Comparison of the Precursor and Mature Forms of Rat Heart Mitochondrial Malate Dehydrogenase†

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ABSTRACT: The complete amino acid sequence of mitochondrial malate dehydrogenase from rat heart has been determined by chemical methods. Peptides used in this study were purified after digestions with cyanogen bromide, trypsin, endoproteinase Lys C, and staphylococcal protease V-8. The amino acid sequence of this mature enzyme is compared with that of the precursor form, which includes the primary structure of the transit peptide. The transit peptide is required for incorporation into mitochondria and appears to be homologous to the NH_2-terminal arm of a related cytoplasmic enzyme, pig heart lactate dehydrogenase. The amino acid differences between the rat heart and pig heart mitochondrial malate dehydrogenases are analyzed in terms of the three-dimensional structure of the latter. Only 12/314 differences are found; most are conservative changes, and all are on or near the surface of the enzyme. We propose that the transit peptide is located on the surface of the mitochondrial malate dehydrogenase precursor.

In mammalian cells, two distinct enzymes catalyze the NAD^+-dependent[1] oxidation of L-malate to form oxaloacetate. These enzymes have different subcellular localizations. Mitochondrial malate dehydrogenase (mMDH) is found in the matrix fraction of mitochondria. A second form of malate dehydrogenase (sMDH) may be isolated from the cell cytoplasm (Banaszak & Bradshaw, 1975). There are several other enzyme pairs with heterotopic cellular locations. For example, aspartate aminotransferase, adenylate kinase, and aldehyde dehydrogenase also have isoenzymes in both the cytoplasmic and mitochondrial compartments. The heterotopic localization of these enzymes is critical to cellular function. The compartmentation of the MDH's provides a mechanism for the transfer of reducing equivalents, in the form of malate, across the inner mitochondrial membrane. In addition, mMDH has a metabolic role in the tricarboxylic acid cycle, where it catalyzes the formation of oxaloacetate.

The malate dehydrogenases are a good model for detailed analysis of structural determinants of mitochondrial sequestration because the high-resolution structures of both mMDH and sMDH have been determined by X-ray diffraction studies (Roderick & Banaszak, 1986; Hill et al., 1972). In addition to the structural data, preliminary studies of the biogenesis of mMDH are consistent with the generally accepted model of mitochondrial import [for a review see Hay et al. (1984)]. Like the majority of mitochondrial enzymes, mMDH is encoded by nuclear DNA and synthesized on cytoplasmic polysomes as a precursor bearing an NH_2-terminal extension (Aziz & Freeman, 1981; Mihara et al., 1982; Grant et al., 1983). This extension is removed upon import by isolated mitochondria (Chien & Freeman, 1984; Chien et al., 1984). In addition to the NH_2-terminal extension, there may be other structural features of mMDH that contribute to mitochondrial targeting and membrane translocation.

In this paper, we report the complete chemical amino acid sequence of mMDH purified from rat myocardium. The sequence of mMDH from rat heart is 96% homologous to the porcine heart sequence described earlier in preliminary form (Birktoft et al., 1982). Each subunit of the isolated homodimer is a polypeptide chain 314 amino acids in length. A comparison of the chemical sequence described in this report with the nucleotide sequence of pre-mMDH (Grant et al., 1986) has proven that mMDH is initially synthesized bearing a 24-residue NH_2-terminal extension.

A comparison of the primary structure of rat mMDH with homologous structures of other 2-hydroxyacid dehydrogenases shows conservation of residues important in catalysis and substrate binding. Correlation of the chemical sequence of rat heart mMDH with the α-carbon model of the pig heart enzyme permits an analysis of the three-dimensional structure. From this analysis, we conclude that differences between rat and porcine sequences occur primarily on the surface of the enzyme. By comparison with the homologous lactate dehydrogenase α-carbon model, it is possible to speculate regarding the structure of the transit peptide that directs the uptake of mMDH by mitochondria.

Materials and Methods

Mitochondrial malate dehydrogenase was purified from rat hearts obtained from 150-g male Sprague-Dawley rats. Pure enzyme was obtained after ammonium sulfate precipitation from a crude homogenate and consecutive chromatography on (carboxymethyl)-Sepharose, blue Sepharose, and 5'-AMP–Sepharose as previously described (Glatthaar et al., 1974; Kuan et al., 1979). Sixty-six milligrams of mMDH with a specific activity of 183 units/mg was obtained from 400 g of rat hearts. Purity was established on the basis of SDS/polyacrylamide gel electrophoresis, specific activity, and the presence of a single NH_2-terminal sequence upon Edman degradation.

Most of the studies were performed with the carboxymethylated form of mMDH, which was prepared as follows. A portion of purified mMDH (100 nmol) was dissolved in 6 M guanidinium hydrochloride in 100 mM Tris buffer at pH 9.5. To this solution, a 5-fold molar excess of dithiothreitol

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[1] Abbreviations: NAD^+, oxidized nicotinamide adenine dinucleotide; mMDH, mitochondrial malate dehydrogenase; sMDH, cytoplasmic malate dehydrogenase; MDH, malate dehydrogenase; LDH, lactate dehydrogenase; HPLC, high-performance liquid chromatography; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid; PTH, phenylthiohydantoin; Tris, tris(hydroxymethyl)aminomethane.

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was added relative to the estimated cysteine content (Kuan et al., 1979). The reduction reaction was incubated for 3 h at 25 °C. After acidification to pH 8.0 with 0.1 N hydrochloric acid, a 5-fold excess of iodoacetic acid was added relative to dithiothreitol. Fifty microcuries of iodo[1-^{14}C]acetic acid was included in the carboxymethylation reaction, which was allowed to proceed for 2 h. After this period, the *S*-([^{14}C]carboxymethyl)-mMDH was dialyzed against 50 mM ammonium bicarbonate, pH 8.5.

Several methods were used to generate peptides from mMDH. The rat heart enzyme has six methionine residues, and one set of peptides was obtained after overnight cleavage of 100 nmol of *S*-([^{14}C]carboxymethyl)-mMDH by cyanogen bromide (100 mg) in a 70% formic acid solution. The reaction mixture was then concentrated by lyophilization. With only six cleavage sites, it was expected that some peptides would be quite large, and therefore, the digest was fractionated by gel filtration. The cyanogen bromide peptides were redissolved in 200 μL of 0.1% TFA, and insoluble material was removed by centrifugation. The acid-soluble peptides contained in the supernatant were loaded onto a TSK gel G-3000 SW column (Bio-Rad; 0.75 cm × 30 cm) and separated by HPLC in 0.05% TFA at a flow rate of 0.5 mL/min. Peptide fractions were pooled, as indicated in Figure 1 of the supplementary material (see paragraph at end of paper regarding supplementary material), and concentrated.

Pooled fractions were then rechromatographed at 0.25 mL/min in 0.5% TFA to obtain homogeneous preparations. Individual pools were numbered with Roman numerals in the order of elution. Portions from individual pools were analyzed for *S*-([^{14}C]carboxymethyl)cysteine content and subjected to amino acid sequent analysis.

A second cyanogen bromide digest was prepared as described above, and this time, peptides were purified by reverse-phase HPLC. During elution, a linear gradient of 0.01% TFA in H_2O to 0.01% TFA in acetonitrile was developed at a rate of 0.25% acetonitrile/min and peptides were numbered by elution time. The flow rate was 1 mL/min. Comparison of the reverse-phase elution profile shown in Figure 2 of the supplementary material with the gel filtration separation shows that considerable material was retained on the reverse-phase column and that gel filtration gave a better separation of the large, relatively hydrophobic peptides generated by cyanogen bromide digestion.

Trypsin and staphylococcal protease V-8 were selected for the preparation of small peptides because of differing cleavage specificities. For tryptic digestion, 150 nmol of *S*-([^{14}C]-carboxymethyl)-mMDH was dissolved in 0.1 M Tris, pH 8.5. Two nanomoles of TPCK-treated bovine trypsin (Worthington) dissolved in 0.001 M hydrochloric acid was added to the mMDH, and the mixture was incubated for 1 h at 25 °C. A second aliquot of trypsin (2 nmol) was then added, and proteolysis continued for an additional 16 h at 25 °C. Following acidification with TFA, the peptide mixture was concentrated and resuspended in 0.5% trifluoroacetic acid.

The conditions of digestion with staphylococcal protease V-8 were selected in an attempt to favor cleavage after glutamate residues (Drapeau, 1977). Fifty nanomoles of *S*-([^{14}C]-carboxymethyl)-mMDH was digested by 2 nmol (25 units) of staphylococcal protease V-8 (Worthington) in 100 mM ammonium bicarbonate containing 1 mM ethylenediaminetetraacetic acid overnight at 37 °C. The mixture was acidified with TFA, concentrated, and resuspended in 0.5% TFA.

Finally, proteolytic digestion with endoproteinase Lys C was selected to generate larger peptides for determination of sequence overlaps. This protease cleaves after lysine residues. One hundred nanomoles of *S*-([^{14}C]carboxymethyl)-mMDH was dissolved in 1.7 mL of 50 mM ammonium bicarbonate, pH 7.8, and digested for 16 h at 37 °C with 2 nmol (1 unit) of endoproteinase Lys C (Boehringer Mannheim). The mixture was then acidified, concentrated, and resuspended in 0.5% TFA.

All of the proteolytic digests were first fractionated by reverse-phase HPLC. After centrifugation to remove acid-insoluble peptides, the samples were loaded onto a 4.6 mm × 25 cm C_{18} (μBondapak; Waters) column equilibrated with 0.01% TFA. The peptides were eluted with a linear gradient from 0.01% TFA in H_2O to 0.01% TFA in acetonitrile. The acetonitrile concentration was increased at a rate of 0.25%/min. The flow rate for all separations was 1 mL/min. Fractions of 1-mL volume were collected, and the elution of each peptide was monitored by the absorbance at 229 nm. Purified peptides were numbered according to elution time, concentrated by lyophilization, and dissolved in 50% TFA for Edman degradation.

For analysis of amino acid composition, rat mMDH was treated with 6 N hydrochloric acid in evacuated, sealed glass tubes at 110 °C for 18 h. After removal of HCl, hydrolysates were resuspended in 50 mM HCl and analyzed on a Waters HPLC. For many peptides, we proceeded directly to sequence determinations by using a gas-phase sequenator. Purified fractions from the different proteolytic digests were selected for Edman degradation on the basis of their positions in the HPLC elution profiles and the content of *S*-([^{14}C]carboxymethyl)cysteine.

The amino acid sequences of purified mMDH peptides were determined by sequential Edman degradation on a Beckman 890C or an Applied Biosystems 470A protein sequencer. A small amount (5–10%) was removed from each cleavage cycle for liquid scintillation counting when radiolabeled peptides were analyzed. These data were used in the assignment of cysteine residues. Phenylthiohydantoin amino acids were uniquely identified by reverse-phase HPLC analysis on an Altex Ultrasphere ODS-PTH column (Grant & Chiappinelli, 1985). The identities of PTH-amino acids were assigned on the basis of the elution position relative to standards. Yields were calculated by peak integration. All the reported values were 3-fold above background levels, unless otherwise noted.

Results

The complete amino acid sequence of rat mMDH was unambiguously derived from automated Edman degradation of the intact protein and the peptides obtained by cyanogen bromide cleavage or proteolytic digestion as described in the previous section. The amino acid sequence of rat heart mMDH and the peptides that were used to establish the primary structure are shown in Figure 1. Peptides yielding redundant sequences were not included in this figure. Edman degradation of intact mMDH identified 34 of the first 35 residues. Thr-30 was not identified in this analysis but was assigned from the amino acid sequence of an NH_2-terminal cyanogen bromide peptide.

Cyanogen bromide should cleave at the six methionine residues and thereby generate seven peptides. However, upon HPLC separation, only five cyanogen bromide peptides were found. Inspection of the locations of methionine residues in the final amino acid sequence shows that the complete digestion by cyanogen bromide would yield the following peptides: residues 1–85, 86–112, 113–227, 228–242, 243–292, 293–313, and an isolated residue 314. Lysine-314 was not recovered from the cyanogen bromide digest. All other pep-

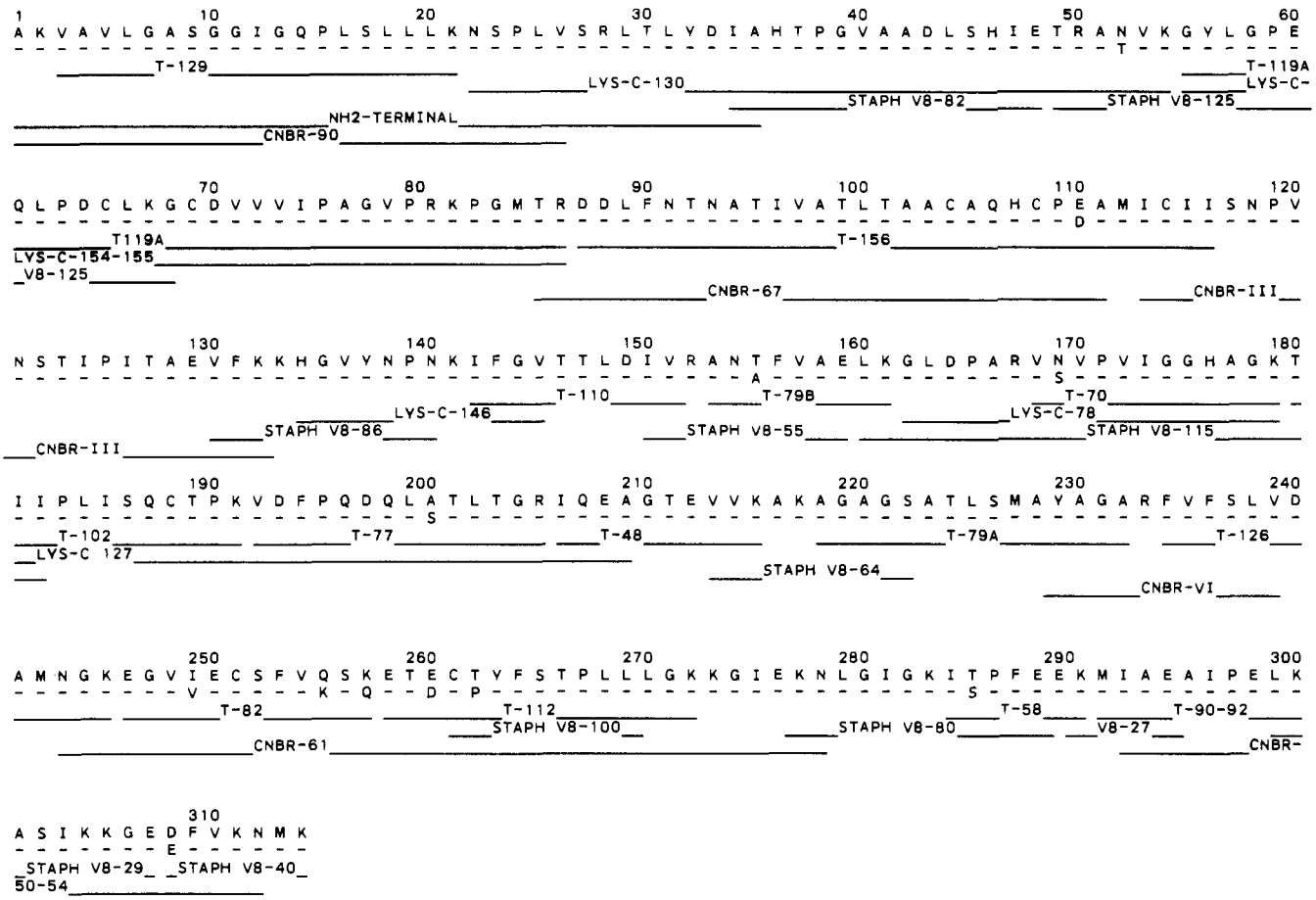

FIGURE 1: Complete amino acid sequence of rat heart mMDH. The amino acid sequence of rat mMDH is depicted above the preliminary sequence of the porcine enzyme (Birktoft et al., 1982), with identities indicated by a dash and differences by the single-letter code. The sequence of rat mMDH has been deduced from the sequences of overlapping peptides which are also given in the figure and described more fully in the text. The bars represent the extent of sequence determination by automatic Edman degradation of peptides obtained from cleavage of mMDH by cyanogen bromide (CNBr), endoproteinase Lys C (Lys-C), staphylococcal protease V-8 (Staph V8), and trypsin (T). Data were also obtained by Edman degradation of the mature enzyme (NH_2 terminus). The numbers used in peptide identification correspond to HPLC fractions as shown in the supplementary material.

tides except the one beginning at residue 86 were found. In a second cyanogen bromide digest, this peptide (86–112) was recovered. Although it was not possible to obtain the complete sequence of each cyanogen bromide peptide, nearly 40% of the total amino acid residues were assigned by using data from the two sets of cyanogen bromide peptides.

In order to generate the remaining amino acid sequence and for the establishment of overlaps with the cyanogen bromide peptides, further proteolytic digestions of rat mMDH were necessary. Staphylococcal V-8 protease cleaves predominantly at glutamic acid and was expected to give at least 20 peptides, on the basis of the amino acid composition (Table I). Endoproteinase Lys C cleaves after lysine residues only and should yield 26 peptides. Trypsin, which cleaves after lysine and arginine, was selected to generate numerous small peptides to establish overlapping sequences. Sequence information was obtained for 19 tryptic peptides, 7 peptides generated by endoproteinase Lys C, and 17 peptides from staphylococcoal V-8 proteolysis. In four instances, Edman degradation of a single HPLC elution peak yielded two simultaneous amino acid sequences. In each case, the amounts of coeluting peptides differed considerably and permitted unambiguous amino acid sequence determination of both.

As seen in Figure 1, the mMDH polypeptide chain was 314 amino acids and has a calculated molecular weight of 33 156. As shown, 83% of the residues were identified in two or more peptides. Only one residue, Thr-262, was identified ambiguously in Edman degradation of two different peptides. In one cleavage cycle, residue 262 was identified as a mixture of proline and threonine. The nucleotide sequence of a cDNA clone encoding rat heart mMDH revealed threonine at position 262 (Grant et al., 1986). These data do not rule out the possibility of allelic polymorphism. The peptide sequence data supporting the identification of all residues in rat heart mMDH are included in Table II, which is available as supplementary material.

The assignment of the COOH terminus is based on the amino acid sequence of peptide 40 from the staphylococcal protease V-8 digest. Residue 314 was shown to be lysine by recovery of 1.2 nmol of PTH-lysine and by noting that three additional cleavage cycles yielded no PTH-amino acids. This COOH-terminal lysine could not result from proteolysis, as staphylococcal protease V-8 cleaves specifically after acidic residues. In addition, the homologous sequence of porcine mMDH (Birktoft et al., 1982) ends at residue 314 (see alignment in Figure 1). Finally, in the nucleotide sequence of the cDNA clone for rat heart mMDH, a TGA stop codon immediately follows the AAG codon encoding Lys-314 (Grant et al., 1986).

The amino acid composition calculated from the chemical sequence is given in Table I. For comparison, values calculated from hydrolysates of the intact enzyme are also shown. The two methods are in good agreement. The polypeptide chain is comprised of 9% acidic residues and 12% basic residues.

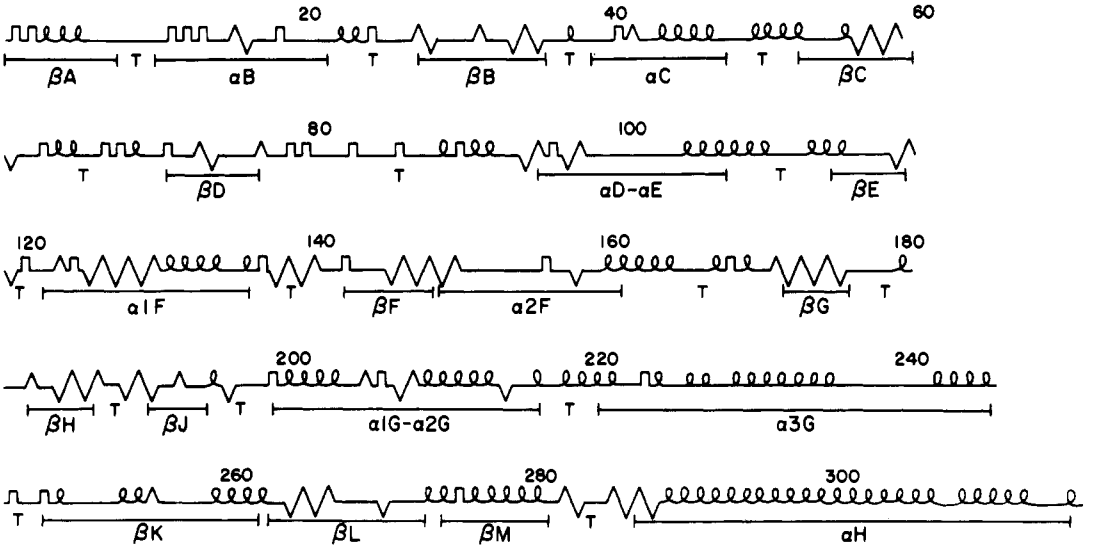

FIGURE 2: Comparison of secondary structure predicted by Chou–Fasman method with X-ray crystallographic structure. This figure shows two linear representations of the secondary structure of mMDH with every 20th residue numbered above the figure. The upper line is a schematic representation of the Chou–Fasman prediction of secondary structure, using the amino acid sequence of rat heart mMDH. Loops, zigzags, and square waves are used to designate α-helices, β-pleated sheets, and β-turns, respectively. Straight line segments represent regions where no secondary structure could be reliably predicted. Immediately below this representation is a series of labeled bars that delineate discrete elements of secondary structure found in the three-dimensional structure of porcine heart mMDH determined by X-ray diffraction studies. These elements are named by convention, with the prefix α representing helical stretches, the prefix β representing β-structures, and the letter T representing major directional changes in the polypeptide chain (Hill et al., 1972).

Table I: Amino Acid Composition of Rat Heart Mitochondrial Malate Dehydrogenase[a]

amino acid	I	II	III
lysine	25	27	25
histidine	5	5	5
arginine	8	8	8
aspartic acid	27	26	12
asparagine	*b*	*b*	13
threonine	22	22	23
serine	17	14	15
glutamic acid	29	25	17
glutamine	*c*	*c*	8
proline	29	29	28
glycine	44	32	32
alanine	*d*	4	8
cysteine	24	30	27
valine	5	6	6
methionine	19	21	24
isoleucine	33	28	27
leucine	6	5	5
tyrosine	10	11	11
phenylalanine	10	11	11
tryptophan	*e*	*e*	0

[a] Amino acid compositions of rat heart mMDH are expressed as number of residues per subunit. Included in the table are values from an 18-h hydrolysis of rat mMDH at 110 °C (I), results from acid hydrolyses as described by Kuan et al. (1979) (II), and results from the chemical sequence of the entire protein (III). [b] Measured as aspartic acid. [c] Measured as glutamic acid. [d] Measured as cysteic acid after performic acid oxidation. [e] Not determined.

These values contrast with the composition of cytoplasmic MDH (sMDH), which contains more acidic and basic residues and has a lower isoelectric point than the mitochondrial form (Banaszak & Bradshaw, 1975; Kuan et al., 1979). Rat mMDH has eight cysteine residues, and like the mMDH enzymes from many other species, it is devoid of tryptophan.

Previous studies suggested that rat mMDH was composed of nonidentical subunits on the basis of isolation of multiple active forms by electrophoresis (Mann & Vestling, 1970; Kuan et al., 1979). Multiple electrophoretic forms of mMDH had also been found in other species (Kitto et al., 1966; Glatthaar et al., 1974). On the basis of physical findings, it was concluded that these multiple forms resulted from modification of amino acids after biosynthesis rather than differences in genomic sequences. The lack of microheterogeneity in the amino acid sequence of rat heart mMDH supports the conclusion that mMDH is a dimer of identical subunits encoded by a single gene.

Discussion

In order to study the features of mitochondrial MDH that are responsible for the posttranslational incorporation of this protein into mitochondria, but preclude uptake of the cytoplasmic form of MDH, we have continued the structural characterization of mMDH by determination of the complete chemical sequence of the rat heart enzyme. Although the primary sequences of mMDH and sMDH differ considerably, the structures are quite similar. For this reason, we have focused interest on the 24 amino acid NH_2-terminal extension, or transit peptide, which likely mediates uptake into mitochondria. The sequence of this region has been determined from a full-length cDNA clone and has been used together with comparisons to a partially homologous portion of lactate dehydrogenase to propose plausible configurations of the precursor enzyme.

The primary amino acid sequence of the rat heart mMDH reported here has 96% sequence identity with the preliminary sequence of the pig heart enzyme (Birktoft et al., 1982) and, therefore, may be described in terms of the known crystallographic structure of the pig heart protein (Roderick & Banaszak, 1986). The polypeptide chain of mMDH from pig heart is known to consist of two domains, each with well-defined secondary structural elements (Roderick & Banaszak, 1983). This structure was compared with that of the rat heart mMDH which was predicted by the Chou–Fasman rules (Chou & Fasman, 1978). The results of the prediction are depicted schematically in Figure 2 and are aligned with a linear representation of the conformation of porcine heart mMDH deduced from the X-ray crystallographic studies. In this comparison, the elements of secondary structure in the COOH-terminal (catalytic) domain were predicted more reliably than those in the NH_2-terminal, nucleotide-binding, domain. The conformation of mMDH includes 16 locations

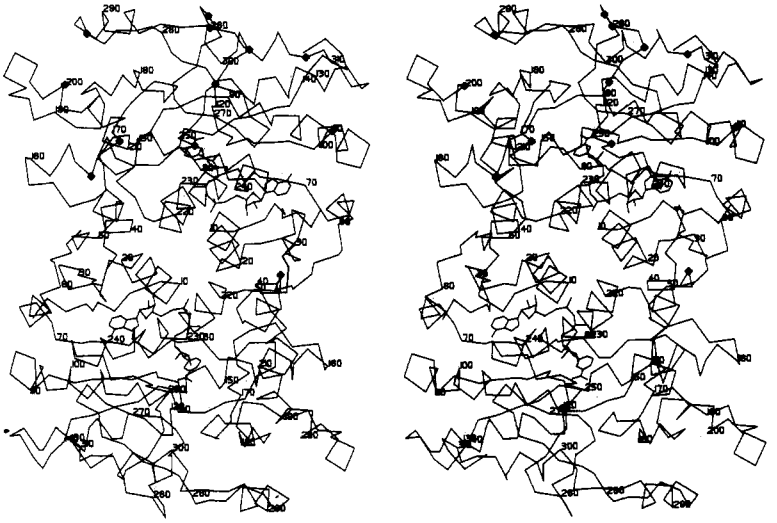

FIGURE 3: Stereo diagram of the α-carbon model of dimeric porcine mitochondrial malate dehydrogenase. A stick model of the α-carbon atoms of porcine mMDH is shown in the stereo diagram with every tenth residue numbered beginning at the NH_2 terminus. The two identical subunits of the dimeric protein are shown, and each subunit includes a stick model representation of the bound nucleotide cofactor NAD^+. Amino acids that are different between rat and porcine sequences are indicated by filled circles.

where the polypeptide chain undergoes a major directional change. The positions of these changes are designated by T for "turn" in Figure 2. The Chou–Fasman representation includes 15 predicted β-turns, but only 7 (44%) are near actual turns. In general, for rat heart mMDH, the predicted secondary structure was not in good agreement with the conformation as determined by X-ray crystallographic studies of the pig heart enzyme.

Comparison of structures of lactate dehydrogenase (LDH), mMDH, sMDH, alcohol dehydrogenase, and glyceraldehyde-3-phosphate dehydrogenase has shown that the nucleotide-binding domains are similar in both their overall conformation and mode of NAD^+ binding (Birktoft & Banaszak, 1984). In this domain, the polypeptide chains of mMDH, LDH, and sMDH are folded into a structure containing six parallel β-strands interconnected by four α-helices (Rossmann et al., 1975). The catalytic domains, which are primarily responsible for substrate specificity, are more diverse in structure. However, dehydrogenases with similar substrate specificities have been shown to exhibit homology in both domains (Birktoft & Banaszak, 1984; Rossmann et al., 1975). For example, LDH, sMDH, and mMDH, which utilize 2-hydroxyacid substrates, are structurally homologous throughout most of both the nucleotide-binding and catalytic domains.

This homology among the 2-hydroxyacid dehydrogenases is not reflected by extensive similarity of the amino acid sequences, which reveal less than 25% sequence identity (Birktoft et al., 1982). Despite this absence of overall sequence homology, certain residues are absolutely conserved in mMDH, sMDH, and LDH. His-176 and Asp-149 in the rat mMDH sequence are analogous to the His–Asp pair found at the active sites of sMDH and LDH (Birktoft & Banaszak, 1983). It has been proposed that these residues participate in a charge relay system which facilitates proton transfer from the solvent to the substrates of sMDH and LDH. Arg-171 in LDH and Arg-155 in sMDH have been implicated in the binding of the respective substrates in the ternary enzyme complexes (Holbrook et al., 1975; Birktoft & Banaszak, 1983). The corresponding residue in the rat mMDH sequence is Arg-152. The placement of these three residues in the three-dimensional structure shows that they are near the active site of mMDH (Roderick & Banaszak, 1986). This active-site conservation in rat mMDH is further evidence of the similarity of catalytic mechanisms among 2-hydroxyacid dehydrogenases. Four critical residues in the nucleotide-binding domain are also preserved in the rat mMDH sequence; they are Gly-7, Gly-11, Asp-33, and Gly-77 (Birktoft & Banaszak, 1984).

Compared with the low degree of amino acid sequence homology among the 2-hydroxyacid dehydrogenases, alignment of the rat and porcine mMDH sequences shows that 96% of the residues are identical. Differences are found in only 12 positions, and several represent conservative substitutions. For example, Asp-110 is replaced by glutamic acid, Glu-308 by aspartic acid, and Ser-285 by threonine. The virtual identity between rat and porcine sequences allows correlation of the rat primary structure directly with the α-carbon structure of porcine mMDH (Figure 3). All of the differences occur near the surface of the enzyme. Five substitutions clustered between residues 249 and 262 lie within a segment of two twisted antiparallel β-strands separated by an exposed turn. In this region, the LDH sequences all have short deletions with respect to the mMDH sequence (Birktoft et al., 1982). The variability of sequence and structure in this region of mMDH and LDH suggests that this loop on the molecular surface can accomodate evolutionary changes without compromising catalytic function.

In view of the structural similarity of mature mMDH and sMDH with the other 2-hydroxyacid dehydrogenases, the factor that must determine the sequestration of pre-mMDH by mitochondria is the transit peptide (Grant et al., 1983; Aziz & Freeman, 1981). In general terms, the problem of mitochondrial protein uptake is poorly understood at a molecular level. However, the importance of transit peptides in mitochondrial targeting has been demonstrated experimentally. In gene-fusion experiments, NH_2-terminal sequences from mitochondrial transit peptides were sufficient to direct cytoplasmic protein hybrids into mitochondria (Hurt et al., 1985; Horwich et al., 1985). It is evident that, to understand the molecular processes involved in mitochondrial targeting, it is necessary to determine what unique structural features of transit peptides are responsible for recognition by mitochondria. Recently, sequences of several mitochondrial precursors have been deduced from cDNA clones. In efforts to locate homologous regions within transit peptides that might be responsible for targeting, no consensus sequence was found.

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                -24   -20          -10
Rat Heart mMDH  M L S A L A R P V G A A L R R S F S T S A Q N N
                  *  : : :   : *    :  : * : : : * *
Pig Heart LDH     A T L K E K L I A P V A Q Q E T T I P N N
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FIGURE 4: Alignment of rat mMDH transit peptide and NH_2-terminal sequence of LDH. Structural comparison of mature mMDH and LDH from pig heart reveals that LDH has about 20 additional amino acids at the NH_2 termius. The diagram shows the 24 amino acid NH_2-terminal transit peptide of pre-mMDH aligned with the NH_2-terminal "arm" of pig heart LDH. The similarity between sequences is marked in terms of invariant residues (*) and conservative substitutions (:).

However, all transit peptides are similar in composition and have a notable abundance of basic residues. It is likely that the charge and configuration of the transit peptide provide unique features that are critical for interactions with mitochondria, possibly via proteinaceous receptors.

Mitochondrial protein precursors exist, at least transiently, in cytoplasmic pools. The state of precursor molecules prior to import, especially with regard to association with cytoplasmic components that may promote uptake, is presently unknown. Therefore, consideration of the configuration of precursor proteins is necessary for the eventual understanding of the molecular interactions involved in the recognition process. Knowledge of the amino acid sequence of the rat heart mMDH transit peptide (Grant et al., 1986), the structural homology among the 2-hydroxyacid dehydrogenases, and the crystallographic structure of mature pig heart mMDH allows speculation concerning the conformation of the mMDH precursor involved in binding to mitochondria. Comparison of the rat heart enzyme with the structure of the pig heart mMDH shows that the NH_2 terminus of mature mMDH is found near the molecular surface. This can be seen in Figure 3, where residue 1 is shown behind the αC-helix between residues 60 and 70. Although it is difficult to make precise predictions based on the molecular structure of the mature enzyme, if the precursor conformation resembles the mature form, the location of the NH_2-terminal residue in the three-dimensional structure shows that this peptide bond is likely to be sterically accessible for proteolysis. On the basis of the chemical sequence of the mature mMDH reported here and the nucleotide sequence of the protein precursor, the proteolytic cleavage site that generates the mature enzyme involves the peptide bond between an asparagine and an alanine residue.

It is not known whether mitochondrial enzyme precursors are catalytically active in the cytoplasm prior to mitochondrial import and processing. With the homology of mMDH with LDH and sMDH and with 314 amino acids contributing to the stability of the three-dimensional structure, dramatic changes in the tertiary structure due to the presence of the transit peptide seem unlikely. Thus, there is no apparent reason why the enzyme with the transit peptide should not retain enzymatic activity. A variety of potential surface locations for the transit peptide are all well away from the active site, which is marked by the model of NAD^+ in Figure 3.

The mMDH transit peptide, like those of other mitochondrial proteins, is predominantly polar and contains several basic residues. Chou–Fasman prediction and analysis of hydropathy indices provide no compelling information regarding the secondary structure of the mMDH transit peptide. However, a sequence comparison of either mature mMDH or sMDH with the LDH's shows that the LDH's have an additional 21 amino acids on the NH_2-terminal end (Birktoft et al., 1982). Although the LDH's are cytosolic enzymes, it is possible that the residues in the NH_2-terminal arm are evolutionarily related to the transit peptide of mMDH. Figure 4 contains one possible alignment of the NH_2-terminal arm of pig heart LDH (Kiltz et al., 1977) and the corresponding transit peptide of rat heart pre-mMDH. In this alignment, significant sequence similarity between the COOH terminus of the mMDH transit peptide, near the predicted cleavage site, and the LDH arm seems to exist. The location of this NH_2-terminal arm of LDH is indicated by heavy lines in the stereo diagram given in Figure 5, which shows the so-called *R* axis dimer (Holbrook et al., 1975; Grau et al., 1981). The NH_2-terminal arm of LDH is a factor in formation of the subunit–subunit interactions about

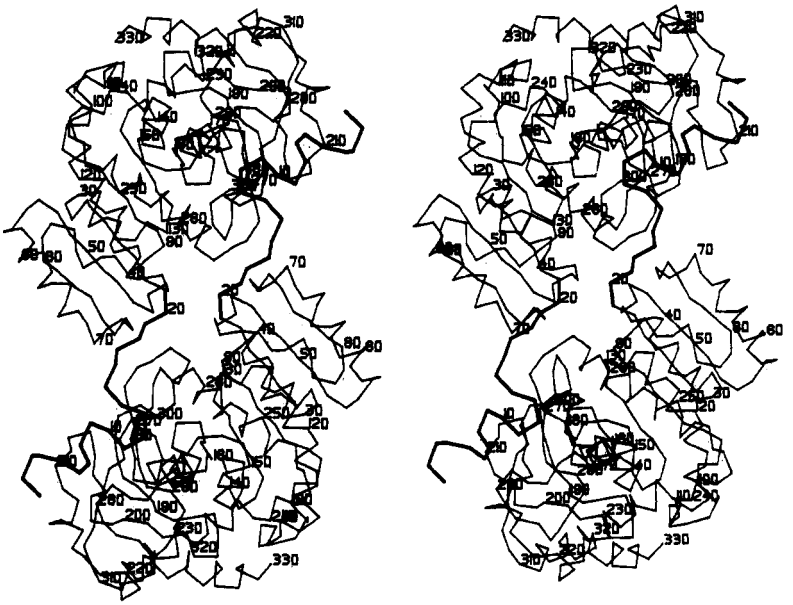

FIGURE 5: Stereo diagram of the α-carbon model of a dimeric portion of the LDH tetramer. The LDH tetramer contains four identical subunits arranged with 222 symmetry. The symmetry axes have been labeled *PQR* (Holbrook et al., 1975; Grau et al., 1981). The dimeric forms of mMDH and sMDH have two subunits in the same arrangement as the pairs of subunits in tetrameric LDH which are related by the *Q* axis. In tetrameric LDH additional subunit–subunit contacts form about the *P* and *R* axes. In this representation, the two subunits of the LDH tetramer in the *R* axis dimer are shown as an α-carbon model with every 20th residue numbered from the NH_2 terminus. In the two subunits of the LDH tetramer selected for this diagram, the major subunit–subunit contacts can be seen to be formed by an "arm" extending from the main body of each subunit. This arm is marked by a heavy line.

this axis resulting in a tetrameric protein. It is possible to speculate upon the quaternary structure of pre-mMDH on the basis of the similarity to LDH. Would the transit peptide extension change the quaternary structure of pre-mMDH such that it would form either monomers or larger multimers rather than dimers? In sedimentation and gel filtration studies, precursors of several mitochondrial enzymes, including pre-mMDH, have been shown to form aggregates (Chien & Freeman, 1983; Kalousek et al., 1984; Zimmerman & Neupert, 1980). The nature of these complexes and their physiological significance, especially with regard to mitochondrial sequestration, have not been determined.

On the basis of these considerations, we propose that the transit peptide is located on the surface of mMDH and is thus available to alter the quaternary structure of the cytoplasmic form of the precursor through subunit–subunit interactions. In addition, this surface location of the transit peptide might promote protein–protein interactions with cytosolic carrier proteins or mitochondrial receptors.

The availability of cDNA clones encoding mitochondrial precursors should enable the expression, isolation, and subsequent study of the precursor forms of mitochondrial proteins. By full characterization of the structures of precursors and through comparison with homologous cytoplasmic enzymes, the conformational determinants of the transit peptide that direct mitochondrial uptake can be defined. Because the structures of several dehydrogenases have been studied in detail, further study of pre-mMDH should prove extremely fruitful.

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Supplementary Material Available

Figures 1–6 showing separation and purification of peptides by gel filtration or HPLC and Table II showing sequence data for rat heart mMDH peptides (14 pages). Ordering information is given on any current masthead page.

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