Pig Heart Triphosphopyridine Nucleotide Specific Isocitrate Dehydrogenase. A Single Polypeptide Chain*

Roberta F. Colman,† Rita Chu Szeto, and Phyllis Cohen

ABSTRACT: Pig heart TPN-specific isocitrate dehydrogenase, with a molecular weight of approximately 58,000, exhibits a single band on polyacrylamide gel electrophoresis under mild and denaturing conditions. It is concluded that this enzyme consists of a single polypeptide chain on the basis of molecular weights determined from equilibrium centrifugation studies in urea and guanidine hydrochloride, from electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate, and from gel filtration in guanidine hydrochloride. The molecular weight data are consistent with "fingerprints" obtained by paper chromatography and electrophoresis of tryptic digests of the enzyme.

Most mammalian tissues have been found to contain two isocitrate dehydrogenases: a TPN-dependent enzyme, isolated chiefly from the cytoplasm and a DPN-dependent enzyme located primarily in the mitochondria (Plaut, 1963; Salgonicoff and Koeppe, 1968; Plaut and Aogaichi, 1967; Higashi *et al.*, 1965). Both types of isocitrate dehydrogenase use the same side of the nicotinamide ring (Chen and Plaut, 1963a) and in both enzymatic reactions a proton from the solvent replaces the carboxyl group with retention of configuration (Lienhard and Rose, 1964; Englard and Listowsky, 1963; Rose, 1966). Marked differences, however, have been noted in the catalytic properties, physical characteristics, and mode of regulation of these two enzymes. The TPN-specific enzyme is a relatively small protein, with a molecular weight of 58,000 as isolated from pig heart (Colman, 1968), which is not known to be subject to control by modifiers. By contrast the DPN-dependent isocitrate dehydrogenase, with a molecular weight of approximately 300,000 (Chen and Plaut, 1963b; Harvey *et al.*, 1970) exhibits more complex kinetic behavior, being activated by ADP, AMP, and citrate (Chen and Plaut, 1963b; Atkinson *et al.*, 1965; Sanwal and Cook, 1966). Analysis of the differences between these enzymes may elucidate the characteristics which distinguish an allosteric from a nonregulatory enzyme.

Allosteric characteristics have generally been associated with proteins containing more than one polypeptide chain and indeed preliminary evidence has been presented for the existence of subunits in the DPN-specific enzyme of yeast and bovine heart (Harvey *et al.*, 1970; Kuehn *et al.*, 1970). The question as to the number of polypeptide chains composing the TPN-specific enzyme has not heretofore been definitely answered.

* From the Biochemical Research Laboratory, Massachusetts General Hospital, and the Department of Biological Chemistry, Harvard Medical School, Boston, Massachusetts 02114. *Received July 13, 1970.* This work was supported by United States Public Health Service Grants 2-R01-AM 12379, FR 05486-07, and United States Public Health Service Career Development Award 7-K4-GM-9576 to one of us (R. F. C.).

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Experimental Procedure

Materials. The TPN-specific pig heart isocitrate dehydrogenase supplied by Boehringer Mannheim Corp. was further purified tenfold by column chromatography on carboxymethylcellulose, followed by gel filtration on Sephadex G-150, as previously described (Colman, 1968). It was demonstrated to be homogeneous in the ultracentrifuge and on cellulose acetate and disc electrophoresis from pH 6.2 to 9.5.

The trypsin obtained from Worthington Biochemical Corp. had been treated with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone to inhibit any contaminating chymotryptic activity. Mann Research Laboratory supplied the urea (Ultra Pure), guanidine hydrochloride (Ultra Pure), and lauryl sodium sulfate (M. A.).

Preparation and Analysis of Tryptic Digest of Native Enzyme. Isocitrate dehydrogenase (2 mg) was incubated with 0.1 mg of trypsin for 72 hr in 0.2 M ammonium bicarbonate buffer, pH 7.7 at 40°. A capillary tube containing toluene was included in the reaction vessel to minimize bacterial contamination. After desalting by evaporation, descending paper chromatography was conducted in 1-butanol–acetic acid–water (4:1:5) for 8.2 hr, followed by electrophoresis in pyridine acetate buffer, pH 6.4, for 50 min at 2500 V.

Gel Electrophoresis in Sodium Dodecyl Sulfate. Polyacrylamide gel electrophoresis of isocitrate dehydrogenase was conducted in 2% sodium dodecyl sulfate by the method of Fairbanks (1969). Enzyme (0.2 mg/ml) was incubated in 1% sodium dodecyl sulfate containing 0.025 M dithiothreitol, pH 7.5, for 1 hr to 4 weeks prior to loading (at a concentration of 0.1 mg/ml) on the gels. Gels (8% acrylamide) contained 2% sodium dodecyl sulfate in 0.2 M Tris acetate buffer, pH 7.5. The electrode buffers were 0.2 M Tris acetate, pH 7.5; but, in addition, 2% sodium dodecyl sulfate was added to the cathodic reservoir. Electrophoresis was conducted for 1.5 hr and 5 mA/tube. Staining was accomplished with coomassie brilliant blue by the method of Weber and Osborn (1969) after the sodium dodecyl sulfate was first removed by soaking the gels in 7.5% acetic acid. Protein standards, treated in the same manner as was isocitrate dehydrogenase, were bovine serum albumin (molecular weight 66,000), glutamate dehydrogenase (53,000), carboxypeptidase A (34,400), chymotryp-

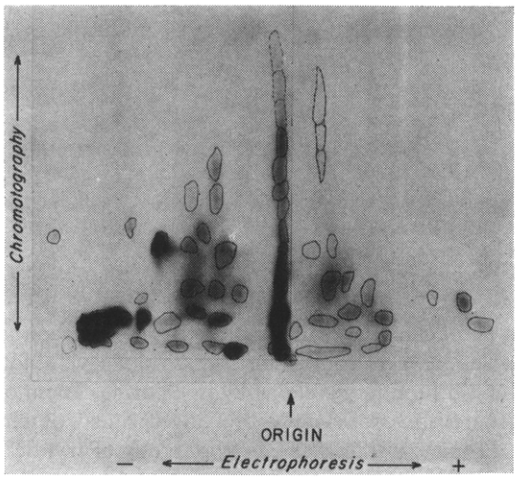

FIGURE 1: "Fingerprint" of tryptic digest of native TPN-specific isocitrate dehydrogenase.

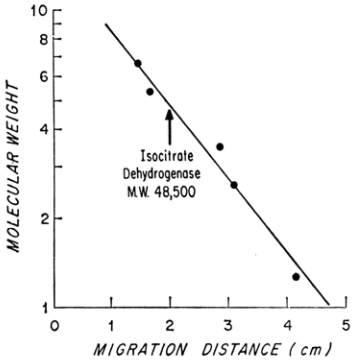

FIGURE 3: Determination of molecular weight of isocitrate dehydrogenase on polyacrylamide gels containing 2% sodium dodecyl sulfate. Isocitrate dehydrogenase and the protein standards were treated as described in Experimental Procedure. Each point represents the average of at least 5 determinations.

sinogen A (25,700), and cytochrome *c* (12,400). Cytochrome *c* was included in all gels as a reference protein and was used to correct for minor variations between the gels.

Equilibrium Centrifugations. Sedimentation equilibrium experiments were conducted by the meniscus depletion method of Yphantis (1964) at 35,600 or 47,660 rpm and 20.0° in 6.5 M urea and 5.89 M guanidine hydrochloride containing 0.1 M mercaptoethanol, pH 7.4. The densities of the solutions were 1.108 g/ml and 1.143 g/ml, respectively (Kawahara and Tanford, 1966).

Gel Filtration in Guanidine Hydrochloride. The molecular weight of denatured isocitrate dehydrogenase was determined by gel filtration on Sepharose 6B equilibrated with 6 M guanidine hydrochloride containing 1 mM dithiothreitol, pH 7.4, in accordance with Davison (1968) and Fish *et al.* (1969). Protein (5 mg/ml) was preincubated in the denaturing solvent for at least 4 hr prior to application of 1.0 ml to the column (2.2 × 35.5 cm). The position of the peak was determined from the absorbance at 280 mμ. The void volume was measured with Dextran Blue and the protein standards were the same used for the polyacrylamide gel electrophoresis experiments.

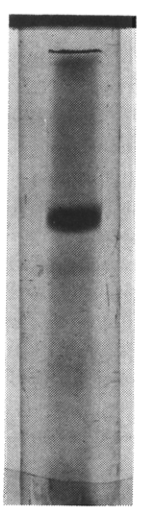

FIGURE 2: Polyacrylamide gel electrophoresis of isocitrate dehydrogenase in 2% sodium dodecyl sulfate by the method of Fairbanks (1969). The origin is at the top and the anode at the bottom of the illustrated gel.

Results

"Fingerprint" of Tryptic Digest. The pig heart TPN-specific isocitrate dehydrogenase contains 58 lysyl and arginyl residues (Colman, 1967). Digestion with trypsin should therefore yield up to 59 peptides if the entire sequence is nonrepeating, but only 30 peptides if there are two identical subunits. Figure 1 shows the "fingerprint" which results from paper chromatography followed by electrophoresis at pH 6.4; 54 ninhydrin-positive spots are readily detectable as indicated by the solid outlines and there is the suggestion of up to seven more spots, shown by the dotted outlines. These results are consistent with either a single polypeptide chain of unique sequence or nonidentical subunits.

Gel Electrophoresis. Electrophoresis on polyacrylamide gels containing 0.1% sodium dodecyl sulfate was developed by Shapiro *et al.* (1967), Weber and Osborn (1969), and Dunker and Rueckert (1969) as a useful method for determining the molecular weight of polypeptide chains. However, it has been demonstrated that in the case of certain proteins, dimers persist in 0.1% sodium dodecyl sulfate (Blumenthal and Heinrikson, 1970) whereas they are not observed in 1–2% detergent. In order to achieve greater assurance of complete dissociation to any existent subunits, the present investigation follows the modifications of Fairbanks (1969) based on electrophoresis in 2% sodium dodecyl sulfate. Isocitrate dehydrogenase is inactivated by concentrations greater than 0.2% sodium dodecyl sulfate, and all of its 13 sulfhydryl groups become available for reaction with the Ellman reagent (Colman, 1968). Figure 2 illustrates the single-band characteristic of isocitrate dehydrogenase as denatured in the detergent. Similar results were obtained when electrophoresis was conducted after 1 hr or 4 weeks of incubation of enzyme with sodium dodecyl sulfate and dithiothreitol. A comparison of the mobility of isocitrate dehydrogenase with that of protein standards (Figure 3) suggests a molecular weight of 48,500 for the denatured enzyme.

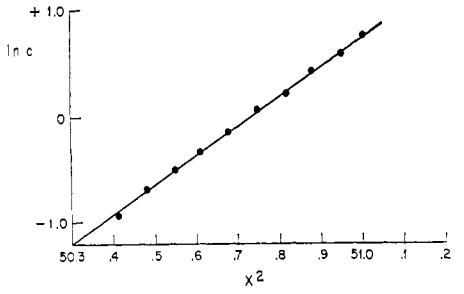

FIGURE 4: Equilibrium sedimentation of isocitrate dehydrogenase in in 6.5 M urea. In this experiment the initial protein concentration was 0.43 mg/ml. Equilibrium was established by sedimentation for 24 hr at 35,600 rpm.

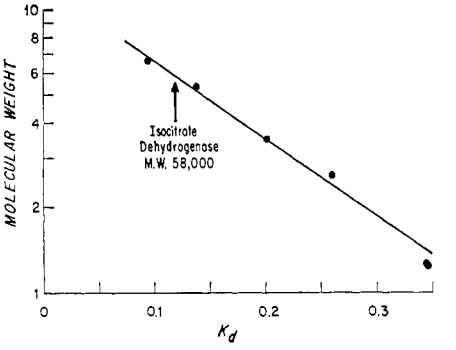

FIGURE 5: Gel filtration of isocitrate dehydrogenase and protein standards on Sepharose 6B equilibrated with 6 M guanidine hydrochloride, containing 1 mM dithiothreitol, pH 7.4.

Equilibrium Sedimentation. Another measurement of the molecular weight of isocitrate dehydrogenase was obtained in sedimentation equilibrium experiments in the denaturing solvents 6.5 M urea and 5.89 M guanidine hydrochloride containing 0.1 M mercaptoethanol. As shown in Figure 4, the plot of ln c *vs.* x^2 was linear, indicating homogeneity of the enzyme denatured in urea. A value of 0.74 ml/g for the partial specific volume can be calculated from the amino acid composition (Colman, 1967) by the method of Cohn and Edsall (1943). If it is assumed that the $\bar{v}$ does not change appreciably in solutions containing urea or guanidine hydrochloride (an assumption which will be considered in the Discussion), the molecular weight for the denatured enzyme in both solvents is 52,900.

Gel Filtration. As an additional check on the size of denatured isocitrate dehydrogenase, the molecular weight was determined by gel filtration on Sepharose 6B equilibrated with 6 M guanidine hydrochloride containing 1 mM dithiothreitol, pH 7.4. This method is not subject to the uncertainties surrounding an appropriate choice of the partial specific volume but depends, rather on the assumption that all proteins possess the same gross conformation in the reduced state in concentrated solutions of guanidine hydrochloride. Figure 5 shows the calibration curve of the logarithm of the molecular weight of standard proteins as a function of the distribution coefficient (K_d) with K_d defined as

$$K_d = \frac{V_e - V_0}{V_i - V_0}$$

where V_e is the elution volume of the solvent corresponding to the peak concentration of the particular sample, V_0 is the void volume, and V_i is the volume of solvent within and without the gel matrix. Isocitrate dehydrogenase yields a single peak with a molecular weight of 58,000 by this method.

Discussion

Apparently conflicting data have been presented regarding the subunit structure of the pig heart TPN-dependent enzyme. Magar and Robbins (1969) reported that this enzyme contains two subunits on the basis of an observed molecular weight of 32,000 in 6.5 M urea. However, it has been found that for a species of molecular weight 58,000 this enzyme binds 1 mole each of α-ketoglutarate and isocitrate (Colman, 1969) and is inactivated by the alkylation of a single methionyl residue (Colman, 1968). The most direct interpretation of these chemical data is that the enzyme consists of a single polypeptide chain. It is possible, however, that negative cooperactivity in binding would produce a reduction in the apparent number of substrate sites observed over the particular concentration range examined (Conway and Koshland, 1968). Alternatively, there may be two subunits, either identical or dissimilar, but only one substrate binding site, in analogy to the case of the DPN-dependent isocitrate dehydrogenase for which there appear to be only four substrate binding sites for an enzyme composed of eight subunits (Harvey *et al.*, 1970). Additional evidence favoring the single-chain nature of the TPN-dependent isocitrate dehydrogenase comes from the isolation of only one amino-terminal alanine in the Edman reaction (Colman and Chu, 1970); although it is possible that a blocked amino-terminal could account for these results. In order to distinguish between these various postulates, this investigation sought to clarify the subunit structure of the enzyme.

The molecular weights of pig heart isocitrate dehydrogenase denatured in three solvents and determined by three different methods are summarized in Table I; these data yield an average value of 53,000. Each of these methods depends on particular assumptions and is subject to its own peculiar errors. In the case of electrophoresis in sodium dodecyl sulfate, Dunker and Rueckert (1969) have pointed out that a positively charged polypeptide may bind slightly more detergent than would a comparable neutral polypeptide, leading to a value for molecular weight that is erroneously low. It is notable that native isocitrate dehydrogenase exhibits a net positive charge at the pH of the electrophoresis experiments (pH 7.5), and in fact has an isoelectric point above pH 9.0 (Colman, 1968). This effect may account for the value of 48,500 found for denatured isocitrate dehydrogenase by this method as compared to 58,000 obtained by sedimentation equilibrium measurements on the native enzyme (Colman, 1968). Nevertheless, this molecular weight is too far from 29,000 to be consistent with the postulate (Magar and Robbins, 1969) of two polypeptide chains composing the native enzyme.

In calculating the molecular weight from the equilibrium sedimentation data, the assumption was made that the partial specific volume does not change appreciably in concen-

TABLE I: Molecular Weight of Native and Denatured Isocitrate Dehydrogenase.

Method	State of Enzyme	Solvent	Molecular Weight
Sedimentation equilibrium	Native	0.1 M triethanolamine chloride, pH 7.7–0.3 M Na_2SO_4–1 mM EDTA	58,000
	Denatured	6.5 M urea, pH 7.4	52,900
	Denatured	5.89 M guanidine hydrochloride–0.1 M mercaptoethanol, pH 7.4	52,900
Polyacrylamide gel electrophoresis	Denatured	2% sodium dodecyl sulfate, pH 7.5	48,500
Gel filtration on Sepharose 6B	Denatured	6.0 M guanidine hydrochloride–1 mM dithiothreitol, pH 7.4	58,000

trated solutions of urea and guanidine hydrochloride. The question of the effect of a three-component system on the apparent partial specific volume of a protein has received considerable attention in recent years (Woods *et al.*, 1963; Marlek *et al.*, 1963; Noelken and Timasheff, 1967; Hade and Tanford, 1967; Seery *et al.*, 1967; Ullman *et al.*, 1968; Reisler and Eisenberg, 1969; Schachman and Edelstein, 1966). Solvent binding, if present, has been shown to change the apparent molecular weight of proteins by only 5–10%. The molecular weight of 52,900 calculated in the present experiments is obviously much closer to that of the native enzyme, which was determined by equilibrium sedimentation using the same value for $\bar{v}$, than it is to a possible subunit of molecular weight of 29,000.

The explanation for the discrepancy between these results and the value of 32,000 in 6.5 M urea reported by Magar and Robbins (1969) is uncertain. However, the evidence for the homogeneity of their preparation was not presented thus making it impossible to assess the comparability of the samples used. By contrast, the isocitrate dehydrogenase used in the studies here reported has been demonstrated to yield one peak in sedimentation velocity studies, a linear plot of $\ln c$ *vs.* x^2 in nondenaturing and denaturing solvents, and a single band in electrophoresis in buffers over a wide range of pH (Colman, 1968) and in 2% sodium dodecyl sulfate. Furthermore, an unusually low value of $\bar{v}$ was assumed in the study of Magar and Robbins (0.70) and it is unclear whether the appropriate correction for the temperature dependence of the density was made for the ultracentrifuge run at 5°.

It is interesting that the existence of a single polypeptide chain in the pig heart TPN-specific isocitrate dehydrogenase differs from the observation of two subunits in the enzyme from *Bacillus stearothermophilus* (Howard and Becker, 1970). The bacterial and mammalian enzyme vary in other properties as well, such as their electrophoretic behavior and the molecular weight of the native enzyme. Further comparisons between these enzymes may aid in the designation of those features of the enzyme which are indispensable for function.

Acknowledgments

We are appreciative of the assistance given by Drs. Linda and Alexander U. Bertland, II in the ultracentrifugation experiments.

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Subunit Homologies in Bacterial Luciferases*

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ABSTRACT: The tryptic peptides of the nonidentical subunits (α and β) of luciferase from both *Photobacterium fischeri* (Pf) and a second strain (MAV) were compared to evaluate possible homologies.

Three identical small peptides were found in one of the subunits from each of the strains.

This relationship, together with previous supporting evidence, led to a relabeling of the subunits of MAV, so that homologous pairs from the two strains are given the same Greek letter designation.

Bacterial luciferase from *Photobacterium fischeri* (Pf) is a bioluminescent protein which contains two nonidentical peptide chains (α and β) each with a molecular weight of about 4×10^4 (Friedland and Hastings, 1967). Recently, luciferase has been isolated from a different strain of luminous bacteria (designated as strain MAV) and likewise shown to contain two nonidentical polypeptide chains of similar molecular weights (Hastings *et al.*, 1969). Furthermore, neither of the luciferase chains from MAV was identical with those from Pf. This was deduced from amino acid compositions, electrophoretic mobilities, molecular weights, and complementation experiments. However, since both luciferases utilize the same substrates, it might be expected that certain sequences from the different polypeptide chains would be identical, especially for those residues essential for enzyme activity. In order to investigate possible homologies, tryptic maps of the individual subunits were compared. These experiments led to the identification of two dipeptides, Gly-Arg and Met-Lys, and more importantly, a tetrapeptide, Gly-(Trp,Gln)-Arg in one of the subunits from each strain.

The subunits from both strains were originally assigned α and β designations on the basis of their relative electrophoretic mobilities in 8 M urea (Hastings *et al.*, 1969). At that time there were indications (molecular weight and extinction coefficient) that Pf α-MAV β and Pf β-MAV α might be the more closely homologous pairs. Strong evidence in support of this relationship has been obtained in this work and we have therefore relabeled the MAV subunits so that the homologous pairs are given the same Greek letter designation. Thus, MAV α (formerly MAV β) is homologous with Pf α and MAV β (formerly MAV α) is homologous with Pf β.

Materials and Methods

Materials. Bacterial luciferase from Pf and MAV was prepared as described previously (Hastings *et al.*, 1965, 1969). The separated polypeptide chains (α and β) of both Pf and MAV were prepared by chromatography on DEAE-cellulose in 8 M urea as described by Friedland and Hastings (1967). The respective fractions were pooled and the urea removed by dialysis. All chemicals were reagent grade unless otherwise noted.

Tryptic Digestion. The separated polypeptide chains were dialyzed into 0.05 M *N*-ethylmorpholine (pH 8.0) to give a partially soluble suspension. The absorbance at 280 nm of the protein solutions was determined by dissolving an aliquot of the suspension in 8 M urea. Prior to digestion with trypsin, the protein (approximately 10 mg/ml) was incubated for 30 min in a boiling-water bath. This was found to be necessary to achieve complete tryptic digestion of the native enzymes. The solutions were then cooled to room temperature and L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone trypsin was added at a ratio of 1 mg/50 *A* units at 280 nm (as determined in 8 M urea). The solution was incubated at 37° for 5 hr, a small amount of insoluble matter removed by centrifugation, and the digestion terminated by freezing the solution.

Peptide Maps. The tryptic digests were electrophoresed on Whatman No. 1 paper (0.5 mg/cm) in pH 6.5 buffer (100 ml of pyridine–3 ml of glacial acetic acid–879 ml of water) for 45 min at 3000 V. The resulting sample was then stitched onto a second sheet of Whatman No. 1 paper; electrophoresis in pH 1.8 buffer (2% formic acid–8% acetic acid) was con-

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