Purification, Characterization, and Immunological Properties of Fumarase from *Euglena gracilis* var. *bacillaris*

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A rapid three-step procedure utilizing heat treatment, ammonium sulfate fractionation, and affinity chromatography on Matrex gel Orange A purified fumarase (EC 4.2.1.2) 632-fold with an 18% yield from crude extracts of *Euglena gracilis* var. *bacillaris*. The apparent molecular weight of the native enzyme was 120,000 as determined by gel filtration on Sephacryl S-300. The preparation was over 95% pure, and the subunit molecular weight was 60,000 as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, indicating that the enzyme is a dimer composed of two identical subunits. The pH optimum for *E. gracilis* fumarase was 8.4. The Kₘ values for malate and fumarate were 1.4 and 0.031 mM, respectively. Preparative two-dimensional gel electrophoresis was used to further purify the enzyme for antibody production. On Ouchterlony double-immunodiffusion gels, the antifumarase serum gave a sharp precipitin line against total *E. gracilis* protein and purified *E. gracilis* fumarase. It did not cross-react with purified pig heart fumarase. On immunoblots of purified *E. gracilis* fumarase and crude cell extracts of *E. gracilis*, the antibody recognized a single polypeptide with a molecular weight of approximately 60,000, indicating that the antibody is monospecific. This polypeptide was found in *E. gracilis* mitochondria. The antibody cross-reacted with an *Escherichia coli* protein whose molecular weight was approximately 60,000, the reported molecular weight of the *fumA* gene product of *E. coli*, but it failed to cross-react with proteins found in crude mouse cell extracts, *Bacillus subtilis* extracts, or purified pig heart fumarase.

Exposure of dark-grown resting *Euglena gracilis* to light or a utilizable carbon source induces a transient increase in the specific activity of the mitochondrial enzyme fumarase (EC 4.2.1.2) (13, 14). Enzyme levels increase for 8 to 12 h after the addition of inducer and then decline (13, 14). Although in mammals fumarase is found in both the mitochondria and cytoplasm (16), it appears to be localized exclusively in the mitochondria of phototrophically grown *E. gracilis* species (12). To extend our studies of fumarase to the molecular level, it became necessary to purify *Euglena* fumarase to prepare antibodies to this protein.

Fumarase purified from pig heart (2, 9, 15), pig liver (2), rat liver (16), chicken heart (3), and *Ascaris suum* (23) is a tetramer composed of four identical subunits having a molecular weight of 48,000. A *Bacillus subtilis* gene, *citG* (18), and an *Escherichia coli* gene, *fumA* (8, 17), have been cloned based on their ability to restore fumarase activity to a fumarase-negative mutant of *E. coli*. The *citG* gene codes for a polypeptide with a molecular weight of 49,000 (18), whereas the *fumA* gene codes for a polypeptide with a molecular weight of 61,500 (8, 17). Although a 48,000-molecular-weight form of fumarase has been purified from a number of sources and the purified enzyme is relatively stable (15), initial attempts to purify *Euglena* fumarase by procedures successfully used in other systems were uniformly unsuccessful, presumably owing to the extreme lability of the partially purified enzyme. This instability suggested that the *Euglena* enzyme was significantly different from the fumarases which had been purified from other organisms. In this paper, we report the development of a rapid, three-step procedure for the purification of *Euglena* fumarase by using affinity chromatography on Matrex gel.

Orange A *E. gracilis* fumarase was found to be a dimer composed of two subunits with a molecular weight of 60,000. Immunologically, *E. gracilis* fumarase is related to an *E. coli* polypeptide with a molecular weight of approximately 60,000, the approximate molecular weight of the polypeptide coded by the *fumA* locus (8, 17). It is immunologically unrelated to vertebrate fumarases and *B. subtilis* fumarase, which have subunit molecular weights of 48,000 (3, 16, 18, 29). The catalytic properties of *E. gracilis* fumarase are, however, similar to those of other characterized fumarases. Because in contrast to other affinity purification procedures for fumarase, such as those which use pyromelatic acid Sepharose (2, 3), ATP Sepharose (3, 9), or 2-(5′-phenylpentyl) fumaric acid Sepharose (6), the Orange A matrix is stable, relatively inexpensive, and most importantly, commercially available, Matrex gel Orange A should be useful for the purification of fumarase from a variety of sources.

**MATERIALS AND METHODS**

**Cell growth.** *E. gracilis* Klebs var. *bacillaris* Cori maintained in the dark for many years was grown in the dark at 26°C on the pH 3.5 medium of Hutner (7). Cells were harvested at mid- to late logarithmic phase by using a Sorval TZ-28 continuous flow system, washed with buffer 1 (50 mM triethanolamine-HCl [pH 7.8], 10 mM malate, 5 mM EDTA, 10 mM 2-mercaptoethanol) containing 20% (vol/vol) glycerol and either used immediately or stored at −20°C. *E. gracilis* mitochondria were isolated from vitamin B₁₂-deficient cells by the trypsin digestion method (28; B. Gomez-Silva, E. Delorme, A. I. Stern, and J. A. Schiff, Plant Physiol., in press) and purified by Percoll density gradient centrifugation (Gomez-Silva et al., in press).

**Purification of fumarase.** Because all of the fumarase in *E. gracilis* appears to be found in the mitochondria (12), it was isolated directly from whole cells without prior isolation of
mitochondria. All operations were performed at 0 to 5°C. For the purification of E. gracilis fumarase, 50 g of cells was thawed, suspended in 70 ml of buffer 1, and disrupted by passage through a French pressure cell at 10,000 lb/in², and this lysate containing all of the fumarase activity was clarified by centrifugation at 27,000 × g for 30 min. This crude cell extract was heated to 50°C and maintained at that temperature for 3.5 min. Denatured protein was removed by centrifugation at 27,000 × g for 20 min, the pellet was washed once with buffer 1, and the pH of the pooled supernatants was adjusted to 7.8 with 2 M triethanolamine. The heat-treated extract was fractionated with ammonium sulfate, and the material precipitating between 40 and 60% saturation was dissolved in approximately 2 ml of buffer 2 (10 mM triethanolamine-HCl [pH 7.8], 1 mM EDTA, 10 mM 2-mercaptoethanol) and dialyzed against 2 liters of buffer 2 for at least 3 h. The enzyme as an ammonium sulfate precipitate could be stored for at least 2 weeks at −20°C without any loss of activity. After dialysis, insoluble material was removed by centrifugation, and NAD⁺, NADH, NADP⁺, and NADPH were added to the supernatant for a final concentration of 250 μM of each pyridine nucleotide. The extract (ca. 10 ml) was applied to a Mono Q 7.5 cm × 0.5 cm column equilibrated with buffer 2. The column was washed first with 100 ml of buffer 2 made 100 μM with respect to each pyridine nucleotide, 20 mM with respect to KCl, and then with buffer 2 (about 70 ml) until no more A₂₅₀ material was eluted. Fumarase was selectively eluted with 2.5 mM fumarate in buffer 2. Active fractions were pooled and concentrated on an Amicon PM 10 membrane. For the preparation of crude cell extracts from small amounts of cells, cells were sonicated in buffer 1 (13).

**Fumarase assay.** Fumarase was routinely assayed at 30°C in 2 ml of 50 mM potassium phosphate (pH 7.3)–50 mM malate containing 15 to 60 μM of enzyme by directly monitoring the production of fumarate as a change in absorption at 250 nm. With an extinction coefficient of fumarate of 1.45 mM⁻¹cm⁻¹ at 250 nm (1), 1 μU of enzyme activity is defined as the amount of enzyme catalyzing the formation of 1 μmol of fumarate per min. For phosphate and ATP inhibition studies and the determination of the Kₘ for malate, the assay was performed in 10 mM Tris-acetate (pH 7.3) at 30°C, and the malate concentration was varied from 0.025 to 2.5 mM. The Kₘ for fumarase was determined by monitoring the malate-dependent reduction of NAD⁺ by using malic enzyme (10). The reaction was performed in 2 ml of 25 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)-KOH (pH 7.5)–0.4 mM NAD⁺–4 mM MgCl₂–0.2 U of NAD⁺ malic enzyme–2.5 to 250 μM fumarate–53 mM of fumarase in a total volume of 2 ml. The pH dependence of fumarase formation was determined over the pH range of 5.6 to 8.8 by using 4-morpholineethanesulfonic acid-KOH (pH 5.6 to 6.8), HEPES-KOH (pH 6.8 to 8.4), and N,N,N-tris(hydroxymethyl)glycine-KOH (pH 8.0 to 8.8). For the determination of Kₘₚ, pH optima, and phosphate and ATP inhibition, a desalted ammonium sulfate-purified fraction was used. Protein concentration was determined by the dye-binding assay of Bradford (5), with bovine serum albumin (BSA) as a standard.

**Molecular weight determination.** Subunit molecular weight was determined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis on 10% gels (21), and the molecular weight was calculated from the relationship between log molecular weight and the relative mobility of the following molecular weight standards: phosphorylase B (92,500), BSA (68,000), ovalbumin (43,000), chymotrypsin (25,700), and lactoglobulin (18,400). Proteins were detected by staining with Coomassie blue R-250 (20) or silver (19, 20). Native molecular weight was determined with an ammonium sulfate-purified fraction by gel filtration through a column of Sephacryl S-300 (2.5 by 90 cm) equilibrated with 50 mM potassium phosphate (pH 7.4)–10 mM malate–1 mM EDTA–10 mM 2-mercaptoethanol. The eluent was monitored at 280 nm on an ISCO model UA-5 monitor, and 5.5-ml fractions were collected. The molecular weight of E. gracilis fumarase was calculated from the relationship between log molecular weight and the Kₘ of standard proteins calculated by the method of Reiland (25) by using the following molecular weight standards: ferritin (450,000), catalase (240,000), aldolase (158,000), BSA (68,000), chymotrypsinogen A (25,000), and cytochrome c (12,500). The void volume was determined with blue dextran, and vitamin B₂ was used to determine the total column volume.

**Immunological methods.** For antibody production, E. gracilis fumarase purified by Orange A chromatography was further purified by preparative nonequilibrium two-dimensional gel electrophoresis (22) with 3.2% (vol/vol) pH 3.5 to 10 and 10 amphotelins in the first dimension, and SDS-polyacrylamide gel in the second dimension. The gels were stained with Coomassie blue R-250, the fumarase subunit was excised from the gel, and the protein from several gels was eluted electrophoretically (31). The eluted protein was precipitated in the agarose gel with acetone, the gel slice containing the protein was placed in 1 ml of 136.9 mM NaCl–2.7 mM KCl–1.5 mM KH₂PO₄–10.6 mM Na₂HPO₄ (phosphate-buffered saline; PBS), and the agarose was melted with intermittent vortexing by incubation at 80 to 90°C over a 30-min period. The solubilized protein was mixed with an equal volume of Freund complete adjuvant, and subcutaneous injections of 100 to 150 μg were performed four times at 4-week intervals. The amount of protein injected per rabbit was 550 μg. One week after the last booster, serum was collected and stored at −20°C. Control serum was collected before the first injection. Ouchterlony double-immunodiffusion tests were run on plates containing 1% agar in PBS. Precipitin lines were visualized by using Coomassie blue R-250.

Immunoblots were performed by a modification of the procedure of Towbin et al. (29). Immediately after electrophoresis, the gel was equilibrated in 192 mM glycine–25 mM Tris (pH 8.3)–20% (vol/vol) methanol for 5 min. Polypeptides were electrophoretically transferred at constant voltage to a nitrocellulose membrane by using this buffer in a Hoeffer TE 52 Transfer unit over a 45-min period at 100 V. After transfer, unbound sites on the blot were blocked by incubation at 40°C for 1 h in a solution of 3% (wt/vol) BSA prepared in PBS. After being rinsed with five changes of PBS over a 30-min period, the blot was incubated in a sealed plastic bag for 2 h at 40°C with 20 ml of 3% (wt/vol) BSA in PBS containing 20 μl of rabbit antifumarase serum. The blot was then rinsed three times with PBS containing 0.1% (vol/vol) Triton X-100 and two times with PBS over a 30-min period, incubated at room temperature in a sealed plastic bag for 30 min with 20 ml of 3% (wt/vol) BSA in PBS containing 2 × 10⁷ cpm of ¹²⁵I-labeled protein A, rinsed with five changes of PBS containing 0.1% (vol/vol) Triton X-100–0.05% (wt/vol) SDS–0.1% (wt/vol) BSA over a 30-min period, and dried, and the antibody-antigen reaction was visualized by autoradiography by using Kodak X-OMAT XAR-5 film. ¹²⁵I-labeled protein A was prepared by using the glycoluril procedure of Smith and Hall (26). Purified protein A was suspended in 100 mM NaPO₄ (pH 7.5) for a final concentra
tion of 500 μg/ml. A 20-μl sample of this solution was radioiodinated with 1 mCi of 125I. The radioiodinated protein A solution was diluted with 1 ml of 100 mM NaPO4 (pH 7.5), dialyzed against three changes of PBS over a 3-day period, and stored at -20°C.

Enzyme-linked immunosorbent assays were performed in 96-well vinyl microtiter plates precoated overnight at room temperature with 50 μl of antigen suspended in PBS per well. The wells were blocked by filling with 2% BSA-0.02% sodium azide in PBS and incubated for 2 h at room temperature. The plates were washed three times with PBS, and then 50 μl of the diluted antiserum or control serum was added to each well. After incubation for 2 h at room temperature, the plates were washed three times with PBS containing 0.1% Nonidet P-40 and three times with PBS. To each well was added 50 μl of alkaline phosphatase-coupled goat anti-rabbit immunoglobulin G, which was diluted 400-fold with 2% BSA-0.05% Nonidet P-40-0.02% sodium azide in PBS. After incubation at 4°C overnight, the plates were again washed three times with PBS containing 0.1% Nonidet P-40 followed by three washes with PBS, and 200 μl of p-nitrophenyl phosphate solution (10 mg of substrate in 25 ml of phosphate reagent buffer) was added to each well. After the plates were incubated for 20 min, the reactions were measured spectrophotometrically at 405 nm by using a Titertek Multiskan MC 405. The results presented are the average of triplicate determinations and represent a typical experiment.

Materials. Matrex gel Orange A was purchased from the Amicon Corp., Lexington, Mass. Sephacryl S-300 and blue dextran were obtained from Pharmacia Fine Chemicals, Piscataway, N.J. The following were obtained from the Sigma Chemical Co., St. Louis, Mo.: NADP+, malic enzyme, alkaline phosphatase-coupled goat antibody against rabbit immunoglobulin G, protein A, and purified pig heart fumarase. Iodo-gen (1,3,4,6, tetrachloro-3a,6a-diphenylglycoluril) was obtained from Pierce Chemical Co., Rockford, Ill. 125I (IMS 30) was obtained from Amersham Corp., Arlington Heights, Ill. Molecular weight markers for gel filtration were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Molecular weight markers for polyacrylamide gel electrophoresis were purchased from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Nitrocellulose sheets were purchased from Bio-Rad Laboratories, Richmond, Calif. Coster microtiter plates were purchased from Belco Glass, Inc., Vineland, N.J.

RESULTS

Purification of fumarase. A rapid three-step purification procedure was developed for the purification of E. gracilis fumarase (Table 1). The greatest purification was obtained by affinity chromatography of the enzyme on a Matrex gel Orange A column (Fig. 1). A fivefold purified enzyme fraction was loaded onto an Orange A column in a buffer containing all four pyridine nucleotides to prevent the binding of pyridine nucleotide-require enzymes. When all of the unbound protein had passed through the column, the pyridine nucleotide concentration and ionic strength were lowered by washing the column with buffer lacking pyridine

<table>
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<th>TABLE 1. Purification of E. gracilis fumarase</th>
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<td>Fraction</td>
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<td>Crude cell extract*</td>
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<td>Ammonium sulfate precipitation</td>
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<td>Matrex gel Orange A column*</td>
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* Extract from 50 g of cells (wet weight).

* Fumarate (2.5 mM)-eluted fraction.
nucleotides and KCl. Fumarase was specifically eluted with 2.5 mM fumarate (Fig. 1). Further washing of the column with 10 mM fumarate eluted an additional 3.2% of the bound fumarase activity, whereas 2 M KCl eluted a large amount of protein but only 1.2% of the fumarase which had bound to the column. A significant amount of fumarase activity did not bind to the Orange A column (Fig. 1) under the conditions used. However, this activity did stick to and could be recovered from a second Orange A column, indicating that the column was overloaded. We found that, owing to the instability of purified \textit{E. gracilis} fumarase, higher specific activities were obtained if the Orange A column was overloaded. Malate could also be used to elute fumarase, but the fold purification achieved was lower, presumably because other malate-requiring enzymes were bound to the column. Because the ionic strength of the washing buffer was lowered before elution with fumarate, elution was not simply due to an increase in ionic strength. There is probably an interaction between the dye and the substrate-binding sites on the enzyme. A summary of a typical purification is shown in Table 1. From 50 g of \textit{E. gracilis} cells, 0.78 mg of fumarase was obtained with a 623-fold purification and an overall yield of 18.3%. The recovery of the unbound material is not included in the yield data of Table 1.

**Molecular weight of fumarase.** The progress of the purification, purity, and subunit molecular weight of \textit{E. gracilis} fumarase were determined by SDS-gel electrophoresis (Fig. 2). Many prominent bands were found in the crude cell extract (lane 1) and in the heat-treated ammonium sulfate fraction (lane 2). Over 95% of the protein eluted from the Orange A column with 2.5 mM fumarate migrated as a single band (lane 3), and a protein with identical mobility was the major protein eluted with 10 mM fumarate (lane 4). Many proteins were eluted by 2 M KCl (lane 5), indicating that although many proteins were bound to the column, a single protein was specifically eluted with fumarate. The mobility of \textit{E. gracilis} fumarase was distinctly lower than the mobility of pig heart fumarase (lanes 6 and 7). Based on its mobility relative to a series of molecular weight standards (data not shown), \textit{E. gracilis} fumarase has an apparent subunit molecular weight of 60,000, whereas the apparent subunit molecular weight of pig heart fumarase was 48,500, in agreement with reported values (2, 15). The native molecular weight of \textit{E. gracilis} fumarase was found to be 120,000 by gel filtration chromatography (data not shown), indicating that it is a dimeric enzyme. This is in contrast to vertebrate fumarase which is a tetrameric enzyme (15, 16).

**Stability of fumarase.** Attempts to use DEAE-cellulose chromatography, ATP Sepharose chromatography, Matrex gel Red A chromatography, and gel filtration to further purify the enzyme eluted with fumarate from the Orange A column were unsuccessful, as recoveries were low and the specific activity of the recovered enzyme was lower than that of the starting material. These results suggested that purified \textit{E. gracilis} fumarase was extremely unstable. As pig heart fumarase is cold labile (24, 30), the stability of purified \textit{E. gracilis} fumarase at 4 and 20°C was compared with the stability of the pig heart enzyme (Fig. 3). Orange A-purified \textit{E. gracilis} or pig heart fumarase was incubated at 4 or 20°C in a buffer containing 10 mM triethanolamine-HCl (pH 7.8), 10 mM malate, 1 mM EDTA, and 20 mM 2-mercaptoethanol, and after various times, the activity remaining was determined. Purified pig heart fumarase (7.5 U/ml; 26.8 μg of protein per ml) was relatively stable at both temperatures, losing only 10 to 20% of its activity over a 9-h period. In agreement with previous reports (24, 30), the pig heart enzyme was more stable at 20 than at 4°C. In contrast to pig
heart fumarase, purified *E. gracilis* fumarase was extremely unstable, but it was more stable at 4 than at 20°C. At an enzyme concentration of 7.7 U/ml (43.8 μg of protein per ml; Fig. 3), the half time for inactivation was 1.6 h at 20° and 4.6 h at 4°C. At a lower concentration (1.7 U/ml; 29.4 μg of protein per ml), the half time of inactivation was 1.2 h at 20°C and 4.8 h at 4°C (data not shown). The lability of purified *E. gracilis* fumarase contrasted with the stability of the enzyme in crude extracts or after ammonium sulfate fractionation in which the enzyme was stable at 4°C in the presence of malate for at least 48 h at a concentration of 0.7 U/ml. A concentrated preparation (390 U/ml; 905 μg of protein per ml) of purified *E. gracilis* fumarase lost 42% of its original activity after incubation at 4°C for 2.5 h, suggesting that the lability of the purified enzyme is not simply due to low amounts of protein.

**Kinetic properties of fumarase.** When measured in the direction of fumarate formation, *E. gracilis* fumarase had maximum activity at pH 8.4, whereas maximal activity for the pig heart enzyme was at a lower pH, 8.0. Pig heart fumarase is activated by phosphate anions at concentrations less than 5 mM but inhibited at higher concentrations (1). At a malate concentration of 0.1 mM, 5 mM phosphate produced a small (14%) but reproducible increase in the activity of *E. gracilis* fumarase, whereas at 50 mM phosphate there was a 60% inhibition of enzyme activity. At a malate concentration of 1 mM, 10 mM phosphate produced an 80% stimulation of fumarase activity, and although phosphate concentrations above 10 mM produced progressive decreases in enzyme activity, the reaction rate at 50 mM phosphate was 15% higher than the rate in the absence of phosphate. ATP is also an inhibitor of pig heart fumarase (24). In 10 mM Tris-acetate (pH 7.3) and at a malate concentration of 1 mM, 32.2 μU of *E. gracilis* fumarase was inhibited 50% by 0.35 mM ATP.

The *K_m* values of *E. gracilis* fumarase for malate dehydrogenation and for fumarate hydration were determined in Tris-acetate and HEPES buffer, respectively, to avoid the influence of phosphate on the kinetic constants. The *K_m* values were determined from double reciprocal plots (data not shown). The *K_m* for malate was 0.14 mM, which is comparable to the *K_m* of rat liver (16) and *A. suum* fumarase (23) but higher than the *K_m* of pig heart fumarase (11). The *K_m* of *E. gracilis* fumarase for fumarate was 0.031 mM, which is comparable to the *K_m* for *A. suum* fumarase (23) but higher than the *K_m* for the mammalian enzymes (11, 16).

**Immunological studies.** For the production of antibodies, the Orange A fraction was further purified by preparative nonequilibrium two-dimensional gel electrophoresis (22). The predominant polypeptide formed a single spot with a molecular weight of 60,000. This polypeptide was electroeluted from the gel (31) and used to produce antiserum in rabbits. On an Ouchterlony double-immunodiffusion gel, a precipitin reaction was not observed with control serum (data not shown), whereas a single fused precipitin band was formed between both the antiserum and purified *E. gracilis* fumarase and the antiserum and a crude cell extract from *E. gracilis* (Fig. 4). This indicates that the crude cell extract contained only a single protein species which reacts with the antifumarase serum. Thus, the antibody appears to be monospecific. A cross-reaction could not be detected against pig heart fumarase and BSA (Fig. 4), providing further evidence for the specificity of the antibody.

The antifumarase serum was further characterized by the more sensitive technique of immunoblotting (Fig. 5). The antifumarase serum reacted with a single protein of 60,000 molecular weight (lane 2), which corresponds to the major stainable protein present in preparations of purified *E. gracilis* fumarase (lane 1). The antifumarase antibody reacted only with the major stainable protein when Orange A-purified fumarase was analyzed by nonequilibrium two-dimensional gel electrophoresis (data not shown). Extracts of *E. gracilis* total cellular protein (lane 3) also contained a

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**FIG. 4.** Ouchterlony double-immunodiffusion analysis of antifumarase serum. Wells: 1, *E. gracilis* crude cell extract (20 μl containing 30 U of fumarase per ml); 2 and 5, Orange A-purified *E. gracilis* fumarase (5 μg); 3, purified pig heart fumarase (6.3 μg); 4, *E. gracilis* fumarase eluted from a nonequilibrium two-dimensional gel (2.4 μg); 6, BSA (10 μg). Well A contains rabbit antiserum against *E. gracilis* fumarase which was purified by preparative nonequilibrium two-dimensional gel electrophoresis.

**FIG. 5.** Immunoblot analysis of antifumarase serum. Proteins were separated by SDS-polyacrylamide gel electrophoresis on 10% gels and electrophoretically transferred to nitrocellulose membranes. The membranes were incubated with antifumarase serum, and the antibody-antigen reaction was detected by using 125I-protein A. Lanes: 1, silver-stained polyacrylamide gel loaded with purified *E. gracilis* fumarase (3.0 μg); 2 to 8, autoradiographs of immunoblots containing purified *E. gracilis* fumarase (0.5 μg; lane 2), crude cell extract of *E. gracilis* fumarase (20.0 μg; lane 3), crude cell extract of mouse cell line L5178y (20 μg; lane 4), crude cell extract of *B. subtilis* (20 μg; lane 5), *E. gracilis* mitochondrial protein (10 μg; lane 6), purified pig heart fumarase (20 μg; lane 7), and crude cell extract of *E. coli* (20 μg; lane 8).
60,000-molecular-weight protein which was the only protein recognized by the antifumarase serum, providing further evidence that the antibody is monospecific. This 60,000-molecular-weight protein is the major mitochondrial protein which was recognized by the antifumarase serum, showing that the protein recognized by the antibody is a mitochondrial protein. The weakly reacting lower-molecular-weight proteins sometimes found in mitochondrial preparations probably represent proteolytic breakdown products of fumarase which are formed during mitochondrial isolation. The fumA gene of E. coli codes for a polypeptide that has a molecular weight of 61,500 (8, 17), and the antibody to E. gracilis fumarase reacted with an E. coli protein whose molecular weight was slightly larger than that of E. gracilis fumarase (lane 8). B. subtilis (18) and vertebrate (2, 15, 16) fumarases have an approximate molecular weight of 48,000. Antibody to E. gracilis fumarase failed to cross-react with purified pig heart fumarase (lane 7) and total cell protein extracted from B. subtilis (lane 5) or mouse cell line L5178y (lane 4).

The extent to which antibody to E. gracilis fumarase cross-reacts with E. coli protein was estimated by using a sensitive enzyme-linked immunosorbent assay. When crude cell extracts of E. gracilis or E. coli containing equal amounts of fumarase activity (250 mU per well) were incubated with antibody, the reactivity of the E. gracilis extract was about 4.5-fold greater than the reactivity of the E. coli extract. As expected, BSA and pig heart fumarase exhibited no cross-reaction. It appears that E. gracilis and E. coli fumarase are immunologically similar but not identical.

DISCUSSION

Studies of fumarase from a variety of organisms have identified two physically distinct forms of this ubiquitous enzyme. B. subtilis (18), A. suam (23), and vertebrate fumarases (2, 15, 16) have a subunit molecular weight of approximately 48,000, and at least in the latter two cases, the native enzyme is a tetramer. The subunit molecular weight of the fumA gene product of E. coli (8, 17) and E. gracilis fumarase is approximately 60,000, and in the case of E. gracilis, the native enzyme is a dimer. Antibody to pig heart fumarase cross-reacts with fumarase from chickens (3), a distantly related species. Antibody to E. gracilis fumarase cross-reacted with an E. coli protein, presumably the fumA gene product, whereas it failed to cross-react with the enzymes from mouse cells, B. subtilis, or pig heart, enzymes whose subunit molecular weight is 48,000 (2, 15, 16, 18).

Thus, the limited immunological data suggests that the two physically distinct forms of eucaryotic fumarase may have arisen independently from distinct procaryotic ancestors and that structural features of the ancestral forms have been conserved in modern procaryotes.

The structural differences between E. gracilis fumarase and other fumarases are not reflected in the catalytic properties of the enzyme. As found for all other fumarases studied (1, 24), E. gracilis fumarase is regulated by phosphate and by ATP. The K_m value of E. gracilis fumarase for fumarate was similar to those of the other fumarases which have been studied. The K_m value of E. gracilis fumarase for malate was similar to the reported values for rat liver (16) and A. suam fumarase (23), which are an order of magnitude larger than for the pig heart enzyme (11). Because fumarate specifically elutes fumarase from the Orange A column owing to a specific enzyme-substrate interaction, this column should provide a convenient method for the rapid purification of fumarase from a variety of sources.

Under appropriate conditions, the tetrameric pig heart fumarase can be dissociated into its subunits (27, 32) and then reassocitated into the active enzyme (32). Dissociation produces a significant structural change, as evidenced by a loss of most of the alpha helical structure of the polypeptide (32), a significant increase in the trypsin susceptibility of the subunits relative to the tetrameric active enzyme (32), the exposure to the environment of all of the sulfhydryl groups which are normally hidden in a hydrophobic environment in the tetrameric enzyme (27, 32), and a complete loss of all of the antigenic determinants present in the native enzyme (3). Thus, although antibody to native pig heart fumarase cross-reacts with the chicken heart enzyme, the antibody does not cross-react with denatured pig heart fumarase (3). The antibody to E. gracilis fumarase was made against the denatured enzyme, and it failed to react with native E. gracilis and E. coli fumarases as determined by immunoprecipitation of fumarase activity by using protein A containing Staphylococcus aureus to precipitate the antigen-antibody complexes (unpublished data). This contrasts with the positive enzyme-linked immunosorbent and immunodiffusion assays which used antigen which had not been denatured with SDS, suggesting that the antibody does in fact react with the native enzyme. Because E. gracilis fumarase is extremely labile when partially purified or in the absence of substrate, it is not unreasonable to think that the enzyme becomes denatured during the 3 days required for the immunodiffusion assay or when absorbed overnight at room temperature onto the surface of a microtiter plate. Thus, although a native enzyme was used as antigen for the enzyme-linked immunosorbent and immunodiffusion assays, the enzyme had probably been denatured during the assay so that the antibody was in fact recognizing a denatured rather than a native antigen. Taken together with the structural and immunological studies of pig heart fumarase, the reaction of antifumarase serum with denatured but not native enzyme suggests that the conformation of native E. gracilis fumarase is significantly different from the conformation of the denatured subunit, so that different antigenic determinants are exposed on the two proteins.

Reassociation of fumarase subunits into an active tetrameric enzyme proceeds through a number of distinct steps (32). The unfolded subunits are thought to refold and assemble into an inactive dimer which in turn can be converted into an active tetramer by incubation at 25°C (32). The cold lability of mammalian fumarase (16, 24, 30) may represent the dissociation of the active tetramer into the inactive dimer. E. gracilis fumarase was inactivated more rapidly at 20 than at 4°C, indicating that although it is extremely unstable, it can not be considered cold lable. The rapid inactivation of E. gracilis fumarase is probably due to a conformational change, dissociation of the active dimer, or both. It has been shown that fumarase can specifically associate with malate dehydrogenase, and it has been proposed that this association is found in vivo (4). The stability of E. gracilis fumarase in crude cell extracts and the marked instability of the purified enzyme could in fact be due to an interaction between fumarase and another enzyme. Purification of fumarase would remove the stabilizing protein, the dimer would rapidly dissociate, and enzyme activity would be lost. The characterization of fumarase from a wide range of organisms is necessary to determine whether the dimeric form of fumarase is inherently less stable than the tetrameric form.
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LITERATURE CITED


