monooxygenase). No phenylglyoxalate was formed in the absence of an electron acceptor, either PMS or DCPIP.

Substrate Specificity. Both wild-type MDH and MDH-GOX were specific for the (*S*)-enantiomer of mandelate. The k_{cat} of wild-type MDH for (*S*)-mandelate is ~100-fold greater than that for MDH-GOX (Table II) while the K_ms are the same. Small α-hydroxy acids, e.g., glycolate, lactate, and α-hydroxybutyrate, were neither substrates nor inhibitors. (*R*)-Mandelate, phenylacetate, and phenyl-1,2-ethanediol inhibited both enzymes at low substrate concentrations.

The k_{cat} for (*S*)-phenyllactate is comparable for wild-type MDH and MDH-GOX (Table II). However, for wild-type MDH, (*S*)-phenyllactate was a poor substrate compared to (*S*)-mandelate, with a k_{cat} ~0.2% that of (*S*)-mandelate. In contrast, for MDH-GOX, the k_{cat} for (*S*)-phenyllactate was ~50% that measured for (*S*)-mandelate.

DISCUSSION

MDH from *P. putida* is the only well-characterized member of the FMN-dependent, α-hydroxy acid dehydrogenase/oxidase family that is membrane-associated. The remaining members of the family, including the structurally characterized GOX and flavocytochrome b_2 from yeast, are all soluble. Although other α-hydroxy acid dehydrogenases are known that are membrane-associated, utilize FMN as a cofactor, and have physical and chemical properties analogous to this family of homologous proteins, no sequence data are currently available for these enzymes. These membrane-bound enzymes include (*S*)-mandelate dehydrogenase from *A. calcoaceticus* (Allison et al., 1985), L-lactate dehydrogenase from *E. coli* (Futai & Kimura, 1977), and L-pantoyl lactone dehydrogenase from *Nocardia asteroides* (Kataoka et al., 1992). All three of these membrane-associated dehydrogenases oxidize an α-hydroxy acid substrate by transferring electrons to FMN which, in turn, is reoxidized by passing electrons to a membrane-associated electron transport chain.

The goal in this study was to locate the membrane-binding region of MDH from *P. putida*. Like the enzyme isolated from *P. putida*, the recombinant wild-type protein expressed in *E. coli* was membrane-associated. Mutagenesis experiments involving the amino and carboxy termini of MDH produced membrane-associated enzymes, thereby ruling out the participation of either end of the protein in membrane-binding and implicating an interior sequence. Examination of the sequence of MDH did not reveal any long hydrophobic stretch of residues that might serve as a membrane anchor. However, it was evident from modeling that a segment of residues in the middle of MDH that had no sequence similarity with the other proteins in the family was likely to be located on the surface of the αβ-barrel structure of the enzyme. We, therefore, adopted the strategy to delete this portion of the membrane-associated MDH and replace it with the corresponding region from the *soluble* homologous enzyme GOX (Figures 1 and 2). We succeeded in constructing a chimeric mutant, MDH-GOX, which was predominantly in the soluble fraction (Table I and Figure 3). The reason that ~30% of MDH-GOX is located in the membrane fraction is perhaps because it is expressed to high levels in the *E. coli* host and is also somewhat hydrophobic. The majority of the MDH-GOX is found in the soluble fraction.

The chimeric MDH-GOX, in which 53 residues have been deleted from the middle of MDH and replaced by 34 residues from GOX, retains the ability to dehydrogenate (*S*)-mandelate, although at a lower rate than that of the membrane-associated wild-type MDH (~1%; Table II). Several residues in the active site of GOX and flavocytochrome b_2 have been implicated in substrate and cofactor binding and catalysis (Lindqvist et al., 1991). Examination of the MDH sequence shows that these residues are all conserved, and none of them has been deleted in creating the chimeric mutant. Thus, it is not surprising that wild-type MDH and MDH-GOX have some kinetic properties in common: the K_ms for (*S*)-mandelate are similar, both are inhibited by phenylacetate and phenyl-1,2-ethanediol, and, most importantly, both are FMN-dependent dehydrogenases. In addition, the chimeric enzyme did not produce any detectable amounts of hydrogen peroxide. These results indicate that the loops are not important in either substrate or cofactor binding. However, since the loops are in close proximity to the active sites, the conformation of the active site of MDH-GOX may be altered, thereby affecting the rate of flavin reduction and, therefore, the overall rate of the reaction.

The soluble MDH-GOX catalyzes dehydrogenation of (*S*)-mandelate much more slowly than wild-type MDH. However, with (*S*)-phenyllactate as substrate, the two enzymes are almost equally active; in fact, MDH-GOX is 3-fold more active than wild-type MDH. Wild-type MDH catalyzes dehydrogenation of (*S*)-phenyllactate at $\sim 10^{-3}$ the rate of dehydrogenation of (*S*)-mandelate. It has been proposed that abstraction of the α-proton of the substrate is the first step in the reaction mechanisms of these enzymes (Ghisla & Massey, 1989). Since the pK_a of the α-proton of (*S*)-phenyllactate is expected to be several units greater than the pK_a of the α-proton of (*S*)-mandelate (Gerlt et al., 1991), it is expected that MDH should utilize (*S*)-phenyllactate much more slowly than (*S*)-mandelate. Further studies are in progress to understand how the reaction mechanism differs for wild-type MDH and the soluble MDH-GOX.

The mode of membrane association of wild-type MDH deserves special mention. The homology of MDH with both GOX and flavocytochrome b_2 suggests that MDH has an αβ-barrel structure with a segment in the *middle* of the protein extending into the membrane. MDH is tightly associated with the membrane since it can only be released from the membranes by detergents. The membrane-binding segment has a number of polar residues and does not appear to have a sequence that can form a hydrophobic, α-helical structure. To the best of our knowledge, MDH is the first example of a membrane-associated protein for which it has been directly demonstrated that the membrane anchoring is associated with a structural segment in the *middle* of the amino acid sequence, with the amino- and carboxy-terminal halves residing in the cytoplasm.

Biophysical studies of mutants of the D-lactate dehydrogenase from *E. coli*, a membrane-associated, FAD-containing enzyme, indicate that the membrane-binding domain is also located in an internal region of the primary sequence (between amino acids 228 and 369; Peersen et al., 1990). Furthermore, these studies suggest that separated segments of this internal region are responsible for the membrane association (Sun et al., 1993; Pratt et al., 1993). Since the region of MDH that we have implicated as being primarily responsible for membrane association is much smaller than this region of D-lactate dehydrogenase, the structural elements of these two proteins that are responsible for the membrane association are necessarily different.

Conclusions. We have replaced an internal segment in the membrane-associated MDH with a segment from the soluble GOX that is structurally analogous but has no sequence identity. This segment was identified as a potential membrane-binding sequence in MDH on the basis of similarities and differences in the primary sequences within a family of FMN-

dependent, α-hydroxy acid dehydrogenases/oxidases, including the structurally characterized GOX. The resulting chimeric enzyme is both soluble and catalytically active. We, therefore, conclude that a 50 amino acid residue segment in the interior of MDH anchors the protein to the membrane. This is an unprecedented topology for a membrane-binding protein. The kinetic mechanism of the soluble MDH-GOX chimeric enzyme appears to differ from that of wild-type MDH.

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