Substrate Independence of Molecular Weight of Triphosphopyridine Nucleotide-specific Isocitrate Dehydrogenase*

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SUMMARY

The techniques of gel filtration and light scattering were used to evaluate a report that the molecular weight of the TPN-specific isocitrate dehydrogenase of pig heart can be influenced by its substrates. The results here presented indicate that the elution position of the enzyme from a column of Sephadex G-150 is not appreciably altered by the addition of isocitrate, manganous ion, TPN, α-ketoglutarate, or TPNH. The Stokes radius is estimated as 39.3 Å. Similarly the weight average molecular weight of the enzyme, as measured by light scattering, does not vary significantly from 58,000 when isocitrate, α-ketoglutarate, metal ion, and the coenzymes are added. It is concluded that the pig heart TPN-dependent isocitrate dehydrogenase exists as a single molecular weight species independent of the presence of substrates.

Pig heart TPN-specific isocitrate dehydrogenase has been reported to consist of a single polypeptide chain with a molecular weight of approximately 58,000 on the basis of equilibrium centrifugation studies in a nondenaturing buffer as well as in urea and guanidine hydrochloride solutions, electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate, and gel filtration in guanidine hydrochloride (1). However, Kemper and Kaplan (2) have recently reported that the active species catalyzing the reduction of TPN is a 30,000 molecular weight monomer, whereas the species catalyzing the oxidation of TPNH is a 120,000 molecular weight species; these conclusions were drawn from the sedimentation constants measured in the presence of substrates. The mechanisms of regulation of enzymes which undergo reversible association-dissociation reactions (3, 4) are quite distinct from those characteristic of a single peptide chain enzyme with an invariant molecular weight. It, therefore, seemed important to assess by independent methods which of these categories best describes the TPN-dependent isocitrate dehydrogenase. This paper presents gel filtration and light scattering experiments demonstrating that the molecular size of this enzyme is not influenced by the addition of its substrates isocitrate, TPN, TPNH, α-ketoglutarate, or manganous ion.

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The TPN-specific pig heart isocitrate dehydrogenase was obtained from the Boehringer Mannheim Corp. and was purified 10-fold by column chromatography on carboxymethylcellulose followed by gel filtration on Sephadex G-150 as described previously (5). The resulting preparation is homogeneous in the ultracentrifuge and on cellulose acetate and polyacrylamide gel electrophoresis under mild and denaturing conditions (1, 5).

The peak of the elution profile from a gel filtration column reflects the molecular size of the protein. Fig. 1 records the elution position of isocitrate dehydrogenase when filtered in buffer alone, with added isocitrate and manganous ion, with α-ketoglutarate, MnSO₄, and TPNH, and with isocitrate, MnSO₄, and TPN. The last experiment represents a complete reaction mixture and the enzyme catalyzes the oxidative decarboxylation of isocitrate to its final equilibrium position during the course of its migration through the column. In all of these cases, the buffer contains 10% glycerol which has been cited by Kemper and Kaplan (2) as stabilizing the tetramer. It is notable that the peak positions and the width of the elution profiles shown in Fig. 1 do not vary significantly with the composition of the buffer and thus no change in the molecular size of the enzyme seems to be induced or stabilized by the addition of these substrates.

The data of Fig. 1 were used to calculate a distribution coefficient, $K_d$, which is defined as:

$$K_d = \frac{V_e - V_o}{V_e - V_o - m \cdot \mu}$$

where $V_e$ is the total volume of the gel bed, $V_o$ is the elution volume of the sample, $m$ is the void volume determined using dextran blue, and $m$ and $\nu$ are the weight and partial specific volume of the gel matrix. The value of $K_d$ for isocitrate dehydrogenase in buffer alone is 0.216 and does not change appreciably in the presence of substrates. The position of elution from a Sephadex column is determined primarily by the Stokes radius ($\alpha$) of the protein (6). Using bovine serum albumin ($K_d = 0.252; \alpha = 3.70$ nm) and cytochrome c ($K_d = 0.542; \alpha = 1.74$ nm) to calibrate the column, the Stokes radius of isocitrate dehydrogenase was estimated by the method of Ackers (8) to be 3.93 nm. Assuming a molecular weight of 58,000, a fractional ratio ($J/J_0$) of 1.62 may be calculated, indicating that the isocitrate dehydrogenase molecule may be somewhat elongated. It is interesting that when the enzyme is denatured, as is the case during gel filtration in buffers containing 6 M guanidine hydrochloride, it is eluted behind bovine serum albumin and exhibits a molecular weight of 58,000 (1).

Gel filtration is a relatively slow process and yields information on the size of the molecule which has been in contact with the substrates for a period of hours. In contrast, the complementary technique of light scattering provides the possibility of observing directly within minutes the effect on the weight average molecular weight of the addition of substrates.

The light scattering measurements were made at 90° at a wave length of 436 nm in a Phoenix-Brice Universal light scattering photometer. The experiments were conducted at 22° in 0.03 M triethanolamine chloride buffer, pH 7.4, under conditions similar to those which have been used for the determination of kinetic
Molecular weight determined by light scattering in presence and absence of substrates

These experiments were conducted at 22° in 0.03 M triethanolamine chloride buffer, pH 7.4. All substrates are present at concentrations that are equal to 10 times their Michaelis constants or greater (8). The values given in this table represent the averages of at least four experiments.

| Experiment | Additions to enzyme solution | Molecular weight |
|------------|-----------------------------|------------------|
|            | 0.44 mM isocitrate           |                  |
| 1          |                             |                  |
| 2          |                             |                  |
| 3          |                             |                  |
| 4          |                             |                  |
| 5          |                             |                  |
| 6          |                             |                  |

The apparent conflict between the conclusions of this paper regarding the lack of influence of substrates on the molecular weight of isocitrate dehydrogenase and the report of Kemper and Kaplan (2) is difficult to resolve. These authors apparently used the procedure of Cohen and Mire (14) in which a thin barrel of enzyme solution is layered onto a substrate solution in a rotating ultracentrifuge cell. The analysis by this method assumes that the substrate is present at saturating concentrations throughout the enzyme band. Cohen and Mire caution that the failure to achieve these conditions may yield an apparent sedimentation constant that is erroneously high. Since the Michaelis constants for α-ketoglutarate and carbon dioxide are several orders of magnitude higher than that for isocitrate, adequate conditions would be less readily maintained in the reductive carboxylation reaction. In fact, it is difficult to see how saturation with respect to carbon dioxide could be achieved under the conditions of ultracentrifugation. The details of the experiments of Kemper and Kaplan have not yet appeared, making it impossible to evaluate their methods.

Most mammalian tissues have been shown to contain two isocitrate dehydrogenases, a DPN-specific enzyme located primarily in the mitochondria and a TPN-dependent enzyme found both in the mitochondria and the cytoplasm (15). The two enzymes catalyze similar reactions, but differ in several structural and functional characteristics including the actual form of isocitrate which functions as the substrate (9, 16), the molecular parameters of isocitrate dehydrogenase (5). The enzyme solutions were cleaned by centrifugation for 20 min at 20,000 rpm, followed by filtration through a fine sintered glass filter. Buffer and substrate solutions were filtered separately. The protein concentration was determined from the absorbance at 280 nm (3) using aliquots of the enzyme solutions after filtration. The measured refractive index of the solvent was 1.341. The molecular weight was derived from the following equation (7).

\[ \frac{H_0}{T} = \frac{1}{M_w} + 2\beta c \]

However, since \( H_0/T \) did not vary over the protein concentration range used (0.17 to 0.75 mg per ml), the last term was neglected.

The weight average molecular weights for isocitrate dehydrogenase as determined by light scattering are recorded in Table I. In the absence of substrates (Line 1) the enzyme exhibits a molecular weight of 63,300, which agrees reasonably well with the value of 58,000 measured by equilibrium centrifugation in buffer (5). The manganese complex of tribasic isocitrate has been shown to be the substrate for this enzyme (9) and to bind in the absence of pyridine nucleotide (10). The addition of isocitrate and MnSO_4 (Line 2) to the enzyme solution does not significantly change the measured molecular weight. When TPN is added (Line 3), oxidative decarboxylation of isocitrate proceeds; however, no marked decrease in the molecular weight is noted either immediately after addition or after 30 min, when the reaction has reached equilibrium. The presence of higher concentrations of α-ketoglutarate and TPNH (Lines 4 to 6) produces no large increase in the molecular weight; in fact, the molecular weight observed was remarkably constant throughout these experiments. An average of all of the listed values is 58,400, which is essentially identical with the reported determinations made in the analytical ultracentrifuge under both mild and denaturing conditions in the absence of substrates (1, 5).

These results lead to the conclusion that the TPN-dependent isocitrate dehydrogenase exists as a single molecular weight species whether or not substrates are present. The postulation of an active 30,000 molecular weight species (2) is highly improbable on chemical grounds. On the basis of a molecular weight of 58,000 it has been shown that the enzyme binds 1 mole of isocitrate, α-ketoglutarate, and manganese ion (10, 11); catalytic activity is lost as the result of modification of a single methionyl residue (5); and only one NH₂-terminal alanine has been identified in the Edman reaction (12). Furthermore, the number of peptides detected in a “fingerprint” obtained by paper chromatography and electrophoresis of tryptic digests of the enzyme is consistent with the existence of a single polypeptide chain of molecular weight 58,000 (1). No evidence suggesting the formation of a 120,000 molecular weight species of this enzyme has been found in the present investigation. If the enzyme existed in a monomer-dimer-tetramer equilibrium with preferential binding of isocitrate by the monomer and of α-ketoglutarate by the tetramer, nonlinear Scatchard plots for the binding of these substrates would have been anticipated (3, 13). On the contrary, linear Scatchard plots have been observed for both isocitrate and α-ketoglutarate (11).
weight (17), and the susceptibility to allosteric modification. The insensitivity to the presence of ligands of the molecular weight of the TPN-dependent isocitrate dehydrogenase, which is here demonstrated, stands in contrast to the reported ability of the DPN-specific isocitrate dehydrogenase to undergo self-association promoted by its activator ADP (17). It is apparent that the mode of regulation of the structure and catalytic activity of these two enzymes must be distinct.

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