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AMINO ACID SEQUENCE OF PORCINE HEART FUMARASE

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The complete amino acid sequence of porcine heart fumarase (EC 4.2.1.2) has been determined from peptides produced by cyanogen bromide, endoproteinase Arg-C, *S. aureus* V8 protease, and trypsin. The enzyme is a tetramer of identical subunits with M_r = 50,015 and composed of 466 amino acid residues. Porcine heart fumarase displays 96% identity to human liver fumarase. Prediction of the secondary structural elements of porcine fumarase indicate that the enzyme contains a large amount of alpha helix with very little beta structure.

Fumarase catalyzes the interconversion of L-malate and fumarate, and is a member of a large family of enzymes called hydratases. No conformational information on any member of this family is presently available. In order to determine the three dimensional structure of fumarase, amino acid sequence studies have now been completed on the enzyme purified from porcine heart and X-ray crystallographic studies are in progress (1). In addition, the amino acid sequence of porcine heart fumarase can be compared to the primary structure of six other members of this family.

The enzymatic reaction catalyzed by fumarase is part of the citric acid cycle. Therefore, in eucaryotic cells, most of the enzyme is believed to be located within the inner membrane matrix of the mitochondrion. However a number of workers have reported the presence of two fumarase isozymes in human liver (2), yeast (3), rat liver (4), and pig liver (5). In yeast, there is substantial evidence that all forms of fumarase are coded for by a single nuclear gene (3). There appears to be some heterogeneity at the NH_2-terminal end of the protein but the paradoxical question of one gene and both a mitochondrial and cytoplasmic form of fumarase remains unanswered. In the chemical sequence studies described below, heterogeneity in the NH_2-terminal region was again observed.

Porcine heart fumarase is a tetramer of identical subunits. Analysis of the crystals of fumarase show that the subunits of the tetramer are arranged in the point symmetry group, 222 (1). The crystals diffract to a resolution of 2 Å, and X-ray diffraction data has been measured for the native and several potential heavy atom derivative containing crystals. Both the amino acid sequence and the potential location of secondary structural elements will be vital to the completion of the three dimensional structure.

MATERIALS AND METHODS

Protein Preparation. Porcine heart fumarase (EC 4.2.1.2) was prepared as previously described (1).

Peptide Preparation. Prior to cleavage, porcine heart fumarase was reduced with dithiothreitol and alkylated with iodo[^{14}C]acetic acid (6). The S-[^{14}C]carboxymethyl fumarase was digested with cyanogen bromide(7), *Staphylococcus aureus* V8 protease (Miles), and Endoproteinase Arg-C (Boehringer Mannheim). Sub-digestions of cyanogen bromide fragments were performed with V8 protease and trypsin.

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Digestions with endoproteinase Arg-C and trypsin were performed in tris buffer as previously described for trypsin(7).

All peptides were purified by reverse-phase high performance liquid chromatography on a 4.6 mm X 25 cm C-18 μBondapak column (Waters) which was equilibrated in 0.05% trifluoroacetic acid. All digests were acidified prior to loading and the columns were developed with a linear gradient from 0.05% trifluoroacetic acid to acetonitrile made 0.05% in trifluoroacetic acid after an initial 5 min. wash at starting conditions. The chromatography was monitored at 214 nm and small aliquots were removed for scintillation counting. Peptides were recovered by combining appropriate fractions and were evaporated to dryness under a stream of nitrogen. The peptides were redissolved in 50% trifluoroacetic acid immediately prior to sequence analysis.

Amino Acid Analysis. Samples were hydrolyzed either in 6 N HCl in evacuated sealed glass tubes or in HCl vapor *in vacuo* at 110°C for the indicated times. Hydrolysates were dried by rotary evaporation and redissolved in 0.2 M sodium citrate, pH 2.0. Amino acid analyses were performed either on a Waters HPLC amino acid analyzer equipped for o-phthalaldehyde detection and continuous infusion of hypochlorite for the detection of proline or on a Beckman 6300 amino acid analyzer with ninhydrin detection.

Sequence Analysis. All polypeptides were sequenced by automated Edman degradation on an Applied Biosystems Model 470A gas phase protein sequencer equipped for "on-line" PTH amino acid identification using an Applied Biosystems Model 120A HPLC. Standard columns and conditions were employed.

RESULTS AND DISCUSSION

Porcine heart fumarase consists of 466 residues with a calculated molecular weight of 50,015 daltons. The composition of porcine heart fumarase determined by amino acid analysis is presented in Table 1 and compares well with that determined from the amino acid sequence. A summary of the sequence determination is presented in Fig. 1. In addition to the amino terminal sequence analysis, which identified the first 37 residues, 80 individual peptides were isolated and sequenced. Of these, 33 were needed to solve the primary structure. A complete set of cyanogen bromide peptides (CN-1 to CN-17), including the amino terminal peptide which is not shown in Fig. 1, were recovered. Of these peptides, 10 were sequenced completely to the carboxyl terminal residue in a single run and one other was sequenced to its penultimate residue. Overlaps and the amino acid sequences of the remaining segments were provided by the peptides produced by endoproteinase Arg-C, V8 protease, and trypsin.

Table 1

Amino Acid Composition of Porcine Heart Fumarase

amino acid	calcd[a]	sequence	amino acid	calcd	sequence
Asx	48	46	Met	17	17
Thr	30	30	Ile	25	25
Ser	22	22	Leu	40	39
Glx	44	43	Tyr	9	9
Pro	22	22	Phe	17	16
Gly	40	39	His	14	14
Ala	58	56	Lys	32	32
1/2-Cys	3	3	Arg	15	15
Val	37	36	Trp	ND[b]	2
Total					466

[a] calcd, calculated. Amino acid composition was determined after 24, 48, and 72 h hydrolyses. Serine, threonine, and tyrosine were extrapolated to zero time hydrolysis. Methionine and 1/2 cystine values were determined after performic acid oxidation as methionine sulfone and cysteic acid, respectfully.

[b] ND, not determined.

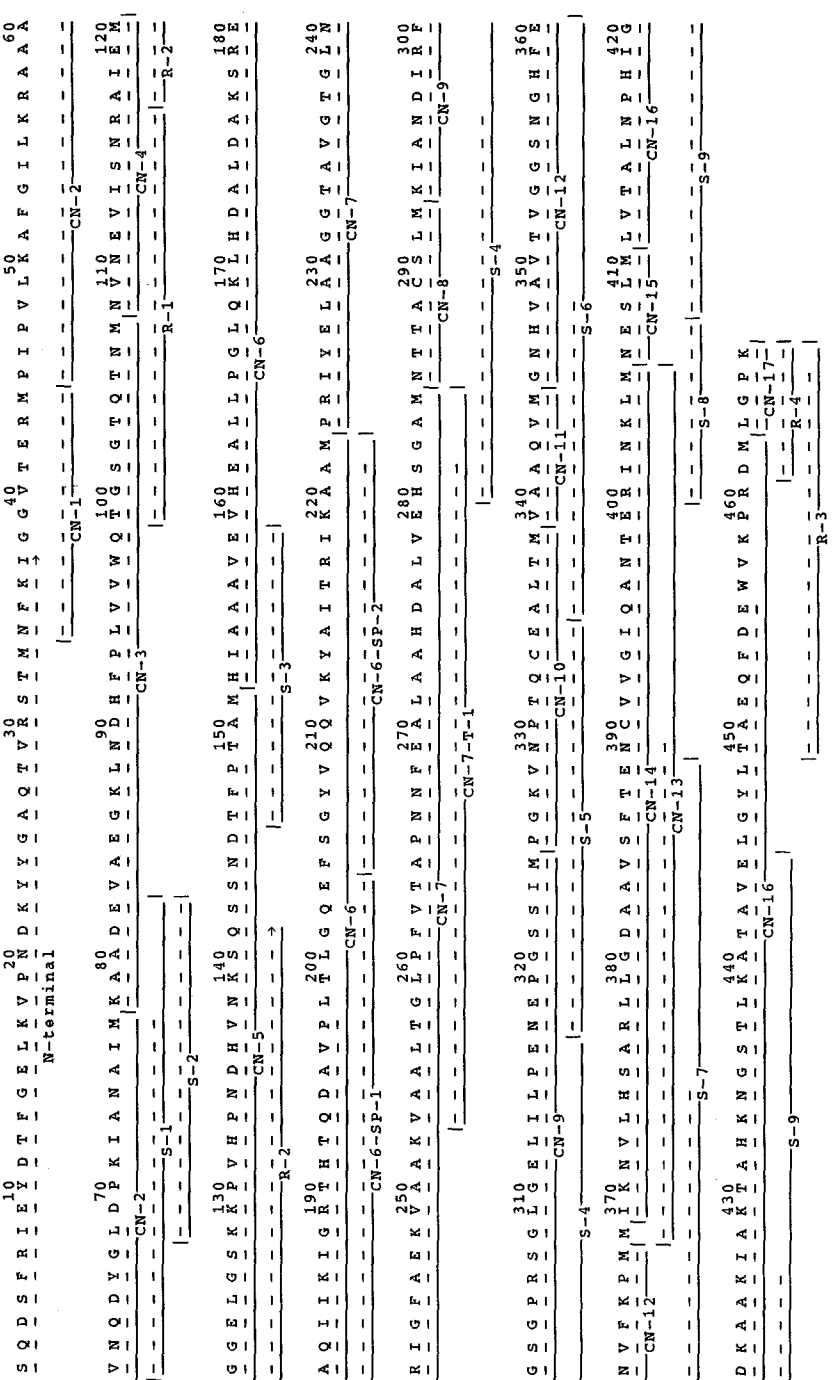

Figure 1: Summary proof of the amino acid sequence of porcine heart fumarase. Peptides were obtained from digests produced by cyanogen bromide (CN), *S. aureus* V8 protease (S), and endoproteinase Arg-C (R). Sub-digests of selected cyanogen bromide peptides were made with V8 protease (SP) or trypsin (T). Isolated peptides are designated with vertical bars and the extent of sequence information derived from each peptide is indicated with dashes. When the size of the peptide is large or unknown the sequence obtained is indicated by dashes terminating with an arrow.

The alignment of the cyanogen bromide peptides was made by the overlaps presented in Fig. 1 and by the extremely high degree of homology (96%) with human fumarase (see Fig. 2). Although overlaps were not found at three positions (residues 44, 203, and 222), there can be little doubt about the overall alignment of peptides at these positions. The complete amino acid sequence is supported by its near identity to the human sequence, the excellent agreement between the deduced sequence and the amino acid composition, and by the positive identification of the carboxyl terminal methionine residues consistent with the set of cyanogen bromide peptides. The positioning of peptides CN-6-SP-1 and CN-6-SP-2 was again indicated by the near identity to the human sequence and confirmed by the isolation of a complete set of V8 protease peptides corresponding to the locations of the glutamyl residues in peptide CN-6. In addition to CN-6-SP-1 and CN-6-SP-2 shown in Fig. 1, these peptides, which are not shown in Fig. 1, are His-152 to Glu-158, Val-159 to Glu-180, and Ala-162 to Glu-180.

It should also be pointed out that several peptides generated by endoproteinase Arg-C and *S. aureus* V8 protease appeared to result from cleavages at residues not usually considered to be primary sites for these proteases. In Fig. 1, these peptides are R-1, R-3, and S-2 and in all cases the identity of the residues at the cleavage site were verified from the sequences of overlapping peptides. The observation of anomalous cleavages, particularly for V8 protease, has been reported previously (8,9). This could be due to non-specific cleavage or to contamination with other proteases.

As seen in Fig. 2, porcine heart fumarase displays significant homology not only with human fumarase (96%), but also with yeast fumarase (67%), two bacterial fumarases (61% and 58%), and two bacterial aspartate ammonia lyases (41% and 38%). A total of 106 amino acids are conserved through all seven enzymes with 19 glycine residues representing the most conserved amino acid. Several highly conserved regions containing potential catalytically reactive residues can also be seen. These areas are Gln-98 to Asn-115, Lys-186 to Glu-203, Gly-231 to Asn-240, Lys-293 to Gly-308, and Gly-320 to Pro-330. One can speculate that the reactive amino acids contained within these conserved areas may possibly function at the active site.

Although there is currently no experimental information available on the tertiary structure of any of these enzymes, the predicted secondary structure of porcine fumarase shown in Fig. 3 indicates a large amount of alpha helix (42%) distributed throughout the protein, and little beta structure. None of the major conserved regions mentioned above occur in areas of predicted alpha helix.

As noted in the introduction, other laboratories (2-5) have suggested that there are two different isozymes of fumarase in eucaryotic cells, a mitochondrial and a cytoplasmic form. Although the precise primary structure of the putative cytoplasmic form of the enzyme has yet to be determined in any tissue, evidence so far indicates that the amino acid sequence of the two forms are nearly identical with the differences confined to the amino terminal region. O'Hare and Doonan (5) have reported that the mitochondrial fumarase from porcine liver has an amino terminal alanine residue while the cytosolic form of the enzyme has a glutamic acid or glutamine amino terminus. Amino terminal sequence analysis of porcine heart fumarase in this study indicated the presence of a second sequence at levels of about 10% in some preparations. This secondary sequence was identical to that of fumarase except that the first two amino acids were missing and the first residue was a glutamic acid instead of the expected glutamine (residue 3 of Fig. 1). It is also important to note that there was no indication of deamination of Gln-3 in the principal polypeptide chain. The heterogeneity, while possibly representing two pre-existing forms, can also be explained by a proteolytic cleavage and deamination of Gln-3 during the purification procedure. Our observations that the secondary sequence was not observed in all instances, and always at low levels, are insufficient to suggest that it is representative of a second, perhaps cytosolic isozyme of fumarase.

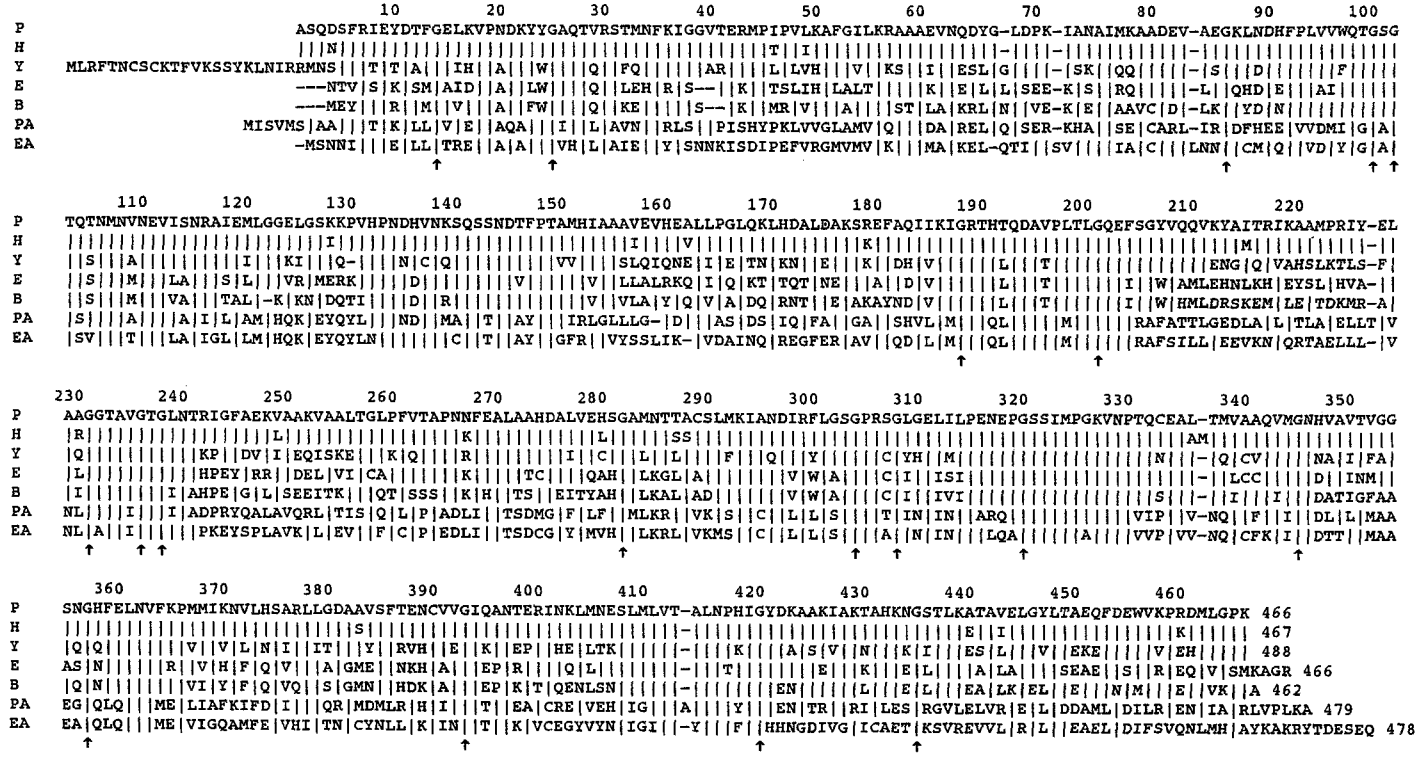

Figure 2: Amino acid sequence homology between porcine heart fumarase (**P**), human fumarase (**H**) (2), yeast fumarase from *Saccharomyces cerevisiae* (**Y**) (3), bacterial fumarase from *Escherichia coli* FumC gene (**E**) (10) and from *Bacillus subtilis* (**B**) (11), and bacterial aspartate ammonia-lyase from *Pseudomonas fluorescens* (**PA**) (12) and from *E. coli* (**EA**) (13). Vertical lines (|) denote homology with porcine fumarase sequence. Dashes (-) denote gaps (insertion or deletion sites) inserted to obtain the best alignment. Arrows (↑) denote the locations of glycine residues conserved in all family members sequenced to date. The numbers follow the porcine fumarase sequence. The homology of porcine fumarase to *B. subtilis* fumarase, and the two bacterial aspartate ammonia-lyases were found through the use of the FASTP data base search program (14). The alignments were refined using the PRTALN program(14).

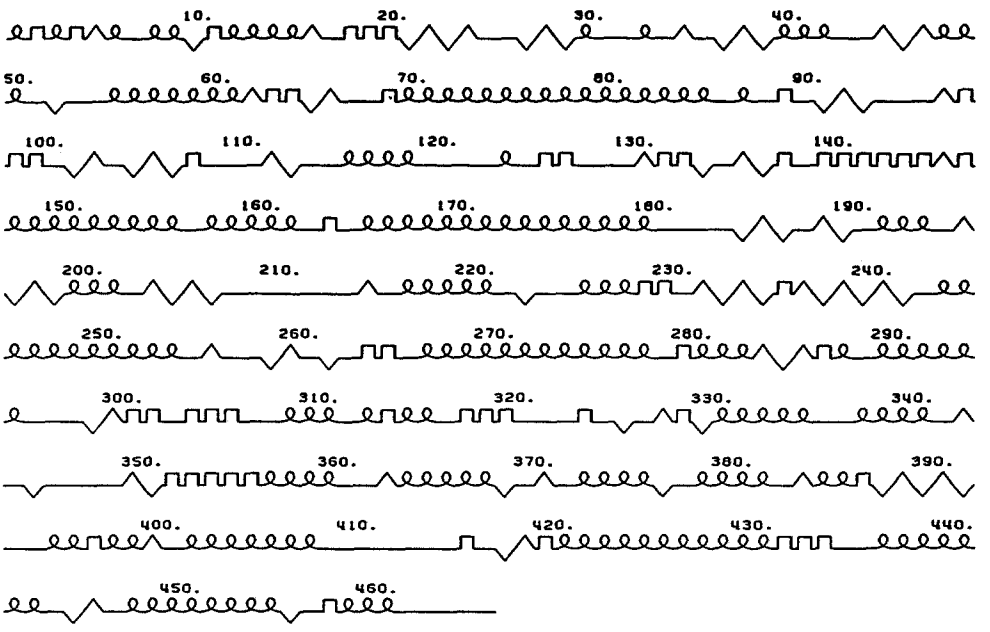

Figure 3: Secondary structure prediction of porcine heart fumarase using the Fasman secondary structure prediction program (15). The loops denote alpha helical regions, the slanted lines denote beta sheet segments, and the squares denote turn regions.

The determination of the amino acid sequence of porcine fumarase was aided significantly by the close similarity to the human enzyme. By extending the list of known primary structures of the hydratase family, highly conserved segments have been identified. In addition to the primary structure determination, the identification of the highly conserved regions should be useful in the X-ray diffraction studies.

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