Evidence for the presence of two nonidentical subunits in NAD-dependent isocitrate dehydrogenase of pig heart

(acylamide gel electrophoresis/NH₂-terminal amino acids/trypsic map)

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ABSTRACT The NAD-dependent isocitrate dehydrogenase [threo-D₃-isocitrate:NAD⁺ oxidoreductase (decarboxylating); EC 1.1.1.41] from pig heart is a multisinubunit enzyme with a molecular weight of approximately 340,000. Electrophoresis of the enzyme in 10% polyacrylamide gels containing sodium dodecyl sulfate reveals two discrete bands with molecular weights of 41,000 and 39,000. The two bands exhibit approximately equal intensity when stained with Coomassie Blue, Amido Black, and Bromophenol Blue, suggesting that these polypeptide chains are present in equimolar quantities in the native enzyme. The same two-band pattern is observed when the sulfhydryl groups of the enzyme are blocked by alkylation with iodoacetate prior to electrophoresis, indicating that sulfhydryl oxidation is not responsible for the observed heterogeneity. Each of the subunits appears as a single band when eluted from the gel and again subjected to electrophoresis under the same conditions. Isocitrate dehydrogenase contains a total of 41 lysine and arginine residues per average subunit of 40,000 daltons. The observation of approximately 80 peptides upon paper chromatography and high voltage electrophoresis of tryptic digests of the enzyme is consistent with the existence of two distinct polypeptide chains. Dansylation yields two NH₂-terminal amino acid derivatives: dansyl-phenylalanine and dansyl-alanine. It is concluded that the NAD-specific isocitrate dehydrogenase is composed of equal numbers of two nonidentical subunits.

The NAD-dependent isocitrate dehydrogenase [threo-D₃-isocitrate:NAD⁺ oxidoreductase (decarboxylating); EC 1.1.1.41] isolated from yeast, bovine heart, and porcine heart is a multisinubunit enzyme with a molecular weight of approximately 300,000–340,000 (1–3). Polyacrylamide gel electrophoresis under denaturing conditions in sodium dodecyl sulfate (NaDodSO₄) or urea in the presence of a reducing agent has been reported to yield a single band with a molecular weight of 39,000–40,000, suggesting that the native enzyme is composed of eight apparently similar subunits. The limited binding studies conducted with this enzyme reveal that there are only four NADH binding sites in the beef heart enzyme (4) and four isocitrate binding sites in the yeast enzyme (1). These results prompted us to investigate the question of whether the subunits in these two enzymes are identical or dissimilar. This paper presents evidence demonstrating that the NAD-dependent isocitrate dehydrogenase isolated from pig heart is composed of two different subunits that are present in equimolar amounts in the native enzyme.

MATERIALS AND METHODS

Enzyme Purification. The NAD-dependent isocitrate dehydrogenase from pig heart was purified from 80 kg of fresh pig heart by the procedures of Weber et al. (5). The enzyme preparation was dialyzed against 0.1 M sodium phosphate buffer (pH 7.2) containing 10 mM EDTA, 0.1 M sodium borate buffer (pH 8.5) containing 6 M guanidine hydrochloride, and 0.5 M sodium sulfate. The solution was made 1% with respect to 2-mercaptoethanol, evacuated, and maintained under nitrogen atmosphere for 1 h at room temperature. Sodium iodoacetate was then added with stirring to yield a final concentration of 0.14 M and the solution was maintained in the dark for 30 min. Excess 2-mercaptoethanol (10%) was then added and the protein solution was dialyzed in the cold against repeated changes of distilled water.

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Abbreviation: NaDodSO₄, sodium dodecyl sulfate.

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boiling water bath for 2 min and then incubated at 40° for 1 hr. Glutamate dehydrogenase (molecular weight 56,000), creatine kinase (41,000), malate dehydrogenase (35,000), chymotrypsinogen (25,600), and cytochrome c (12,400) were treated similarly and used as standards. The gels were stained with Coomassie Blue after they were washed overnight with a solution containing 15% methanol/10% acetic acid to remove NaDodSO₄. In experiments with isocitrate dehydrogenase, the gels were also stained with 1% Amido Black in 7% acetic acid and with 0.2% Bromophenol Blue in ethanol/water/acetic acid (50:45:5, vol/vol).

Localization of Protein Bands and Their Elution from Polyacrylamide Gels Containing NaDodSO₄. In order to localize the protein bands in NaDodSO₄ polyacrylamide gels before they were fixed and stained, we modified the enzyme by treatment with fluorescamine (Roche Diagnostics) prior to electrophoresis. For this purpose, 0.2 mg of enzyme in 1.0 ml of 0.01 M sodium phosphate buffer, pH 7.2/1% NaDodSO₄ was mixed rapidly with 250 μl of a solution of fluorescamine (0.2 mg/ml) in acetone. The mixture was allowed to stand at room temperature for 15 min before the addition of 2-mercaptoethanol and treatment in a boiling water bath as described above. After electrophoresis, the protein bands were localized by their fluorescence under ultraviolet light. They could be cut out and extracted with water containing 0.1% NaDodSO₄.

Paper Chromatography and High Voltage Electrophoresis of Tryptic Digest of Isocitrate Dehydrogenase. Approximately 1.5 mg of unmodified or of reduced and alkylated enzyme was suspended in 1.0 ml of 50 mM ammonium bicarbonate buffer, pH 7.8. Trypsin (75 μg) treated with L-(1-tosylamide-2-phenyl)ethyl chloromethyl ketone (Worthington Biochemical Corp.) was added and the protein solution was incubated at 40° for 24 hr. The tryptic hydrolysate thus ob-

![FIG. 1. Chromatography of NAD-dependent isocitrate dehydrogenase on cellulose phosphate. The cellulose phosphate column (2.5 x 40 cm) was equilibrated with Tris/10 mM citrate buffer, pH 6.5, containing 20% glycerol, 0.1 mM ADP, and 0.1 mM dithiothreitol. After application of the sample (about 1200 enzyme units of specific activity 10–12 units/mg), the column was washed with the starting buffer. Fractions containing 11.4 ml were collected. At fraction 20, a linear gradient to Tris/0.4 M citrate buffer, pH 6.5, containing 20% glycerol, 0.1 mM ADP, and 0.1 mM dithiothreitol was started, using a mixing chamber and reservoir of 500 ml each. The concentration of manganous ion in the fractions was adjusted to 2 mM to stabilize the enzyme.

RESULTS AND DISCUSSION

The purified NAD-dependent isocitrate dehydrogenase of pig heart exhibits two closely spaced protein bands upon electrophoresis in 10% polyacrylamide gels containing NaDodSO₄ (Fig. 2A). The same pattern was obtained with enzyme isolated in the presence or absence of dithiothreitol and ADP. The pattern obtained did not differ appreciably when the electrophoresis was conducted in 15% polyacrylamide gels. Although the resolution was decreased when electrophoresis was carried out in 7% polyacrylamide gels, two bands could also be seen, but only when the protein concentration was low. An earlier report that the enzyme exhibited a single band upon electrophoresis in 7% polyacrylamide gels containing NaDodSO₄ (6) can be explained by the fact that those gels were heavily loaded with protein in order to detect any contaminants in the preparation.

The two bands stained with approximately equal intensity regardless of the stain used to visualize the protein. A densitometric scan of a typical gel stained with Coomassie Blue is shown in Fig. 3. The ratio of area occupied by band I to that of band II was 1.047, 1.063, and 1.245, as determined from densitometric scans of gels stained with Coomassie Blue, Amido...
justed to amounts logarithm of containing NaDodSO₄ re-electrophoresis, one layered which observed major dehydrogenase isocitrate appears dehydrogenase conditions Black, and their mobilities with those of isocitrate dehydrogenase is alkylated and Bromophenol Blue, respectively. These results strongly suggest that the two polypeptide chains are present in equimolar amounts in the native enzyme.

As indicated earlier, the enzyme, when subjected to electrophoresis at pH 7.0 in the absence of NaDodSO₄, yields a major catalytically active protein band. If this band is cut out and layered on top of the same type of gel, it exhibits, upon re-electrophoresis, one protein band, which is also enzymatically active. Alternatively, if the same band is layered on top of a gel containing NaDodSO₄ and is subjected to electrophoresis under the conditions given in Materials and Methods, two bands are observed which are identical to those shown in Fig. 2. It thus appears that both polypeptide chains are intrinsic to the active isocitrate dehydrogenase molecule.

The molecular weights of the two subunits of NAD-specific isocitrate dehydrogenase may be determined by comparison of their mobilities with those of protein standards. A plot of the logarithm of the molecular weight of standard proteins against the migration distance reveals molecular weights of 41,000 for band I and 39,000 for band II.

One possible explanation for the appearance of two bands on the polyacrylamide gels might be that although the enzyme initially contained only one type of subunit, the conditions of the electrophoretic experiment in some way converted the enzyme into two discernible species. If this were the case, one might expect regeneration of the two bands upon re-electrophoresis under the same conditions of the isolated individual bands. In order to test this hypothesis, we labeled the protein with fluorescamine to facilitate detection of the bands on the gels without staining. It was separately determined that the fluorescamine treatment did not alter the mobility of the two bands. The individual bands from 12 replicate gels were cut and extracted by stirring for 2 hr in 2 ml of a solution containing 0.1% NaDodSO₄. After concentration, the extracts were re-electrophoresed either individually or after mixing, as before.

As can be seen from Fig. 2B, band I and band II each retained its characteristic mobility and the mixture of band I and band II yielded a pattern similar to that obtained with the original intact enzyme. This experiment demonstrates that the two bands observed do not result from an artifactual equilibration under the electrophoretic conditions of one type of subunit to generate two. Rather, it appears that the native enzyme contains two distinguishable polypeptide chains.

Another possible explanation for the appearance of two bands on electrophoresis might be that they represent the products of sulphydryl oxidation, resulting in the formation of intra-peptide disulfide bonds which are not reduced by the treatment with dithiothreitol prior to electrophoresis. To examine this possibility we treated native enzyme first with 2-mercaptoethanol and then allowed it to react with iodoacetate in order to block all the free sulphydryl groups. This carboxymethylated enzyme preparation exhibited the same two-band pattern seen in Fig. 2A upon electrophoresis in the presence of NaDodSO₄. Thus, oxidation of sulphydryl groups does not seem to be re-

FIG. 3. Densitometric scan of NaDodSO₄ electrophoretic gel of isocitrate dehydrogenase after staining with Coomassie Blue. The gel was scanned with a Gilford model 2410S linear transport attachment to a model 240 spectrophotometer with full scale of the recorder adjusted to 1.0 absorbance unit.

FIG. 4. Tryptic peptide map of reduced and alkylated isocitrate dehydrogenase. The peptides were visualized after they were sprayed with ninhydrin and are circled in the figure.
isocitrate dehydrogenase. Complete amino acid analysis of the pig heart enzyme shows that it contains a total of 41 lysine plus arginine residues per average subunit of 40,000 daltons (6). If it is assumed that the enzyme consists of identical subunits, its tryptic map would be expected to yield a maximum of 42 peptides. In contrast, as can be seen in Fig. 4, the tryptic digest of the reduced and alkylated isocitrate dehydrogenase yields approximately 80 ninhydrin-positive spots. A similar number of spots was obtained when unmodified isocitrate dehydrogenase was digested by trypsin. These observations indicate the existence of two different polypeptide chains in this enzyme.

The NH₂-terminal amino acids of intact isocitrate dehydrogenase were determined by dansylation of the protein. The two-dimensional thin-layer chromatogram of the dansylated, acid-hydrolyzed enzyme is shown in Fig. 5. The O-dansyltyrosine and e-dansyllysine that appear are the derivatives expected from reaction with the internal tyrosyl and lysyl residues, respectively, and the dansyl hydroxide is the residual decom-pound product of dansyl chloride. Two fluorescent spots corresponding to two NH₂-terminal amino acids are seen with approximately equal intensity: dansylalanine and dansylphenylalanine. No other derivatives were detected when different solvent systems were used. These results demonstrate the existence of two distinguishable polypeptide chains in this pig heart isocitrate dehydrogenase.

The NAD-dependent isocitrate dehydrogenase is an allosteric enzyme that is activated by ADP (13). It is possible, but not demonstrated, that the two polypeptide chains represent catalytic and regulatory subunits of the enzyme.

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