Nicotinamide Adenine Dinucleotide Dependent Isocitrate Dehydrogenase from Beef Heart: Subunit Heterogeneity and Enzyme Dissociation†

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ABSTRACT: Heterogeneity in the subunits of nicotinamide adenine dinucleotide dependent isocitrate dehydrogenase from beef heart mitochondria was investigated using one- and two-dimensional electrophoretic analyses in polyacrylamide gels. Electrophoresis under nondenaturing conditions, at several values of pH and gel concentration, followed by second-dimension electrophoresis in the presence of sodium dodecyl sulfate showed that the active enzyme contains four different subunits. The details of these two-dimensional patterns, re-electrophoresis of the active enzyme band under nondenaturing conditions, together with additional evidence indicate that under certain nondenaturing conditions the enzyme exists partially dissociated into its subunits. The molecular weights of the four subunits, determined from electrophoretic mobilities obtained in the presence of sodium dodecyl sulfate, were different, varying between 39 000 and 41 300. Tryptic peptide maps of the subunits are substantially different.

NAD-dependent isocitrate dehydrogenase from beef heart has previously been shown to contain apparently identical subunits of molecular weight approximately 41 000 (Giorgio et al., 1970). The active species formed by these subunits varies with temperature and pH, i.e., under certain conditions consisting of an octamer and under other conditions a tetramer (Fan et al., 1975).

The activity of the enzyme is modified by a number of nucleotides. NADH, NADPH, and ATP are inhibitory, while ADP stimulates activity (Chen & Plaut, 1963). Binding studies indicate separate sites for NADH and NADPH, while ATP shares a site with NADH (Harvey et al., 1972). Separate sites appear to exist for ADP (Fan, 1975, Harvey et al., 1972).

The several different types of effector sites indicated by these studies suggested the existence of nonidentical subunits for the enzyme. The following study was initiated to investigate this possibility.

Materials and Methods

Chemicals. Acrylamide, bisacrylamide,[1] Temed, ammonium persulfate, and $NaDodSO_4$ were obtained from Bio-Rad Laboratories. Guanidine hydrochloride was obtained from Sigma Chemical Co. and urea (ultrapure) from Schwarz/Mann. Fluorescamine (Fluram) was obtained from Roche Diagnostics. Trypsin-TPCK was from Worthington Biochemical Corp. Amberlite MB-3 was from Mallinckrodt Chemical Works and cellulose-coated plates were from Brinkmann Instruments. Other reagents were reagent grade or better.

Assays. Protein and enzyme activity were determined as described by Plaut (1969). One unit of enzyme activity is defined as the amount of enzyme which catalyzes the formation of 1 μmol of NADH/min at 24 °C.

Enzyme Preparation. The enzyme was prepared using a procedure similar to that of Giorgio et al. (1970) with the following major modifications. The heat step was replaced by gel chromatography on Sepharose 6B in the presence of 10% saturated ammonium sulfate and 0.1 mM ADP, and the final negative ammonium sulfate fractionation was carried out at 4 °C in a column using the procedure of King (1972). The purification procedure is described in detail by Rushbrook (1978). The enzyme was obtained from the negative ammonium sulfate fractionation step in approximately 40% saturated ammonium sulfate solution at approximately 2 mg/mL protein and was stored at −60 °C. The specific activities of preparations used in the study varied between 29.5 and 38.0 units/mg.

Electrophoresis under Nondenaturing Conditions. Electrophoresis was carried out in cylindrical polyacrylamide gels at 10 °C using a continuous (Hjerten et al., 1965) buffer system. Ionic strength and osmolarity were held constant as the pH was varied. At pH 8.4, the buffer was 0.05 M Tris-acetate; at pH 7.4, the buffer was 0.033 M Tris-acetate, 0.017 M sucrose. Mercaptoacetic acid (0.01 M) comprised part of the anion content of the buffer for one preelectrophoresis and in some cases for the electrophoresis. pH values given are those at the temperature of the experiment.

Gels (1.8 mL/tube) were prepared by mixing concentrated Tris buffer (no mercaptoacetic acid), 30% acrylamide, 0.8% bisacrylamide (w/v) stock solution, and ammonium persulfate solution (to give a final concentration of 0.07%) and adding Temed (3 μL/12.5 mL) to initiate the gelation reaction. Gels were preelectrophoresed for 1 h at 4 mA/tube with buffer containing mercaptoacetic acid in order to remove unreacted persulfate and immobilized free radicals formed during the gel-polymerization reaction (Peterson, 1972). A second preelectrophoresis was carried out with buffer lacking mercaptoacetic acid to remove mercaptoacetic acid from the gels.

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[1] Abbreviations used: $NaDodSO_4$, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; bisacrylamide, *N,N'*-methylenebisacrylamide; Temed, *N,N,N',N'*-tetramethylethylenediamine; EDTA, ethylenediaminetetraacetic acid; NAD, nicotinamide adenine dinucleotide; NADH, reduced NAD; NADPH, NADH phosphate.

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Enzyme solution [stock enzyme solution (1.9–2.3 mg/mL) diluted 1:10 in electrode buffer containing 0.01 M mercaptoacetic acid and 5% additional sucrose and dialyzed for 4–7 h at 10 °C against 500 volumes of the same buffer] was then layered onto each gel (40 μL/gel). Electrophoresis was carried out initially at 1 mA/tube for 30 min and then at 3 mA/tube with buffer circulation from 2.7 to 4.2 h according to experimental conditions. Buffer circulation was stopped while the mercaptoacetic acid originally present in the sample solution eluted into the lower buffer chamber, and electrophoresis was stopped briefly while this buffer solution was replaced. Unless indicated, mercaptoacetic acid was absent during electrophoresis. In experiments where mercaptoacetic acid was present during electrophoresis, the second preelectrophoresis and the manipulations during electrophoresis were omitted.

Gels were stained for protein by immersing each gel for 16 h with agitation in 40 mL of a freshly prepared solution containing 20% acetic acid, 20% methanol, 0.03% Coomassie brilliant blue R and were destained by diffusion in 7.5% glacial acetic acid, 5% *tert*-butyl alcohol. Gels were stained for activity essentially as described by Giorgio et al. (1970). The activity staining patterns shown were obtained only when mercaptoacetic acid was absent from the gels. In its presence it was possible to obtain staining of all protein bands in the gel.

Gels stained for protein were scanned using a Gilford spectrophotometer Model 240 with the linear-transport attachment, 0.05-mm slit width, at 580 nm.

Reelectrophoresis of the Active Band under Nondenaturing Conditions. Enzyme dialyzed for 21 h at 10 °C against 0.05 M Tris–acetate, 0.01 M mercaptoacetic acid (pH 8.4) was electrophoresed as described above at 3 mA/tube for 2 h in the same buffer. Control gels were then stained for protein. One gel was stained for activity, and the main active band obtained was used as a guide to cut two unstained gels just in front of the active band. The lower pieces of the cut gels were stained for protein. The upper gel pieces containing the active band were inserted into tubes containing gels lacking protein which had been through the previous preelectrophoresis and electrophoresis. One of the composite gels was then electrophoresed immediately and the other after incubation at 10 °C for 20 h. After staining for protein, all gels were scanned and mobility values relative to the active band calculated. The original position of the active band in the piece of gel applied to the second-stage gel was the starting point for the calculation of relative mobility values for the reelectrophoresis.

Electrophoresis in the Presence of 8 M Urea. Electrophoresis was carried out in cylindrical polyacrylamide gels at 16 °C for 4.2 h at 3 mA/tube. The buffer system, gels, and procedure were identical to those described for electrophoresis under nondenaturing conditions at pH 8.4 with mercaptoacetic acid present in the prerun and run, except that 8 M urea, deionized with Amberlite MB-3, was present in gels and in electrode buffer. Stock enzyme was diluted 1:10 in electrode buffer containing 5% sucrose and dialyzed 6.5 h against the same buffer prior to electrophoresis.

Electrophoresis in the Presence of $NaDodSO_4$. Electrophoresis of the enzyme was carried out directly, and as the second part of a two-dimensional analysis, in $NaDodSO_4$–polyacrylamide slab gels prepared after the procedures of King & Laemmli (1971) and Studier (1973) with minor modifications (Rushbrook, 1978).

For direct application to the gel, stock enzyme solution was diluted 1:10 or 1:20 into sample buffer, made 5% in mercaptoethanol, held in a boiling water bath for 3 min and then dialyzed against sample buffer containing 0.01 M mercaptoethanol. Prior to application to the gel, the solution was again made 5% in mercaptoethanol and held in a boiling water bath for 3 min. Protein was applied at 0.5–4 μg/well. The procedure for the second-dimension electrophoresis of cylindricals gels, electrophoresed in the first dimension under nondenaturing conditions or in the presence of 8 M urea, was modeled after that of O'Farrell (1975). Following electrophoresis in the first dimension, the gels were cut longitudinally into three pieces and frozen. Prior to second dimension electrophoresis, the central gel slice was incubated at room temperature with gentle agitation for 30–40 min in sample buffer containing 5% mercaptoethanol.

Electrophoresis was carried out at room temperature in 15 × 18 cm gels containing a 10% acrylamide separating gel. Slabs 1.5-mm-thick were used when one-dimensional electrophoresis alone was being carried out and 3-mm-thick slabs for two-dimensional electrophoresis. Careful attention to the pH of the various buffers used was essential for the resolution of the four main components of the enzyme preparation. For 1.5-mm-thick slabs, a constant current of 20 mA was applied until the Bromophenol blue dye reached the stacking gel–separating gel interface, at which time the current was raised to 30 mA and electrophoresis continued until the Bromophenol blue dye approached the separating gel–plug gel interface. Slightly higher currents (40 mA followed by 50 mA) could be used for the thicker slab. Gels were stained for approximately 1 h in 0.25% Coomassie brilliant blue R in 20% trichloroacetic acid and destained in 7.5% acetic acid, 5% *tert*-butyl alcohol.

Slab gels were scanned with a Joyce-Loebl microdensitometer 3CS with 575-nm filter and integrator attachment.

Molecular weights in this $NaDodSO_4$ system were calculated according to Shapiro et al. (1967) from duplicate determinations in the same gel. The protein subunits used as standards and their molecular weight values were: bovine pancreas α-chymotrypsinogen A, 23 650; bovine erythrocyte carbonic anhydrase, 30 000; ovalbumin, 43 500 (Smith, 1970); rabbit muscle glyceraldehyde-3-phosphate dehydrogenase, 37 000; bovine liver catalase, 57 500 (Klotz & Darnall, 1970); bovine serum albumin, 66 210 (Peters, 1975); horse liver alcohol dehydrogenase, 40 000 (Jörnvall & Harris, 1970).

Preparation of Tryptic Peptide Maps. Tryptic peptide maps were prepared from the four major components of the enzyme preparation obtained by electrophoresis in $NaDodSO_4$–slab gels. Enzyme solution (approximately 40 μg per well, 0.51 mg of protein total) was applied to 3-mm-thick $NaDodSO_4$–slab gels and electrophoresis carried out as previously described for 1.5 times the period of time required for the Bromophenol blue dye to pass the lower edge of the separating gel. After 10-min staining and destaining periods, the individual bands were cut out and bands containing the same component combined.

Protein in the bands was eluted electrophoretically into a solution containing 0.05 M Tris-Cl, 0.5% $NaDodSO_4$, 0.5% mercaptoethanol (pH 8.0) and precipitated by the addition of 10 volumes of a cold acidified acetone solution (1:40, 1 N HCl–acetone) (Stolzfus & Reuckert, 1972) followed by incubation overnight at −20 °C. Following centrifugation at 8700*g* for 4.5 h at 0 °C, the precipitates were taken up in 1.65 mL of 6 M guanidine hydrochloride, 0.1 M Tris-Cl, 0.002 M EDTA (pH 8.1).

Reduction and alkylation were carried out by a combination of the procedures of Weber et al. (1972) and Hirs (1967) (Rushbrook, 1978). Proteolytic digestion was carried out using 5 μg of TPCK–trypsin per modified subunit dissolved in 3.25 mL of 0.1 M NH_4HCO_3 solution. This amount of TPCK–trypsin did not produce spots on the final peptide maps. Following digestion at 37 °C for 8 h, the samples were acidified

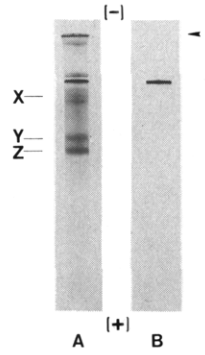

FIGURE 1: Electrophoresis of the enzyme under nondenaturing conditions. Enzyme (specific activity 29.5 units/mg) was electrophoresed at 10 °C (pH 8.4) in 8.5% polyacrylamide gels. Gels were stained for protein (A) or enzyme activity (B). X, Y, and Z identify certain nonactive bands migrating faster than the active band. The arrowhead indicates the top of the gel stained for activity. Electrophoresis was toward the anode.

to pH 4.5 with acetic acid and lyophilyzed three times, and the precipitate was taken up in 35 μL of water. The digests were then applied to 10 × 10 cm cellulose plates, prechromatographed with water–acetic acid–pyridine–1-butanol (302:76:378:144), and chromatography was carried out with the same buffer. Electrophoresis was carried out at 450 V for 50 min in water–acetic acid–pyridine–1-butanol (900:25:25:50) (pH 4.7). The plates were then treated successively with acetone, 1% triethylamine in acetone (freshly prepared), and 0.03% fluorescamine in acetone and photographed under UV light on Panatomic X film using a Nikon Y44 filter. Further details of experimental procedures are described by Rushbrook (1978).

Results

Two-Dimensional Electrophoretic Analysis. Heterogeneity in the subunits of isocitrate dehydrogenase from beef heart mitochondria was investigated by two-dimensional electrophoresis in polyacrylamide gels. Electrophoresis in the first dimension was carried out under nondenaturing conditions and in the second dimension in the presence of $NaDodSO_4$.

Electrophoresis in the first dimension alone (pH 8.4, 10 °C, 8.5% gel) produced the protein and activity staining patterns shown in Figure 1. A single protein staining band exhibited enzymatic activity; bands migrating more slowly than this band were minor in intensity. A number of bands, certain of which are labeled in Figure 1, were present ahead of the band showing activity. Their significance will be discussed below.

Electrophoresis of a gel identical to those shown in Figure 1, in the second dimension in the presence of $NaDodSO_4$, produced the two-dimensional pattern shown in section a of Figure 2A. The active band obtained under the first-dimension nondenaturing conditions was found to contain four main components numbered 1–4, in order of increasing mobility (Figure 2B). A fifth minor component, indicated with an arrow, was absent in preparations of higher specific activity and, therefore, need not be considered as a component of the enzyme. Enzyme was also applied directly to this $NaDodSO_4$–slab gel without prior electrophoresis (sections b of Figure 2A) to permit comparison with the two-dimensional pattern (see later).

In order to determine whether components 1–4 in Figure 2B were intrinsic components of the enzyme, two-dimensional electrophoretic analyses were carried out similar to that in Figure 2A, varying the first-dimension nondenaturing conditions (Figure 3). The major protein staining band in the first-

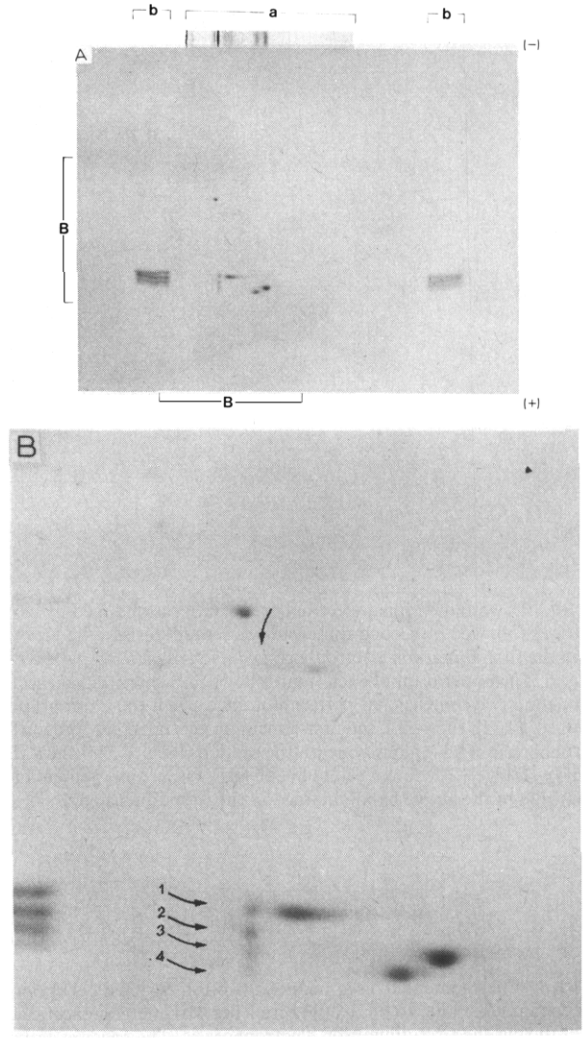

FIGURE 2: Two-dimensional electrophoretic pattern obtained by electrophoresis of the enzyme in the first dimension under the nondenaturing conditions of Figure 1, followed by second dimension electrophoresis in the presence of $NaDodSO_4$. Figure 2A shows the separating gel portion of a Laemmli $NaDodSO_4$–slab gel. The region below the section labeled "a" shows the two-dimensional pattern obtained as described above. A first-dimension gel stained for protein is inserted at the top of this section to show its orientation during the second-dimension electrophoresis. Electrophoresis in the second dimension was toward the anode. The regions of the gel beneath the sections labeled "b" show the single-dimension pattern obtained when the enzyme preparation was applied directly to the $NaDodSO_4$ gel. Figure 2B is an enlargement of the segment of Figure 2A enclosed in the brackets labeled "B". The major components in the two-dimensional pattern which comigrate in the first dimension with the active band are indicated with numbered arrows. A minor contaminant which migrates at this position (see text) is indicated with an unnumbered arrow.

dimension gel in each case was the only one to exhibit enzymatic activity. The two-dimensional patterns show that under each of the first-dimension conditions investigated, the active band contains components 1–4. No other species was present in the active band under all four first-dimension conditions investigated (Figures 2 and 3A,B,C). The results of the two-dimensional analyses thus indicate that the active enzyme contains four distinct components.

In the two-dimensional patterns of Figures 2 and 3, only a small proportion of the total protein is present in the active band. Enzyme of specific activity 29.5 units/mg was used in these analyses. Enzyme preparations of specific activity as high as 38.0 units/mg exhibited similar low proportions of total protein in the active band region of the two-dimensional pat-

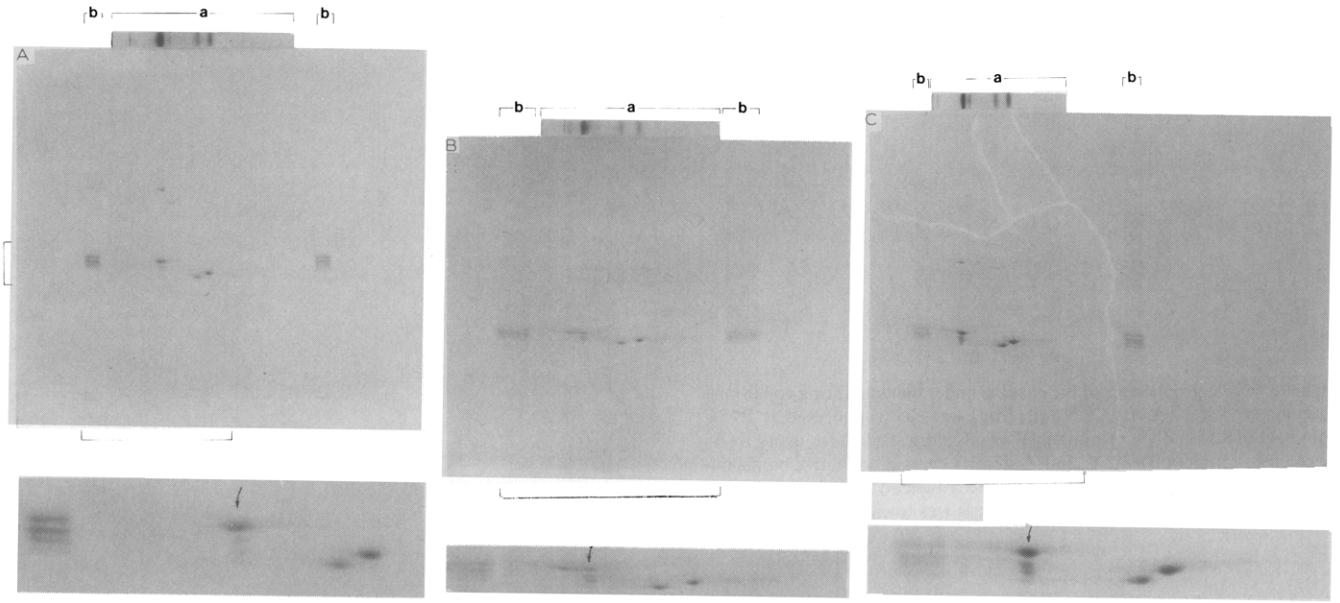

FIGURE 3: Two-dimensional electrophoretic patterns obtained by electrophoresis of the enzyme under various different nondenaturing first-dimension conditions followed by second-dimension electrophoresis in the presence of $NaDodSO_4$. A, B, and C differ as follows in the nondenaturing conditions used in the first dimension electrophoresis: (A) pH 8.4, 6.5% gel; (B) pH 7.4, 6.5% gel; (C) pH 7.4, 8.5% gel. Enzyme of specific activity 29.5 units/mg was used. The upper print of each figure shows the separating gel section of a Laemmli $NaDodSO_4$–slab gel. The region below the section marked "a" contains the two-dimensional pattern obtained when the appropriate first-dimension gel is electrophoresed in the second dimension in the presence of $NaDodSO_4$. In each case, the first-dimension gel stained for protein is inserted at the top of section "a" to show its position during the second dimension electrophoresis. The regions beneath the sections labeled "b" show the single-dimension pattern obtained by applying the enzyme directly to the $NaDodSO_4$–slab gel. The lower print of each figure is an enlargement of the segment in the upper print enclosed in unlabeled brackets. The arrow locates the position of the active band obtained in the first dimension.

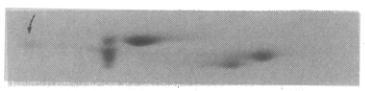

FIGURE 4: Enlargement of a segment of a two-dimensional gel obtained under conditions similar to those of Figure 2 but with mercaptoacetic acid present during the first-dimension electrophoresis. The specific activity of the enzyme preparation was 38.0 units/mg. The segment shown contains the components of the active band and the species comigrating with them in the second dimension. The arrow indicates the discrete material, originating from the top of the first-dimension gel, which comigrates with component 2 of the active band in the second dimension. Orientation of the gel is as in Figure 2.

terns. Approximately 65% of the initial enzyme activity was lost following dilution and dialysis of the stock enzyme solution prior to first-dimension electrophoresis. No protein precipitated. Similar losses have been noted previously (Fan et al., 1975). This loss of activity, together with the small amount of protein present in the active band region of the two-dimensional patterns, suggested that partial dissociation of the enzyme into its subunits was occurring during preparation of the enzyme for first-dimension electrophoresis, with subsequent independent migration of the dissociated species occurring during electrophoresis under nondenaturing conditions.

Examination of the two-dimensional patterns of Figures 2 and 3 reveals that additional species are indeed present in the second dimension at the positions of components 1, 3, and 4 of the active band. Thus, component Y, defined in Figure 1, comigrates in the second dimension with component 4 of the active band. Component Z, defined in Figure 1, comigrates with component 3 of the active band. Under all four first-dimension conditions, components Y and Z migrate much faster in the first-dimension direction than the active band. Component X, defined in Figure 1, comigrates in the second dimension with component 1 of the active band in each case. The position of this species in the first dimension, with respect to the active band, varies with the first-dimension conditions of the figures. It migrates ahead of the active band under the first-dimension conditions of Figure 2, with the active band under the first-dimension conditions of Figure 3A,C, and more slowly than the active band under the first-dimension conditions of Figure 3B where it does not resolve into a distinct species, suggesting partial aggregation.

Strongly staining, discretely appearing material is not present in the two-dimensional figures comigrating in the second dimension with component 2 of the active band. However, under different first-dimension conditions, e.g., with mercaptoacetic acid present during first-dimension electrophoresis (Figure 4) or first-dimension electrophoresis carried out at 22 °C (not shown), a small amount of additional discretely appearing material, comigrating in the second dimension with component 2 of the active band, is present. The position of this material, originating from the top of the first-dimension gel (Figure 4), suggests that under first-dimension conditions it exists as an aggregate and offers an explanation for its small amount compared with components X, Y, and Z in Figure 4 and for its absence from the gels of Figures 2 and 3, i.e., aggregation and subsequent loss from solution prior to first-dimension electrophoresis, perhaps by adhesion to the glassware used in the experiment. Also clearly apparent in Figure 4 is material trailing from each component of the active band to its proposed dissociated species. The existence of this material is most readily explained by continued dissociation of the active enzyme during first-dimension electrophoresis and supports the proposal of dissociation of the enzyme into its subunits. Similar trailing material is visible in Figure 3B,C. In addition, in Figure 3C trailing from all four components of the active band to the top of the first-dimension gel is suggestive of aggregation of the active enzyme under nondenaturing conditions (Giorgio et al., 1970).

Further evidence supporting the existence of dissociated

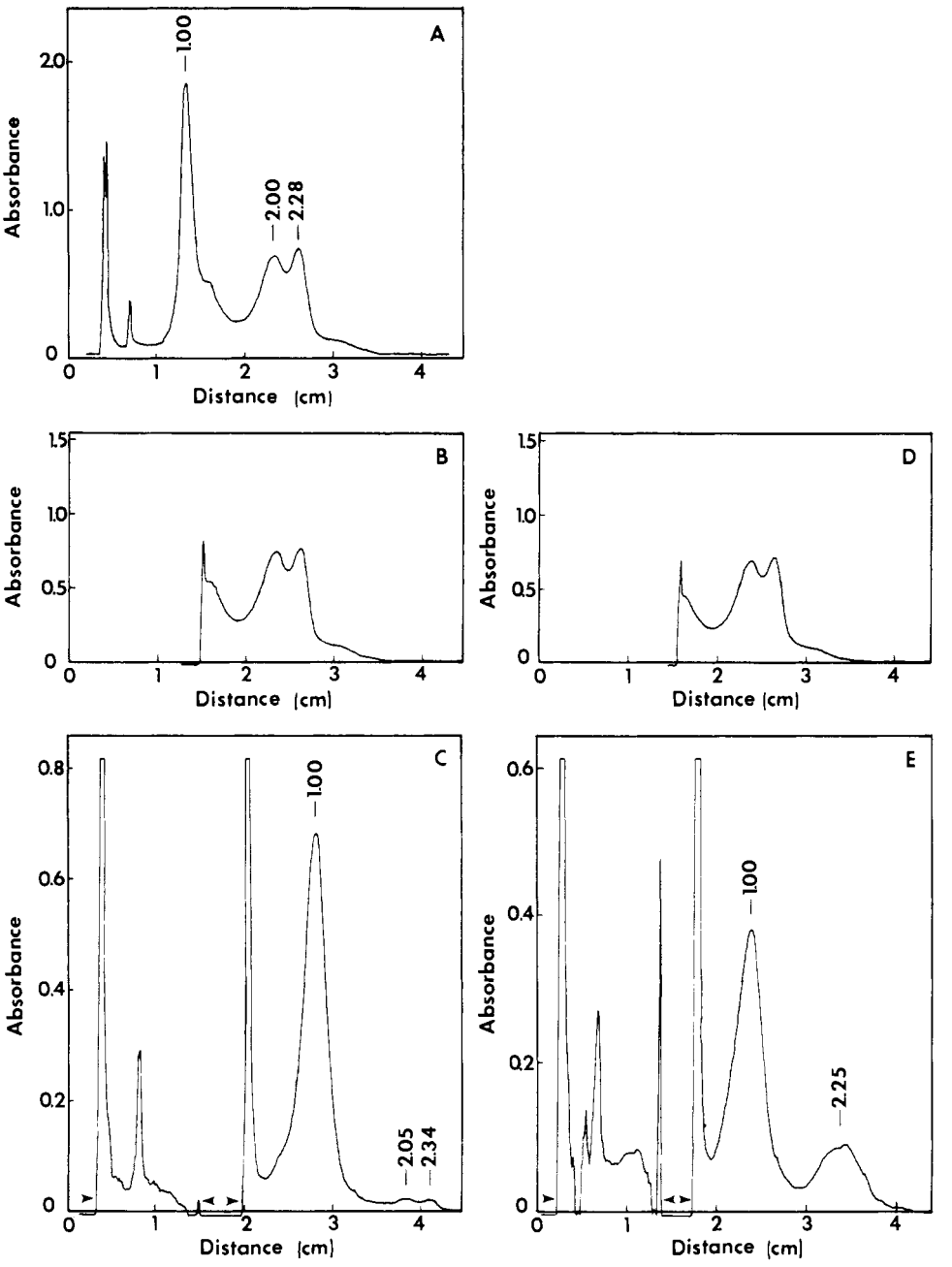

FIGURE 5: Generation by the active enzyme band, under nondenaturing conditions, of two bands with the same relative mobilities as bands Y and Z defined in Figure 1. Dialyzed enzyme (specific activity 38.0 units/mg) was electrophoresed for 2 h at pH 8.4 and 10 °C in a 6.5% polyacrylamide gel containing mercaptoacetic acid (A). Two gels were then cut just below the active band. The lower portions of the gels were stained for protein (B and D) while the upper parts containing the active band were transferred to the tops of fresh gels. One composite gel (containing the upper portion of the gel shown in B) was reelectrophoresed immediately (C). The second composite gel (containing the upper portion of the gel shown in D) was reelectrophoresed following incubation at 10 °C for 20 h (E). The arrowheads in C and E indicate the top and bottom of the applied gel and the top of the fresh gel. Mobility values relative to the main band are indicated in A, C, and E. The peaks obtained at the gel interfaces are not due to protein but to the sudden change in optical density at the gel interface.

subunits under the nondenaturing first-dimension conditions of Figures 2–4 was sought from an experiment whose aim was to determine whether the active band could give rise to components Y and Z. These bands separate suffciently from the active band under nondenaturing conditions to make such an experiment feasible.

The enzyme was first electrophoresed under conditions similar to those of the first dimension of Figure 3A, i.e., pH 8.4 and 6.5% gel, but with mercaptoacetic acid present during the electrophoretic run. Following electrophoresis, several gels were cut just below the active band (Figure 5B,D) and the upper portions containing the active band were applied to the tops of fresh gels. Reelectrophoresis of the composite gels was carried out either immediately or after incubation for 20 h at 10 °C.

Upon immediate reelectrophoresis, two small bands moving much faster than the main band were found (Figure 5C). These bands have the same mobilities relative to the active band as components Y and Z in the control gel following the initial electrophoresis (Figure 5A). When the composite gel containing the active band was incubated for a prolonged period of time prior to reelectrophoresis, a single, broad, fast-moving band was generated (Figure 5E) with mobility relative to the main band intermediate between those of components Y and Z. The proportion of total protein on the gel present in this region was noticeably increased compared with the pro-

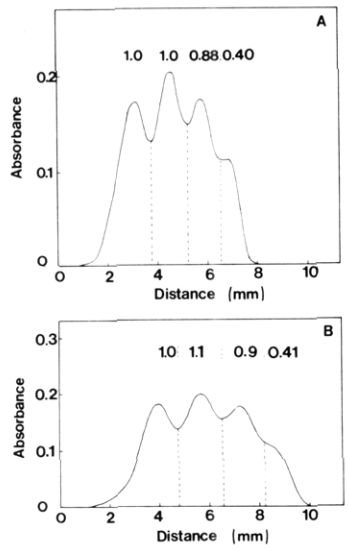

FIGURE 6: Densitometric scans (A) of the four major components present in the active band region of a two-dimensional gel similar to that of Figure 2, and (B) of the four major components obtained when the enzyme is applied directly to the $NaDodSO_4$–slab gel. The direction of electrophoretic migration is from left to right of the scans. The numbers give the ratios of the areas under the corresponding peaks.

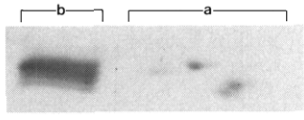

FIGURE 7: Enlargement of segment of gel showing two-dimensional electrophoresis of the enzyme, the first dimension in the presence of 8 M urea and the second in the presence of $NaDodSO_4$. Enzyme of specific activity 38.0 units/mg was used in the first dimension. The segment shown is that with species migrating in the 40 000-dalton region of the second dimension. Section "a" shows the bands present in the two-dimensional pattern; section "b" shows the band obtained by direct application of the enzyme to the gel. Orientation of the gel is as in Figure 2.

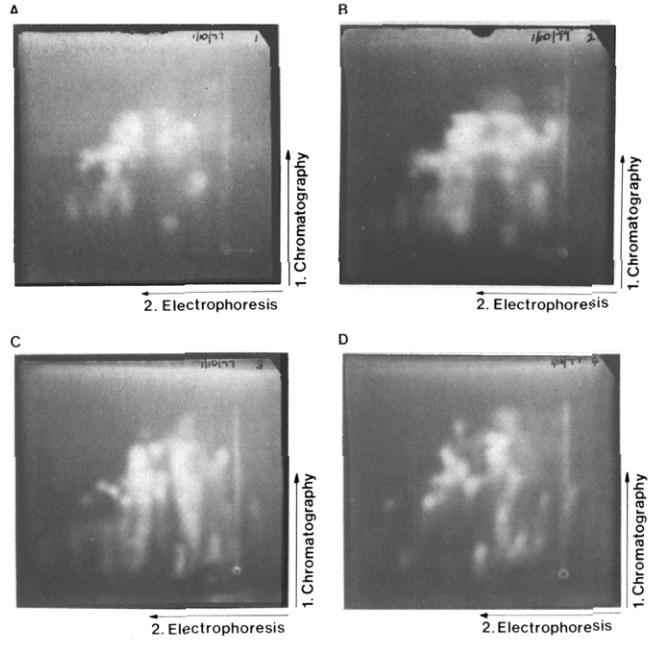

FIGURE 8: Tryptic peptide maps of the four major components of the enzyme preparation. Chromatography was carried out first, followed by electrophoresis, in the directions indicated. The sample was applied in the lower right-hand corner of each plate: (A) component 1; (B) component 2; (C) component 3; (D) component 4.

portion present in this region in the gel reelectrophoresed immediately. Similar results were obtained with gel pieces incubated at room temperature.

The generation of the two fast-moving bands in Figure 5C supports the proposal that the enzyme is able to dissociate into its components. The increased amount of protein generated following prolonged incubation of the active band provides additional evidence for the existence of a dissociation. The appearance of only a single broad band in the latter case is probably due to decreased resolution from the greater amount of generated protein and the longer time available for diffusion. Component X migrates with the active enzyme under the conditions of this experiment (see Figure 3A) and would not be expected to appear as a separate band upon reelectrophoresis of the active band.

Electrophoresis in the Presence of $NaDodSO_4$. Electrophoresis of the enzyme preparation directly on a $NaDodSO_4$–slab gel produced four major components (Figures 2 and 3, sections b). Comparison of the mobilities of these components in this system with those of standard proteins using the procedure of Shapiro et al. (1967) yielded molecular weights of 39 000, 39 600, 40 000, and 41 300. These four components comigrate with the four components of the active bands in the two-dimensional patterns of Figures 2 and 3 (compare sections b and a in each of these figures). The ratio of the staining intensities of the four components is identical to the ratio of the staining intensities of the four components of the active band (Figure 6). (The two-dimensional pattern chosen for this comparison is that where no putative dissociated species comigrates with the active band in the first dimension.) This equality in proportions further strengthens the hypothesis that the species comigrating in the second dimension with the four components of the active bands in Figures 2–4 are indeed subunits dissociated from the active enzyme prior to or during first-dimension electrophoresis.

Electrophoresis in the Presence of 8 M Urea Followed by Second-Dimension Electrophoresis in the Presence of $NaDodSO_4$. Two-dimensional gel electrophoresis, in the first dimension in the presence of 8 M urea and in the second dimension in the presence of $NaDodSO_4$, produced only one component at each of the positions of the four major bands found when the enzyme is applied directly to the $NaDodSO_4$–slab gel (Figure 7). This result supports the proposal of identity of the components of the active bands of the two-dimensional patterns of Figures 2–4 with the species which comigrate with them in the second dimension. In addition, the second slowest moving component in the second dimension of Figure 7 is present in a much smaller proportion than when the enzyme is applied directly to the $NaDodSO_4$–gel. (Compare sections a and b of Figure 7.) This result is consistent with a tendency for this species to aggregate and be lost from solution in the presence of 8 M urea prior to first-dimension electrophoresis. Results consistent with a similar tendency for this species were noted in two-dimensional electrophoretic patterns with the first dimension carried out under nondenaturing conditions (Figures 2–4).

Tryptic Peptide Maps of the Four Main Components of the Enzyme Preparation. Tryptic peptide maps of the four main components of the enzyme preparation were obtained following separation of these components on $NaDodSO_4$–slab gels (Figure 8). The components will be referred to as components 1–4, since the evidence indicates that each consists of that component of the active enzyme which migrates at the same position (Figures 2–4). The maps show a certain amount of diffuseness due probably to the closeness of the original protein

bands in the $NaDodSO_4$-slab gels. The features characteristic of each map are clear however.

Comparison of the four tryptic peptide maps reveals both similarities and differences. Some similarities exist between the maps of components 2 and 3 and the maps of components 3 and 4. The map of component 1 shows similarities to the map of component 2, which, however, contains a number of additional spots.

Discussion

The results of this study show that NAD-dependent isocitrate dehydrogenase from beef heart mitochondria contains four nonidentical subunits. Under certain nondenaturing conditions the enzyme appears to exist partially dissociated into these subunits. This dissociation is probably the origin of the loss of activity previously observed on dilution of the enzyme (Fan et al., 1975).

Detection of the four subunits of the enzyme was dependent on the high resolution of the Laemmli $NaDodSO_4$-polyacrylamide gel system run in the slab mode. In two cylindrical gel systems, the Tris–acetate system used in the present study with the addition of 2% $NaDodSO_4$ and the system of Shapiro et al. (1967), only two bands were readily resolved (Rushbrook, 1978). In the latter system, resolution of two bands was obtained only with 4 μg or less of protein. A previous investigation of subunit composition in this system revealed only one species (Giorgio et al., 1970). This result would seem to have been due to an inadequate resolution due to the large amount of protein applied.

Observation of dissociation of the enzyme into subunits under nondenaturing conditions has not been reported previously. For the enzymes from both beef heart and pig heart, however, diffusely staining material migrating ahead of and behind the major band has been observed in polyacrylamide gels (Giorgio et al., 1970; Shen et al., 1974; Ramachandran and Colman, 1978). In the present study, diffusely staining material, in addition to the major band, was obtained when preelectrophoresis of gels with mercaptoacetic acid and dialysis of the enzyme solution prior to electrophoresis were omitted. Resolution of components Y and Z from the diffuse material was obtained on inclusion of the above two conditions, and resolution of X, with improved resolution of Y and Z, was obtained by lowering the temperature of electrophoresis from 22 to 10 °C (Rushbrook J. I., and Harvey, R. A., unpublished results). Whether the dissociation observed is part of an equilibrium or whether it is due to irreversible denaturation of the enzyme is not clear. An electrophoretic pattern typical of enzyme exposed only to 22 °C was obtained when the enzyme was dialyzed first at 10 °C and then at 22 °C prior to electrophoresis at 22 °C, with mercaptoacetic acid present during dialysis and electrophoresis. Thus, the effect of exposure of the enzyme to the lower temperature was reversible (Rushbrook, J. I., and Harvey, R. A., unpublished observations).

The tryptic peptide maps of the four subunits, while showing similarities, contain a number of differences. The variations in the maps do not appear to be due to proteolysis. The patterns obtained are not those to be expected from limited proteolysis of one or perhaps two original polypeptide subunits. Deamidation occurring during or after purification of the enzyme seems unlikely as a source of the variation in the four peptide maps, since the pH values of solutions employed in the purification procedure and in storage of the enzyme would not promote deamidation and neither nondenaturing nor $NaDodSO_4$-gel electrophoretic patterns varied with storage of the enzyme preparations. Variation in the carbohydrate content of a common protein moiety will not explain the observed heterogeneity. Previous determinations of carbohydrate on this enzyme showed less than 0.5% (w/w) carbohydrate (Harvey et al., 1972), which corresponds to less than one hexose unit per polypeptide subunit. This amount is insufficient to explain the differences observed in the peptide maps. Thus, the four subunits of the enzyme appear to be products of different genes.

Enzymatically active species of the enzyme of approximately 165 000 and 330 000 daltons have previously been observed, depending on the temperature (Fan et al., 1975). Higher aggregates of molecular weight 680 000 and 110 000 (activity undetermined) have been observed under different conditions (Giorgio et al., 1970). In each of these species, the molecular weight is a multiple of approximately 165 000. The finding in the study reported here of four approximately 40 000-dalton species present in the active enzyme, together with the above observations, strongly suggest that the four species are present in an equimolar ratio. The smaller area of the scan of subunit 4 compared with the areas of the scans of the other subunits (Figure 6) may be due to a lower affinity of this subunit for the dye Coomassie brilliant blue R. Unequal degrees of staining of different globular proteins by this dye under nondenaturing conditions have been reported (de St. Groth, 1963) and were also observed in the presence of $NaDodSO_4$ in the present study. This explanation for the unequal staining of the four subunits in the $NaDodSO_4$-slab gels is supported by the observation that the peptide maps of the subunits, derived from equal numbers of bands generated by the enzyme preparation on $NaDodSO_4$-slab gels, have approximately equal intensities.

The presence of four nonidentical subunits is unusual for NAD-dependent dehydrogenases. Multiple identical subunits seem to be the usual finding (Brändén et al., 1975; Holbrook et al., 1975; Smith et al., 1975; Banaszak and Bradshaw, 1975; Harris and Waters, 1976), although in some instances isoenzymes exist and the subunits of the different forms are able to copolymerize to form active species (Holbrook et al., 1975; Pietruszko, 1975). In these enzymes, however, the presence of more than one kind of subunit is not required for activity. Ramachandran and Colman (1978) recently presented evidence that two nonidentical subunits are present in the NAD-dependent isocitrate dehydrogenase from pig heart, with no indications of the existence of isoenzymes. Thus, NAD-dependent isocitrate dehydrogenases appear to differ from other NAD-dependent dehydrogenases in the nature of their subunits. Ramachandran and Colman (1978) suggested that the detection of four nonidentical subunits for the beef heart enzyme (Rushbrook and Harvey, 1977, preliminary report), as opposed to the two found by them for the pig heart enzyme, was due to inadequate reduction of disulfide bonds prior to electrophoresis in the presence of $NaDodSO_4$, based on the observation that multiple bands (more than two) were observed for the pig heart enzyme only on storage of the enzyme for several months followed by inadequate reduction prior to $NaDodSO_4$ electrophoresis. However, the presence of the four bands on $NaDodSO_4$ electrophoresis for the beef heart enzyme was independent of the time of storage of the enzyme, and the reduction conditions were found to be adequate for other proteins. Furthermore, the four species maintained their identities on electrophoresis in the presence of 8 M urea using a different reducing reagent. Finally, if variable sulfhydryl group oxidation were responsible for the observation of two additional subunits in the presence of $NaDodSO_4$, then isolation from $NaDodSO_4$-slab gels followed by reduction using a third type of reducing agent and alkylation would have pro-

duced peptide maps identical in groups of two. This was not the case. Detection of the four subunits of the beef heart enzyme in the presence of $NaDodSO_4$ was critically but reproducibly dependent on the conditions defined under Materials and Methods. The discrepancy between the number of subunits found in this study and that of Ramachandran and Colman may thus be procedural. However, differences due to the species variation in the sources of the enzymes should not be discounted.

The functions of the four subunits are as yet undetermined. It seems almost certain, however, that each will be found to bind one or more of the nucleotides NADH, NADPH, ADP, and ATP. In view of this, the similarities existing among the peptide maps are intriguing. Similarities in the three-dimensional structure have been found in the nucleotide-binding domains of a number of NAD-dependent dehydrogenases and other nucleotide binding proteins, although, in general, sequence homology is low (Rossman et al., 1975). The peptide map patterns found here may indicate both three-dimensional structure and amino acid sequence homologies among the portions of the subunits which bind nucleotides.

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