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CHROMATOGRAPHIC DIFFERENTIATION OF THE MITOCHONDRIAL AND CYTOSOLIC FUMARASES OF BAKER'S YEAST

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ABSTRACT

A new method, designed to avoid the use of harsh conditions, has been developed for separation and purification of the isoenzymes of fumarase from pig liver. Peptide maps using high performance liquid chromatography (HPLC) of tryptic digests of the two isoenzymes had most of the peptides in common, but also showed a small number of differences. These results supported the hypothesis, advanced by O'hare and Doonan[1], that the isoenzymes differ in a small region of their N-termini but have identical C-terminal sequences. It proved not to be possible to confirm this hypothesis by direct N-terminal sequence analysis since the N-terminus of the cytosolic isoenzyme was found to be blocked. A similar result was obtained for the single enzyme from erythrocytes, a purification procedure for which was also developed.

Purification of the cytosolic and mitochondrial fumarases of yeast was also achieved. Unlike the mammalian isoenzymes, those from yeast were found to have different subunit molecular weights. Peptide mapping of digests of the two isoenzymes with trypsin and with S. aureus *protease was carried out. Sets of peptides obtained from the two isoenzymes were mostly identical as judged by retention times on HPLC but some significant differences were observed particularly in the digests with* S. aureus *protease. Amino acid compositions of the two isoenzymes from yeast were also very similar to those of the mammalian forms. It is argued that the yeast isoenzymes may prove to be the more useful pair for establishing the details of the structural relationships between cytosolic and mitochondrial fumarases.*

INTRODUCTION

Both pairs of mitochondrial and cytosolic isoenzymes appear to be coded by separate genes which may be structurally related as is the case for the aspartate aminotransferases or structurally unrelated as for the superoxide dismutases. The existence of a cytosolic fumarase was clearly demonstrated by Tolley[2] and Edwards[3] using cellulose acetate and starch gel electrophoresis. Two major band were observed, the faster moving band (lower pK) predominates in the cytosolic fraction whereas the slower (higher pK) form is localized in the mitochondrial preparations. The situation for the fumarases of animal tissues appears to be different. Genetic evidence[2,3] suggested that the isoenzymes from human tissues, although structurally different, are coded by the same gene. Chemical evidence to support this view was obtained in the case of the fumarases from pig liver by O'Hare and Doonan[1]. These authors reported the complete purification of the cytosolic and mitochondrial fumarases and the results of peptide mapping of the purified proteins. The peptide maps were almost identical but showed some small differences. The results were taken to show that the isoenzymes are identical over most of their structures but different in a small region, probably at the N-terminus. Final confirmation of this proposed site of difference could not be obtained by direct N-terminal sequence analysis because the terminus of the cytosolic isoenzyme appeared to be blocked. Similar results were obtained by Kobayashi and Tuboi[4] for the isoenzymes from rat liver.

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                          20                  40                  60
Hum   ASQNSFRIEYDTFGELKVPNDKYYGAQTVRSTMNFKIGGVTERMPTPVIKAFGILKRAAALVNQQYG-LD
FumC  ----  TV S K SM AID   A   LW    Q  LEH R S---   K    SL H  LALT     K  E L L S
CitC  ----  EY   R  M V    A   FW    Q  KE   C---S   K    MR V   A     ST LA KRL N

                 80                  100                 120
Hum   PKIANAIMKAADEV—AEGKLNDHFPLVVWQTGSGTQTNMNVNEVISNRAIEMLGGELGSKIPVHPNDHVN
FumC  EEK S  RQ      L -   QHD E   AI          S   M   LA   S L    VR MERK     D
CitG  VEK E  AAVC D  LK-    YD N               S   M   VA   TAL -K   KN DQTI    D

     140                 160                 180                 200
Hum   KSQSSNDTEPTAMHIAAAIEVHEVLLPGLQKLHDALDAKSKEFAQIIKIGRTHTQDAVPLTLGQEFSGYV
FunC        V       V  LLALRKQ I  Q KT TQT NE  RA   D V     L    T       I   W
CitG  R             V  VLA Y Q V  A DQ RNT  E A  AYND V     L    T       I   W

                220                 240                 260
Hum   QQVKYAMTRIKAAMPRIYELARGGTAVGTGLNTRIGFAEKLAAKVAALTGLPEVTAPNKFEALAAHDALV
FunC  AMLEHNLKH EYSL  HVA   L              HPEY RRV  DEL  VI  CA             TC
CitG  HMLDRSKEM LE TDKMRA  I       .  I  AHPE  G LVSEEITK     QT  SSS     H  TS  EIT

     280                 300                 320                 340
Hum   ELSGAMNTSSCSLMKIANDIRELGSGPRSGLGELILPENEPGSSIMPGKVNPTQCEAAMTMVAAQVMGNH
FunC  QAH  LKGLAA        V W A    C I   ISI                      -  L    LCC      D
CitG  YAH  LKALAAD       V W A    C I   IVI                    S -  L    I   I    D

                360                 380                 400
Hum   VAVTVGGSNGHFELNVFKPMMIKNVLHSARLLGDASVSFTENCVVGIQANTERINKLMNESLMLVTALNP
FumC    INM   AS  N       R  V H F Q V   A GME  NKH A   EP  R    Q L             T
CitG  ATIGFAA   Q  N         VI  Y F Q VQ  S  GMN  HDK A   EP  K  T QENLSN

     420                 440                 460
Hum   HIGYDKAAKIAKTAHKNGSTLKETAIELGYLTAEQFDEWVKPKDMLGPK
FumC          E   K     E L    AA LA    SEAE  S    R  EQ  V  SMKAGR
CitG       EN       L     E L     A LK EL   E    N  M    E    VK    A
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Fig.1 Alignment of the amino acid sequences of human mitcchondrial fumarase (Hum), the fumarase coded by the fumC gene of *E. coli* (FumC), the fumarase from *B. subtilis* (CitG). Analysis of the results shows that the eukaryotic fumarase and the prokaryotic enzyme share about 57-60% of identical amino acid sequence.

It is not as yet possible to propose a model for how these structurally distinct fumarases arise from a single gene. The two main possibilities are either that there is a single initial translation product from which the two proteins arise by a process of post-translational modification or that transcription of the gene gives rise to two different mRNA molecules each of which is translated to give a single isoenzyme. Preliminary evidence tends to support the second of these possibilities. Kinsella and Doonan[5] showed that cell-free translation systems programmed with total mRNA from rat liver produced two proteins immunoprecipitable with anti-fumarase antiserum, both of these putative precursors being larger than the native isoenzymes. Ono et al[6]. reported similar results except that they found one of the protein products to have the same size as the native isoenzymes as judged by SDS-polyacrylamide gel electrophoresis. The origin of the differences between these two reports is not yet clear.

In order to understand further the genetic and biosynthetic relationships between the fumarase isoenzymes we have embarked on an attempt to clone cDNAs for these proteins. To date a cDNA coding for the complete sequence of the mature mitochondrial isoenzyme from human liver has been obtained and its structure determined[7] but a corresponding cDNA for the cytosolic isoenzyme has yet to be isolated. Other observation of interest were made, however, concerning the relationships between eukaryotic mitochondria and prokaryotic fumarose. This was made possible by reports of the amino acid sequences of the fumarase from *B. Substilis*[8] and of one of the fumarase from *E. Coli*[9] obtained from DNA sequencing. These comparisons are shown in Fig 1.

As a separate approach to the problem of the structural and biosynthetic relationships between fumarases, it seemed to us to be worthwhile to investigate the situation in yeast, and in particular to establish whether in this organism as well as in animals, the two isoenzymes are coded by the same gene. The present paper reports the results of this work.

MATERIALS AND METHODS

Materials

Cake baker's yeast was obtained from the United Yeast Co. Hydroxylapatite was from Bio-Rad Laboratories; pyromellitic acid was from Kock Light; 1,3-diamino-2- hydroxypropanone, cyanogen bromide and1-cyclohexyl-3-(2-morpholino ethyl) carbodiimide metho-p-toluene sulphonate were from Aldrich; solvents for HPLC were from Rathburn; all other chemicals and enzymes were from Sigma. Pyromellitic acid-Sepharose 4B was prepared as described by Beeckmans and Kanarek[10]

Enzyme assays

Fumarase activity was determined spectrophotometrically. The assay solution consisted of 1.5 ml 100 mM sodium phosphate pH 7.3 and 1.5 ml 100 mM L-malate at 25°C. Reaction was started by the addition of enzyme and the increase of absorbance at 250 nm measured as a function of time. Activities were expressed as μmol product/min using a value of 1.45 $mM^{-1}\,cm^{-1}$ for the extinction coefficient of fumarate[11].

Protein concentrations were obtained from measurements of absorbance at 280 nm using A(1%) = 5.1[12].

Purification of yeast fumarases

Step 1. Cell lysis was carried out as described by De la Morena et al[13]. Cake yeast (1000 g) was treated with 250 ml 2 M ammonia for 18 hr at room temperature. The mixture was

then cooled to 4°C and 1200 ml 0.2 M EDTA pH 8.0 added, followed by ammonium sulphate to 48% saturation and stiring for 20 min. The suspension was centrifuged for 20 min at 5000 g.

Step 2. The supernatant from step 1 was cooled to 5°C and brought to 60% saturation with ammonium sulphate. Precipitated protein was collected by centrifugation for 40 min at 5000 g, dissolved in 10 mM sodium acetate buffer pH 5.8 containing 10 μM phenylmethyl sulphonyl fluoride (PMSF) and dialysed overnight against the same buffer.

Step 3. The dialysed protein solution was applied to a column (7 cmx15cm) of carboxymethyl cellulose CM23 equilibrated in the same acetate buffer and the column washed until the absorbance of the effluent was effectively zero at 280 nm. Fumarase activity was then eluted by application of a linear gradient from 0.06 to 0.10 M NaCl in starting buffer over a total volume of 4000 ml.

Step 4. Active fractions from step 3 were pooled and dialysed overnight against 20 mM potassium phosphate buffer pH7.0 containing 10 μM PMSF. The solution was applied to a column (2.0x10 cm) of hydroxylapatite equilibrated in the same buffer and the column was washed until no further protein was eluted. The fumarase isoenzymes were then separately eluted by the application of a linear gradient made from 400 ml each of 20 mM and 350 mM potassium phosphate buffer pH 7.0. Activity in the first peak was due to cytosolic fumarase and that in the second was due to mitochondrial isoenzyme.

Step 5. Active fractions containing the cytosolic isoenzyme were pooled and dialysed against 10 mM Tris-acetate buffer pH 7.0 containing 10 μM PMSF. The dialysed protein solution was applied to a column (2.5x25 cm) of pyromellitic acid-Sepharose 4B equilibrated in the same buffer and the column was washed until no more protein was eluted. Cytosolic fumarase was then eluted by application of a linear gradient of 0-3 mM fumaric acid in starting buffer over a total volume of 700 ml. Active fractions were pooled and transferred into 0.1 M ammonium bicarbonate using a column of Sephadex G25 equilibrated with that buffer. Finally the sample was dried by lyophilization.

Step 6. The mitochondrial fumarase was purified under identical conditions to those described in step 5 for the cytosolic form.

Proteolytic digestion of fumarases

Protein samples were oxidized with performic acid as described by Hirs[14] and dried by repeated lyophilization.

For trypic digestion the oxidized protein (200 μg) was dissolved in 0.2 M ammonium bicarbonate at about 2 mg/ml. Trypsin (1% w/w) was added and digestion allowed to proceed at 37°C for 3 hr. A further aliquot of trypsin was then added and digestion allowed to proceed for another 3 hr. The resulting solution of peptides was dried by lyophilization.

For digestion with the S. *aureus* proteinase, samples (200 μg) were dissolved in 0.1 M ammonium bicarbonate and proteinase added to 5% (w/w). Digestion was allowed to proceed for 18 hr at 37°C after which the peptides were dried by lyophilization.

Peptide mapping

Peptide mapping was by reversed phase HPLC using a Waters μ Bondapak C-18 column. Peptides were dissolved in 150 μl of 0.1 M ammonium bicarbonate for application to the column. Elution was with a linear gradient from 0 to 56% acetonitrile at a flow rate of 1 ml/min. Solvents used were HPLC grade water containing trifluoroacetic acid (TFA, 0.13% v/v/v) and 70% (v/v/v) acetonitrile in HPLC grade water containing 0.1% (v/v/v) TFA. Peptides were detected at 220 nm.

Other methods

Polyacrylamide gel electrophoresis in the presence of SDS was carried out by the method of Laemmli[15] using 15% gels. Standard molecular weight markers were from BDH. N-terminal analysis of proteins was done by using the dansyl method as described by Hartley[16].

RESULTS

Purification of the isoenzymes

The results of a representative purification of the cytosolic and mitochondrial fumarases are summarized in Table 1. After lysis of the cells with ammonia as described by De la Morena et al[13] and fractionation with amonium sulphate, a partial purification was obtained using carboxymethyl cellulose. The isoenzymes were eluted together form this column at about 0.08 NaCl. Separation of the isoenzymes was achieved using hydroxylapatite[15]. The elution profile (Fig 2) showed two peaks of fumarase activity; the first, eluted at about 120 mM phosphate buffer, contained the cytosolic fumarase and the second, at about 250 mM phosphate buffer, contained the mitochondrial isoenzyme. The two peaks were not completely resolved and to avoid cross-contamination only the first 65% of peak 1 and the last 40% of peak 2 were pooled for further purification. Final purification of the two isoenzymes was achieved by affinity chromatography using pyromellitic acid-Sephaose 4B[10]. The purification factor for the two isoenzymes was in excess of 1000 fold and a total yield of 22% of the original activity was obtained; the main loss of activity occurred at step 4 because fractions containing a mixture of isoenzymes were rejected. Both isoenzymes were pure as judged by electrophoresis in the presence of SDS (Fig 3.) On cellulose acetate electrophoresis at pH 8.8 both isoenzymes migrated towards the anode with the cytosolic form having the greater mobility (not shown).

Table 1 Purification of cytosolic and mitochondrial fumarases from yeast.

Step	Volume (ml)	Activity (u/ml)	Total Activity(u)	Protein (mg/ml)	Total Protein (mg)	Specific Activity (u/mg)	Recovery (%)
1	3370	2.3	7750	7.9	26,630.0	0.3	(100)
2	400	12.3	4920	17.1	68,40.0	0.7	63
3	760	5.4	4100	0.2	152.0	27.0	53
4a	130	5.4	700	0.2	26.0	27.0	9
4b	120	10.0	1200	0.2	24.0	50.0	15
5	17	30.5	520	0.08	1.4	380.0	7
6	20	56.0	1120	0.16	3.2	350.0	15

Steps were as described in "Materials and Methods". In step 4, a and b refer to the cytosolic and the mitochondrial isoenzymes respectively. Step 5 was for final purification of the cytosolic isoenzyme and step 6 for the mitochondrial form.

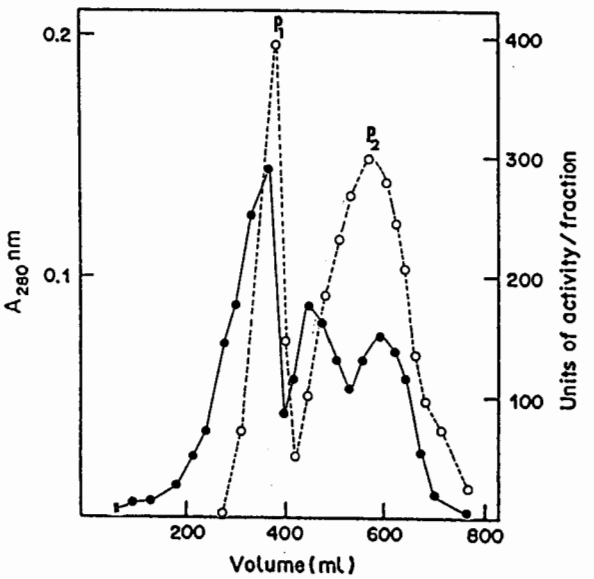

Fig.2 Separation of the cytosolic and mitochondrial fumarases by chromatography on hydroxylapatite. Traces show absorbance at 280 nm (●) and enzyme activity (O). Peak I contained the cytosolic isoenzyme and peak 2 contained the mitochondrial form.

Subunit molecular weights

Subunit molecular weights were estimated by using SDS-polyacrylamide gel electrophoresis in the presence of standard marker proteins (Fig 3). It is immediately apparent that the cytosolic isoenzyme (track 4) had a higher molecular weight than the mitochondrial form (track 3); estimated values were 53,000 and 48,000 respectively. The value for the mitochondrial isoenzyme compared very closely with those for the mitochondrial and cytosolic isoenzymes from pig liver (tracks 4 and respectively) and for the commercially available isoenzyme (presumed to be mitochondrial) from pig heart (track 2).

Peptide mapping of tryptic digests

Oxidized isoenzymes were digested with trypsin and the resulting peptide mixtures analysed by HPLC. Maps of the cytosolic [Fig. 4(a)] and of the mitochondrial [Fig. 4(b)] isoenzymes were almost identical but showed some differences at elution times between about 38 and 42 min [marked with arrows in Fig.4(a) and (b). To confirm the apparent identity of peaks eluting at identical positions in the chromatograms some peaks were collected and the contents subjected to quantitative amino acid analysis; compositions of material in corresponding peaks from the two chromatograms were the same (results not given in detail).

Peptide mapping of digests with S. aureus proteinase

Elution profiles from HPLC of digests of the oxidise cytosolic [fig.5(a)] and mitochondrial [Fig. 5(b)] fumarases were again very similar with the majority of the peaks in common. There was, however,a clear difference in that the map of the cytosolic isoenzyme contained a double peak at about 47 min whereas that of the mitochondrial form contained a single peak at that position, and the map of the mitochondrial isoenzyme contained a peak at about 15 min which was absent from that of the cytosolic form. Again material from corresponding peaks in the two profiles gave the same amino acid compositions.

Attempted N-terminal analysis

Both isoenzymes were subjected to N-terminal analysis by the dansyl method. In neither case was an indentifiable dansyl amino acid obtained.

DISCUSSION

We describe here the first reported purification of the cytosolic and mitochondrial fumarases from yeast. Central to the procedure was use of hydroxylapatite for separation of the isoenzymes. This technique was developed by Hiraga et al[17] who used it to separate the fumarases in crude yeast extracts; they did not, however, attempt further purification of the isoenzymes. An important extention of that work was the demonstration, using subcellular fractions from yeast, that the first isoenzyme eluted from the column (at 130 mM phosphate) was of cytosolic origin and the second (at 210 mM phosphate) was the mitochondrial form [17]. These results allow us to state with confidence that peak 2 of Fig. 2 contained cytosolic fumarase and peak 2 contained the mitochondrial isoenzyme. Final purification of the isoenzymes was achieved by using the established technique of affinity chromatography on pyromellitic acid-Sepharose[10].

The isoenzymes were pure as judged by polyacrylamide gel electrophoresis in the presence of SDS (Fig. 3), and of particular interest is the fact that the cytosolic isoenzyme migrated more slowly than the mitochondrial form; estimates of the subunit molecular weights for the two isoenzymes were 53,000 and 48,000 respectively. This is in marked contrast to the situation with the isoenzymes from pig liver (see also Fig. 3) and rat liver[6] where the two isoenzymes migrate identically on SDS-PAGE.

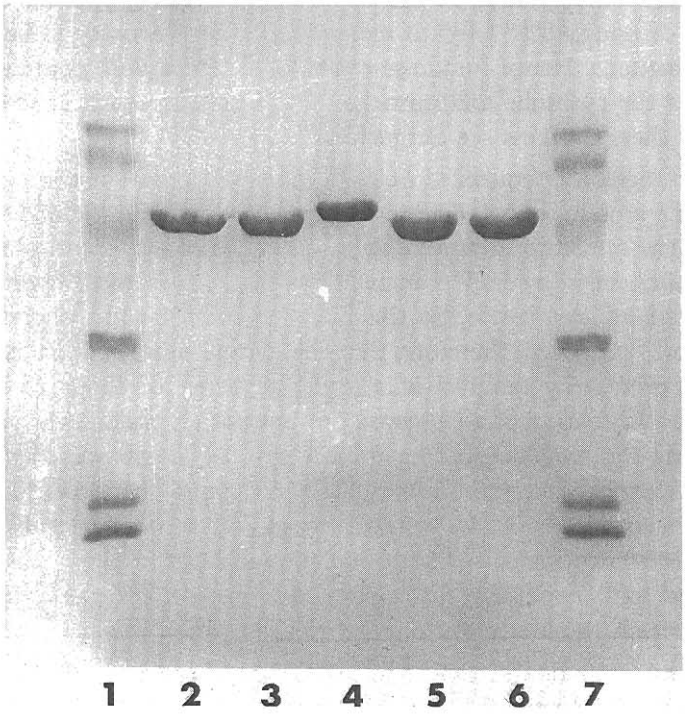

Fig.3 Polyacrylamide gel electrophoresis of fumarases in the presence of SDS. Tracks 1 and 7 contained the marker proteins ovotransferrin (77,000), serum albumin (66,250), ovalbumin (45,000), carbonic anhydrase (30,000), myoglobin(17,200) and cytochrome c (12,300). Positions of fumarase isoenzymes were as follows; pig heart mitochondrial, track 2; yeast mitochondrial, track 3; yeast cytosolic, track 4; pig liver mitochondrial, track 5; pig liver cytosolic, track 6.

As stated previously, both genetic evidence[3,4,18] and chemical evidence[1,4] has shown that mammalian fumarase isoenzymes are coded by the same gene. It was of interest to see if this situation pertained also with the yeast isoenzymes, particularly in view of the fact that the size relationships between mammalian fumarase and yeast fumarases are not the same.

To this end the yeast isoenzymes were digested with trypsin or with proteinase from *S. aureus* and peptide maps [Fig. 4(a) and (b)] it is immediately apparent that most of the peptides were in common; this was confirmed by amino acid analysis of the contents of selected peaks. Some small differences were observed at elution times around 38-42 min where the map of the cytosolic isoenzyme appeared to contain one or two peptides not present in the map of the mitochondrial form.

Even clearer results were obtained with digests produced by the proteinase from S.aureus [Fig. 5(a) and (b)]. In particular an early eluting peak in the map of the mitochondrial isoenzyme was missing from that of the cytosolic form. On the other hand a pair of partially resolved peaks eluting last in the map of the cytosolic isoenzyme was replaced by a single peak in the map of the mitochondrial form. All other peaks were in identical positions and the maps were entirely reproducible.

The results reported here show that the two isoenzymes of fumarase have most of their structures in common but that they also have significant differcences. This is consistent with, but does not prove, the hypothesis that the yeast fumarases are coded by the same gene.

Confirmation of this hypothesis has recently been provided by Wu and Tzagoloff[19]. They have cloned a gene (termed *FUM* 1) from yeast, mutation of which abolished both cytosolic and mitochondrial fumarase activities. Similarly, transformation of a fumarase mutant with a plasmid carrying *FUM* 1 led to increased fumarase activity in both the cytosolic and mitochondrial fractions. Hence fumarase can now be added with confidence to the short list of yeast proteins the cytosolic and mitochondrial forms of which arise from common genes (for a review of these proteins see Surguchov[20]).

Wu and Tzagoloff[19] sequenced *FUM 1* and showed that it contained an open reading frame coding for a protein with 488 amino acid residues. Residues 26-488 could be aligned with the sequence of the fumarase from *Bacillus subtilis*[8] with 56% of identical residues. The first 25 amino acid residues coded by *FUM 1* include 6 basic residues and 5 hydroxylated amino acids but no acidic residues, and hence has the characteristics of a mitochondrial leader sequence. Wu and Tzagoloff[19] proposed, therefore, that the mature mitochondrial isoenzyme is obtained from the larger protein by removal of a leader sequence of about 25 amino acid residues. Consistently, transformation of a fumarase mutant with a plasmid carrying a truncated form of *FUM 1* lacking the sequence coding for the first 17 amino acids led to accumulation of fumarase in the cytosolic fraction but very little in the mitochondria. In addition, residues 21-488 of the protein encoded by *FUM 1* can be aligned with the sequence of human mitochondrial fumarase[7] with 66% identity of structure (our unpublished results). The molecular weight of the putative yeast mitochondrial fumarase is also consistent with the value reported here based on SDS-PAGE within the limits of accuracy of the method.

There seems little doubt, then, that the open residing frame in *FUM 1* codes for the precursor of yeast mitochondrial fumarase from which the mature isoenzyme is obtained by proteolytic removal of an N-terminal peptide of about 20-25 amino acid residues. Our results can not establish the point of cleavage since it was not possible to identify the N-terminal residue of the mature protein by dansylation.

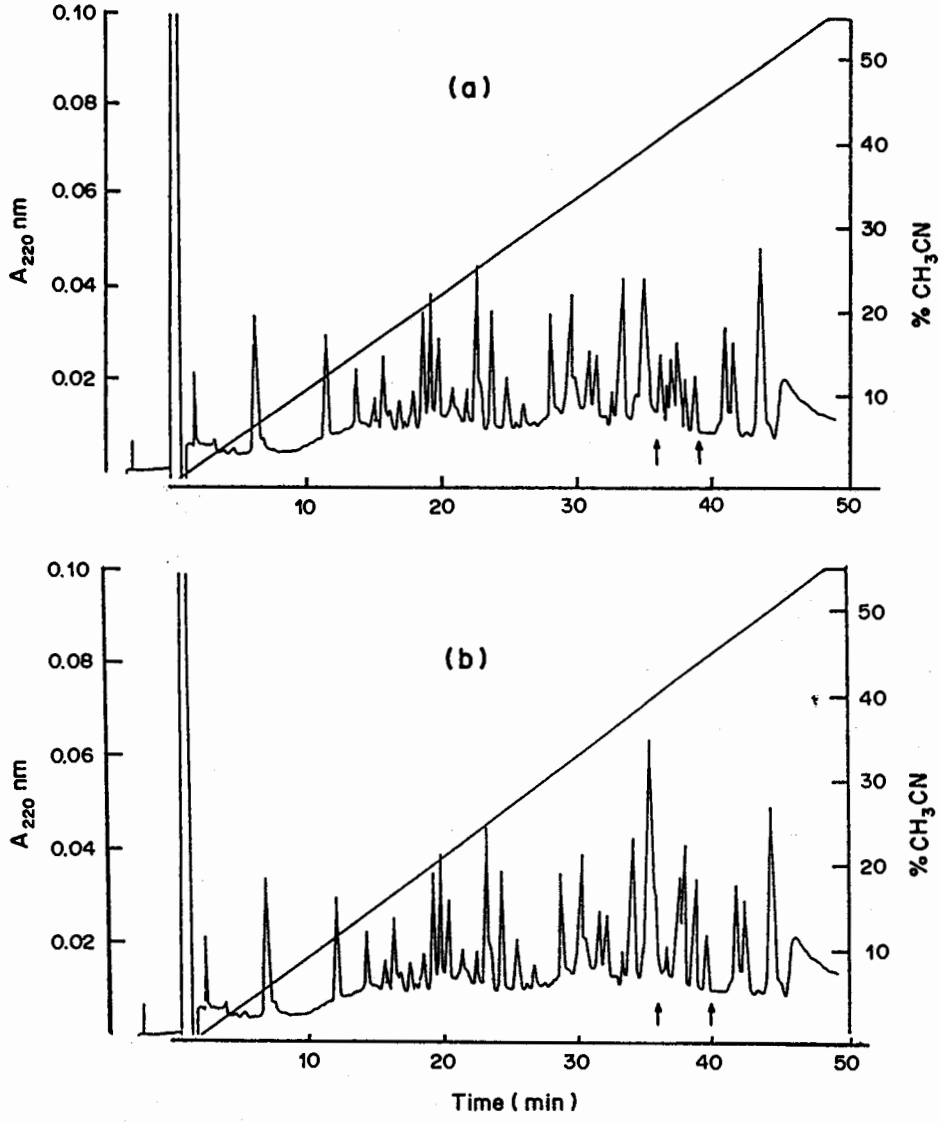

Fig.4 Peptide mapping of tryptic digests of yeast fumarases. Separation of peptides was by reverse phase HPLC on a Waters μ Bondapak C-18 column with a gradient from 0 to 56% acetonitrile (shown by the solid line). Detection of peptides was by absorption at 220 nm. Arrows mark the region of difference between the two maps. (a) Cytosolic isoenzyme; (b) Mitochondrial isoenzyme

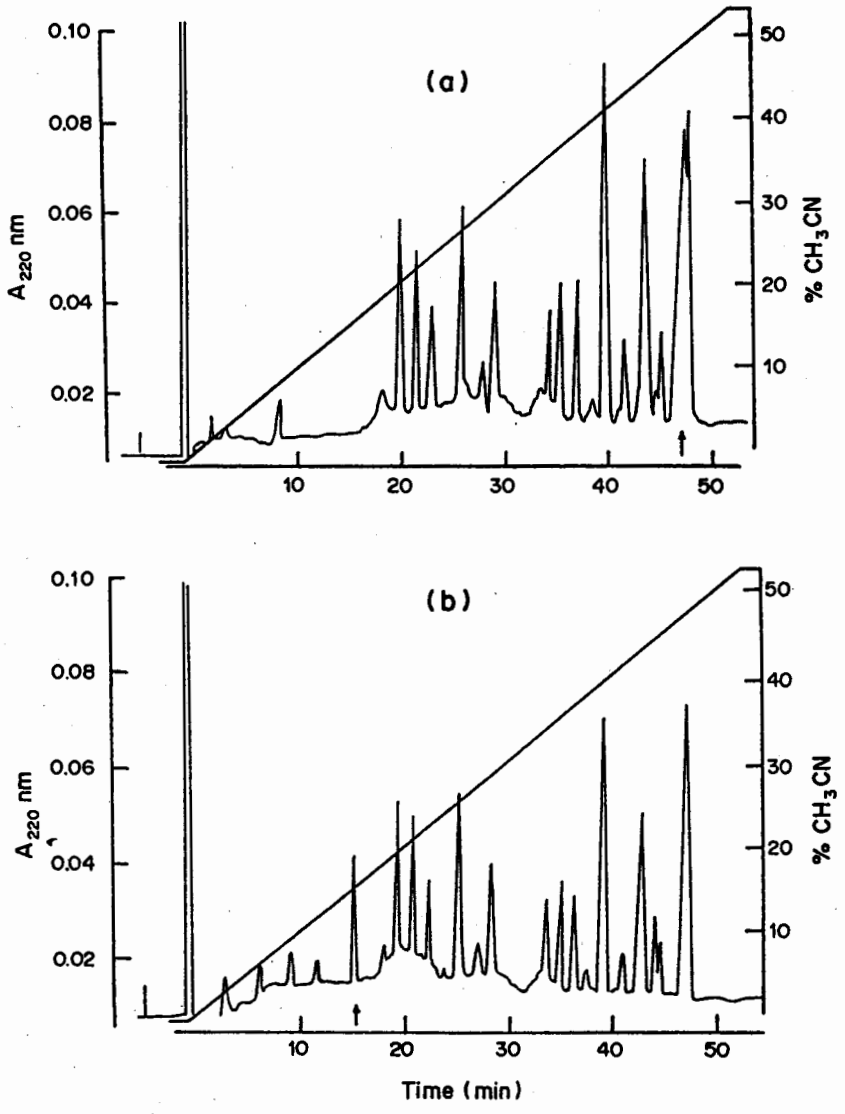

Fig.5 Peptide maps of digests of yeast fumarases with S. aureus proteinase. Conditions were as in (a) Fig 4. Cytosolic isoenzyme; (b) Mitochondrial isoenzyme.

The origin of the cytosolic isoenzyme is by no means clear. Wu and Tzagoloff[17] carried out S1 nuclease mapping of transcripts from the fumarase gene. In addition to a major transcript with a 5¢ terminus upstream of the first ATG initiation codon they found other transcripts with 5¢ termini 57-68 nucleotides downstream from the first ATG. They proposed that translation of these shorter transcripts, lacking the mitochondrial import signal, produces the cytosolic fumarase.

This is not consistent with the chemical evidence presented here. Initiation of translation from the first downstream ATG would yield a protein 23 amino acid residues shorter than the mitochondrial precursor and hence very similar in size to the mature mitochondrial protein. Our results (Fig 3) show, however, that the cytosolic isoenzyme is substantially larger than the mature mitochondrial form. It is not obvious from the sequence of *FUM 1* how such a larger protein arises and the origin of the cytosolic isoenzyme must still be considered unresolved. The solution to this problem will probably require N-terminal amino acid sequence analysis of the cytosolic fumarase. We have not attempted this since, given failure to detect an N-terminal residue for the protein by dansylation, we suspect that the N-terminus may be blocked.

SUMMARY

(1) A method is reported for the complete purification of cytosolic and mitochondrial fumarases from yeast. The method depends on use of chromatography on hydroxylapatite for separation of the isoenzymes[19].

(2) The subunit molecular weights of the isoenzymes measured by SDS-PAGE were 48,000 AND 53,000 for the mitochondrial and cytosolic forms respectively. This is in marked contrast to the situation with mammalian fumarase isoenzymes where the subunit molecular weights are both 48,000.

(3) The cytosolic and mitochondrial fumarases from yeast have most of their amino acid sequences in common as judged by mapping of peptides produced by trypsin or by *S. aureus* proteinase, but also show signification differences.

(4) These structural relationships are consistent with the hypothesis that the cytosolic and mitochondrial fumarases are coded by the same gene. This hypothesis has recently been confirmed by genetic studies reported by Wu and Tzagoloff[19].

(5) The gene isolated by Wu and Tzagoloff[19] codes for a protein of 488 amino acid residues which is thought to represent the precursor of the mitochondrial isoenzyme with a leader sequence of about 25 amino acids. It is not, however, obvious how the gene codes for the cytosolic isoenzyme given that, as shown here, this form has a greater subunit molecular weight. This problem remains to be resolved.

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