Subunit Structure of Inosinic Acid Dehydrogenase from *Escherichia coli*†

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ABSTRACT: Enzymatically active inosinic acid dehydrogenase from *Escherichia coli* is an oligomorphic protein and is derived from two polypeptides of approximately 62,000 and 44,000 molecular weight. These subunits have very similar electrophoretic properties and when separated from each other are enzymatically inactive. Aggregates are enzymatically active with similar specific activities. The smallest enzymatically active species has a molecular weight of 249,000 as determined by gel filtration on Sepharose. The aggregated forms may be disassociated by 8 M urea and 6 M guanidine-HCl. There is some reaggregation after removal of urea.

Inosinic acid dehydrogenase catalyzes the oxidation of inosinic acid to xanthylic acid. The enzyme requires NAD^+ as a cofactor and is activated by potassium and glutathione (Magasanik *et al.*, 1957). Purified inosinic acid dehydrogenase from *Escherichia coli* can be separated into several distinct but enzymatically equivalent fractions by centrifugation or electrophoresis (Powell *et al.*, 1969). Centrifugation of the purified enzyme in a Spinco Model E ultracentrifuge indicated an $s_{20,w}$ value of 12.8 S with a broad shoulder suggesting an associating and disassociating pattern. The disassociating pattern of inosinic acid dehydrogenase was more apparent after centrifugation in a sucrose density gradient (Powell *et al.*, 1969), which indicated at least two distinct fractions. Heterogeneity of even a greater degree was present after electrophoresis of the enzyme on acrylamide gel (Powell *et al.*, 1966), which showed at least six protein bands. To further evaluate the observed heterogeneity the present studies were carried out. The results indicate that heterogeneity of inosinic acid dehydrogenase occurs because of the presence of multiple aggregate forms of two highly acidic polypeptides of approximately 62,000 and 44,000 molecular weight.

Materials and Methods

The materials used in this study were obtained from the following sources. Sodium salts of NAD, GMP, and IMP, guanine and reduced glutathione were obtained from P-L Biochemicals; Tris, glycine, protamine sulfate, phenazine methyl sulfate, and nitro blue tetrazolium from Sigma; urea from Baker; ammonium persulfate and guanidine-HCl from Mann; acrylamide and bisacrylamide from Eastman and Sephadex G-100 and G-200 and Sepharose 6B from Pharmarcia. Haptoglobin β chain, haptoglobin α-1 chain, and haptoglobin 1-1 were gifts from Drs. D. Barnett and M. Rasco.

Purification. A modification of the procedure of Powell *et al.* (1969) was used for purification of inosinic acid dehydrogenase. Growth of the *E. coli*, sonic disruption, protamine sulfate precipitation, differential heat denaturation, and ammonium sulfate precipitation were carried out as previously described (Powell *et al.*, 1969). The ammonium sulfate precipitate was resuspended in 2.5 ml of 0.05 M Tris-HCl (pH 8.0) and chromatographed on a Sephadex G-200 column (83 × 2.5 cm). The enzyme was eluted in 4.6-ml fractions with 0.05 M Tris-HCl (pH 8.0) at a rate of 0.1 ml/min. Except where indicated the studies reported were performed on the enzyme sample from the G-200 column.

Polyacrylamide Gel Electrophoresis. Analytical electrophoresis in 5% polyacrylamide gels was carried out in a continuous buffer system as described by Davis (1964). Optical scanning of the gels was performed using a linear transport attachment on a Gilford 240 spectrophotometer with a linear recorder.

Staining of Disc Gels. Protein staining was accomplished using 1% Buffalo Black in 15% alcohol or 0.01% Coomassie Blue in methanol–acetic acid. Enzymatic staining on the gel was accomplished by coupling the enzyme reaction with phenazine methyl sulfate and precipitating nitro blue tetrazolium at 37°. A control without inosinic acid was included in the enzyme reaction.

Assay of Enzyme Activity. IMP dehydrogenase activity was quantitated by measuring the increase in optical density at 340 mμ caused by the formation of NADH in a 1-ml assay mixture containing 1.0 mM IMP, 1.0 mM NAD, 0.04 M KCl, and 0.05 M Tris-HCl (pH 8.0). A unit of enzyme activity is defined as the quantity of enzyme required to produce an increase in optical density of 1.0/min per cm of the light path at 25°.

Molecular Weight Determination. Molecular weight determinations were carried out by gel filtration on a Sepharose 6B column (2.5 × 91 cm) in 0.05 M Tris-HCl (pH 8.0) at a flow rate of 2.8 ml/hr per cm^2. The column was calibrated with human serum albumin (mol wt 69,000), human γ-globulin (mol wt 160,000), and haptoglobin 1-1 dimer (mol wt 200,000). The void volume was determined with Blue Dextran 2000.

Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was utilized for estimating the subunit molecular weight (Shapiro *et al.*, 1967). Samples of human albumin, human

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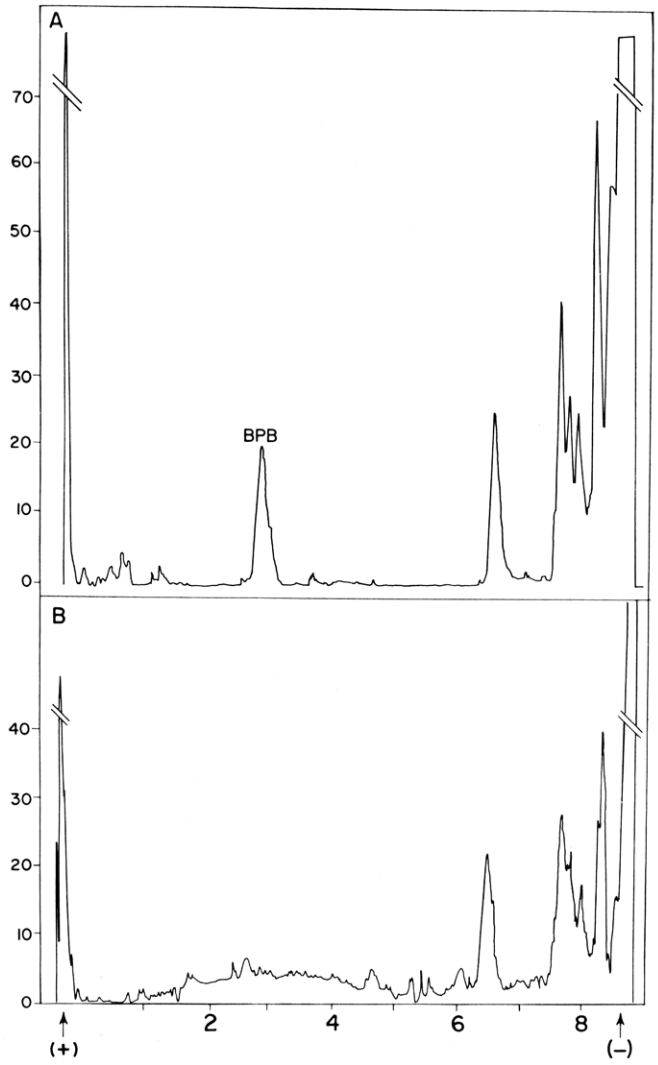

FIGURE 1: Scan of acrylamide gel pattern for specific activity. (A) The gel stained for enzyme activity was scanned at 600 mμ. (B) The enzyme on the unstained gel was scanned at 280 mμ. Bromo Phenol Blue was used as a marker.

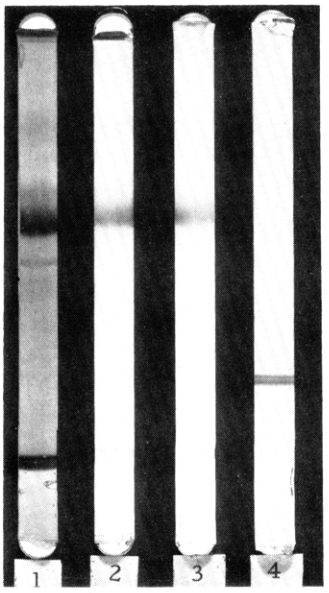

FIGURE 2: Acrylamide gels after exposure of inosinic acid dehydrogenase to 4 M urea. Gel 1: protein stain; gel 2: enzyme stain of the same protein sample as gel 1. The two lower bands give a reddish color unlike that of the usual enzyme stain. This color is independent of NAD^+ or IMP. Gel 3: enzyme stain after cutting the predominantly active band from a gel, removal of the protein from the gel by electrophoresis, reelectrophoresis, and enzyme staining; gel 4: protein stain of the two lower bands from gel 1 after removal of the protein from the gel by electrophoresis and reelectrophoresis on another gel. (Gel 4 was not electrophoresed as long as the other gels.)

IgG, bovine fibrinogen (mol wt 330,000), and haptoglobin 1-1 (mol wt 100,000) were used as reference proteins. Vertical electrophoresis was performed for 6 hr at 95 mA in 5% slab acrylamide gel containing 0.1% sodium dodecyl sulfate and 0.1 M sodium phosphate buffer (pH 7.2) (Weber and Osborn, 1969). Gels were stained for 16 hr with Coomassie Brilliant Blue and destained by diffusion into 7% acetic acid.

Molecular weight determinations of the subunits were also carried out by gel filtration chromatography with Sephadex G-200 (2.5 × 62 cm), 5 M guanidine-HCl and 0.05 M Tris-HCl (pH 7.75). The G-200 had been sieved to obtain particles of 53–74 μ in diameter. The column was calibrated with the α-1 chain of haptoglobin (mol wt 9000), chymotrypsinogen A (mol wt 25,000), the β chain of haptoglobin (mol wt 40,000), human serum albumin (mol wt 69,000), and haptoglobin 1-1 (mol wt 100,000). The enzyme was eluted by upward flow at a rate of 0.7 ml/hr per cm^2 and collected in 2.8-ml fractions.

Results

Activity Pattern on Acrylamide Gel after Various Procedures. To determine whether the heterogeneity of the purified enzyme was produced by the various isolation procedures, protein samples from several steps of purification were subjected to polyacrylamide gel electrophoresis and stained for enzymatic activity. The heterogeneous pattern was present in the crude sonicate and was not affected by the various steps in purification, by concentration or dilution of the enzyme or by the addition of IMP, NAD, or GMP to the enzyme sample and electrophoresis buffer. The use of riboflavin or ammonium persulfate as a catalyst for polymerization of the gels also had no effect.

Specific Activity of the Enzyme Bands. An aliquot of enzyme from the G-200 column was subjected to electrophoresis on 5% acrylamide gels. One gel was stained for enzymatic activity while another was fixed in acetic acid. The pattern of activity staining is demonstrated in Figure 4. The unstained gel was scanned at 280 mμ (Figure 1B) and the enzyme stained gel was scanned at 600 mμ (Figure 1A). All 280-mμ-absorbing bands had enzymatic activity. The specific activity varied from 1.2 for the most highly acidic band to 1.7 for the band with the least mobility.

Effects of Dissociating Agents on Enzyme Aggregation and Activity. UREA. The extent of dissociation of the enzyme was dependent upon the concentration of urea and time of exposure. Limited exposure (1–3 hr) to 4 and 8 M urea resulted in the appearance of a protein which migrated approximately half way toward the anode and which exhibited the majority of the enzyme activity (Figure 2). The remaining enzyme activity resided in proteins which migrated only slightly into the gel, a pattern typical of the enzyme before treatment with urea (Figure 4). In addition to the active proteins, two highly acidic, enzymatically inactive proteins were observed (Figure 2-1). These two bands were removed from the gel by electrophoresis

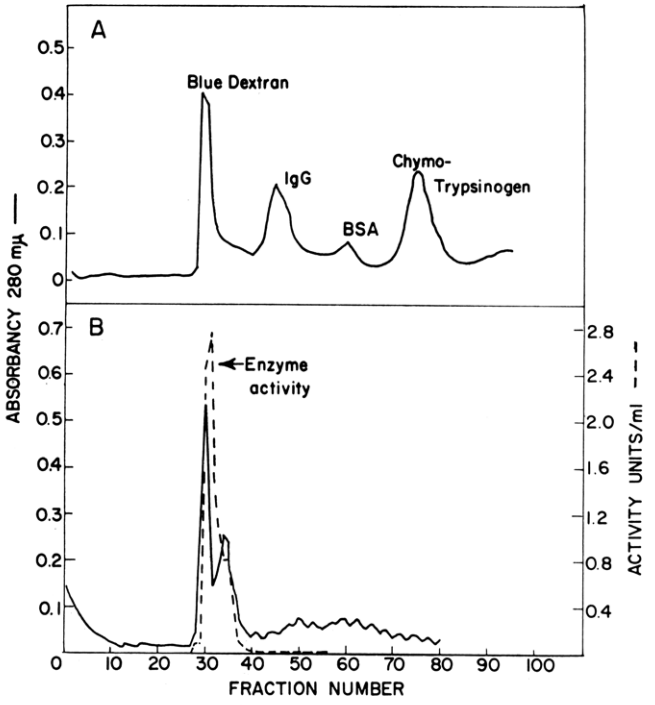

FIGURE 3: Sephadex G-200 elution pattern of inosinic acid dehydrogenase. The enzyme was eluted with 0.05 M Tris-HCl (pH 8.0).

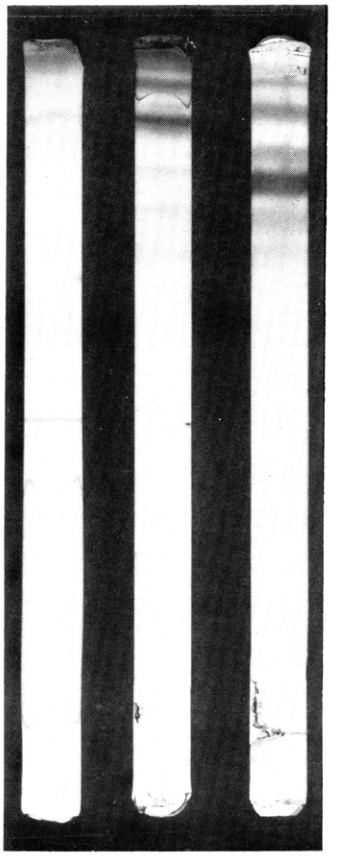

FIGURE 4: Acrylamide gel enzyme stain of inosinic acid dehydrogenase from Sephadex G-200 fractions. Left, fraction 24; center, fraction 29; right, fraction 24; the lower two bands in fraction 24 are not enzymatically active. The reddish color of the two lower bands is independent of NAD^+ or IMP.

and assayed together spectrophotometrically. The specific activity of the mixture was 1.4% of the specific activity of the major aggregate which was removed from the gel and assayed in a similar manner. The two acidic proteins could not be separated from each other sufficiently to test activity individually.

These two inactive proteins were the only ones visible after extensive dialysis of the enzyme against 8 M urea. Reaggregation of the two inactive proteins was accomplished by exhaustive dialysis against 0.05 M Tris-HCl (pH 8.0). Gel electrophoresis of this preparation indicated that the two proteins had partially reassociated into a protein which migrated to the same position as the major enzymatically active protein observed after limited exposure to urea. In this case, however, the protein did not exhibit enzymatic activity.

6 M GUANIDINE. The enzyme was examined by gel electrophoresis after extensive dialysis against 6 M guanidine-HCl (pH 8.0). Two enzymatically inactive protein bands were observed which migrated to the same positions as those seen after exhaustive treatment of the enzyme with 8 M urea. Attempts at reassociation of the proteins by dialysis against 0.05 M Tris-HCl (pH 8.0) were unsuccessful.

Enzyme Pattern on Acrylamide Gel after Sephadex G-200 Filtration. The elution profile of inosinic acid dehydrogenase from a Sephadex G-200 column is seen in Figure 3. Two protein peaks can be observed (3-B), with both peaks displaying enzymatic activity. Two peaks were frequently seen when the column was equilibrated with 0.05 M Tris-HCl or potassium phosphate buffer. Only one broad peak was seen with either increased or decreased buffer molarity. The molecular weight appeared to vary inversely with the salt concentration. But only in columns of 0.05 M Tris-HCl or potassium phosphate buffer did the protein solution elute as two distinct peaks.

Aliquots from fractions 24, 29, and 34 from the Sephadex G-200 column shown in Figure 3 were subjected to electrophoresis on polyacrylamide gels with the resulting pattern of enzyme activity shown in Figure 4. The protein eluted first from the column barely penetrated the gel and showed only a small amount of enzyme activity. Fraction 29 appeared to consist of a number of enzymatically active aggregates, many of which migrated further into the gel. In the last fraction eluted from the column, none of the material remained at the origin and the greatest amount of enzyme activity resided in a more anodal protein. Each of these fractions was dialyzed against 6 M guanidine-HCl (pH 8.0) and then against 0.05 M Tris-HCl (pH 8.0). Electrophoresis showed that the proteins had dissociated into two acidic, inactive proteins (see Figures 2–4). Furthermore, when the three samples were mixed together after guanidine treatment, only two protein bands could be seen on electrophoresis, indicating that the various active forms of the enzyme are composed of the same two highly acidic polypeptides.

Estimation of the Molecular Weight. Estimation of the molecular weight of inosinic acid dehydrogenase and its subunits was accomplished using gel filtration procedures (Whitaker, 1963; Andrews, 1964). The expected linear relationship between V_e/V_0 and log of the molecular weight of the proteins used for calibration was obtained both on Sepharose 6B in 0.05 M Tris-HCl (pH 8.0) and on Sephadex G-200 in 5 M guanidine-HCl in 0.05 M Tris-HCl (pH 7.72). The calculated molecular weight of the enzyme from the Sepharose column was 249,000 ± 12,450. Gel filtration of the two inactive subunits on the Sephadex G-200 column in guanidine

indicated molecular weights of 62,000 ± 3100 and 40,000 ± 2000.

The molecular weights of the two inactive proteins were further estimated by electrophoresis in sodium dodecyl sulfate acrylamide gel after exposure of the enzyme to various dissociating, reducing and alkylating agents. Figure 5 shows the migration of the subunits on a sodium dodecyl sulfate polyacrylamide slab gel after exposure to the following agents: (1) 1% sodium dodecyl sulfate; (2) 1% sodium dodecyl sulfate and 0.1 M mercaptoethanol; (3) 1% sodium dodecyl sulfate and urea; (4) 1% urea and 1% sodium dodecyl sulfate; (5) 1% sodium dodecyl sulfate and dithiothreitol; (6) 1% sodium dodecyl sulfate, dithiothreitol, and iodoacetamide. It is apparent that the enzyme does not dissociate into smaller subunits under any of these conditions. The molecular weights of the two smallest subunits of inosinic acid dehydrogenase was estimated to be 60,600 and 43,800 based on their migration distance compared to that of proteins of known molecular weight (Shapiro *et al.*, 1967).

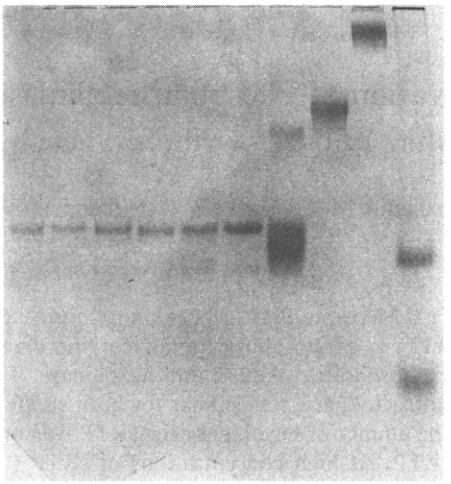

FIGURE 5: Sodium dodecyl sulfate slab acrylamide gel pattern of inosinic acid dehydrogenase and calibrating protein markers. From left or right slots one through six contain inosinic acid dehydrogenase exposed respectively to 1% sodium dodecyl sulfate, 1% sodium dodecyl sulfate and 0.1 M mercaptoethanol, 1% sodium dodecyl sulfate and urea, 1% urea and 1% sodium dodecyl sulfate with 1-hr incubation, 1% sodium dodecyl sulfate and dithiothreitol and 1% sodium dodecyl sulfate, dithiothreitol and iodoacetamide. Slots 7–10 contain human serum albumin, human γ-globulin, fibrinogen, and haptoblobin all with mercaptoethanol. Vertical electrophoresis was performed for 6 hr at 95 mA in 5% slab acrylamide gel containing 0.1% sodium dodecyl sulfate and 0.1 M sodium phosphate buffer (pH 7.2).

Discussion

Enzymatically active inosinic acid dehydrogenase from *E. coli* is an oligomorphic protein derived from two subunits of approximately 62,000 and 44,000 molecular weight. When the subunits are separated from each other by electrophoresis on polyacrylamide gel they are enzymatically inactive as judged by the enzyme-staining procedure. When assayed together spectrophotometrically, however, they exhibit low but distinctly measureable activity. After extensive dialysis against Tris-HCl buffer, the subunits dissociated by 8 M urea demonstrate some reaggregation into an enzymatically inactive form. Subunits prepared by treatment with 6 M guanidine-HCl could not be reassociated even after extensive dialysis against Tris-HCl buffer. Reduction and alkylation of the subunits did not change the migration pattern on sodium dodecyl sulfate polyacrylamide gel, indicating that these subunits each consist of a single polypeptide chain rather than multiple chains held together by disulfide bonds.

The higher molecular weight proteins all exhibit similar specific activities. They result from aggregation involving forces other than covalent linkages since they are easily dissociated by treatment with urea or guanidine-HCl. Furthermore, the degree of aggregation is affected by the molarity of the buffer, suggesting that salt linkages are an important force involved in aggregation. The smallest active form of the enzyme observed has a molecular weight of approximately 249,000. Denaturation resulted in the appearance of two inactive subunits, suggesting that the heterogeneity of the enzyme observed on polyacrylamide gels represents aggregate forms of the enzyme which are all composed of the same two polypeptide subunits. From the observed molecular weights it is conceivable that the smallest active form of the enzyme consists of four subunits, two of each of the two different polypeptide subunits. Further studies will be necessary to determine the precise composition of the smallest active form of the enzyme and to learn how additional subunits are added to it.

The molecular weight of inosinic acid dehydrogenase from *E. coli* varies inversely with the salt concentration as does this same enzyme from *Aerobacter aerogenes* (Brox and Hampton, 1970). The active enzyme from this later organism also has two molecular species but, in contrast to *E. coli*, after exposure to 3 M urea only one inactive species of approximately 100,000 molecular weight is seen on acrylamide gel and on ultracentrifugation.

The subunits differ in molecular weight and in electrophoretic mobility. This would suggest that there are two structural genes for inosinic acid dehydrogenase in *E. coli*.

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