Re-evaluation of Molecular Weight of Pig Heart NAD-specific Isocitrate Dehydrogenase*

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NAD-specific pig heart isocitrate dehydrogenase was earlier reported, on the basis of gel filtration experiments, to have a molecular weight of approximately 340,000. In the present study, the enzyme is shown by equilibrium ultracentrifugation to have a weight average molecular weight of approximately 224,000 which can be attributed to a rapidly associating-dissociating protein system. The results of light-scattering measurements are consistent with the lower value of molecular weight. The enzyme exhibits an average frictional ratio, \( f/f_0 \), of 1.39 as determined from ultracentrifuge experiments, and this deviation from typical proteins may account for the average high molecular weight estimates. An average Stokes radius of 6.0 nm was calculated from the present gel filtration experiments. By use of this value and a sedimentation coefficient of 9.1 S, an average molecular weight of 245,000 has been calculated. Previous studies (Ramachandran, N., and Colman, R. F. (1980) J. Biol. Chem. 255, 8859–8864) have indicated that the enzyme is composed of three different subunits, present in the ratio 2:1:1, each of which has a molecular weight of about 40,000. These results, together with the present observations, lead to the conclusion that, under stabilizing conditions in solution, the NAD-dependent isocitrate dehydrogenase predominantly exhibits a minimum molecular weight of 160,000 but behaves as a mixture of oligomeric species with an average \( M_m \), of about 224,000.

The NAD-dependent isocitrate dehydrogenases (threo-D3-isocitrate:NAD\(^+\) oxidoreductase (decarboxylating), EC 1.1.1.41) purified from beef heart (1) and partially purified from pig heart (2) have been reported to have molecular weights of 333,000 to 340,000 as determined by gel filtration. However, electrophoresis of the purified pig heart isocitrate dehydrogenase on polyacrylamide gels yields several enzymatically active bands (3), and it seemed possible that the enzyme exists in more than one state of aggregation. It has recently been reported (4) that the pig heart enzyme is composed of three distinct subunits, termed \( \alpha, \beta, \) and \( \gamma \), which all have molecular weights of about 40,000 but different isoelectric points. These subunits are present in the ratio 2:1:1, and the suggestion was made that a complete NAD-dependent isocitrate dehydrogenase on polyacrylamide gels yields several enzymatic activities. The results obtained when this extraction procedure is used (4) except that the initial extraction of heart tissue was carried out in Tris, 0.01 M citrate buffer, pH 7.2, containing 10% glycerol and 2 mM MnSO\(_4\). Improved yields were obtained when this extraction procedure is used (4). The final enzyme preparation is comparable to specific activity to that described by Ramachandran and Colman (4, 5) and gives the same amino acid composition and molecular weight.

**EXPERIMENTAL PROCEDURES**

**Materials**—Sepharose 6B was purchased from Pharmacia and Bio-Gel P-300 from Bio-Rad. dL-Isocitrate, NAD, and dithiothreitol were supplied by Sigma. Protein standards used for gel filtration were obtained from Pharmacia.

The pig heart NAD-dependent isocitrate dehydrogenase was prepared as described by Ramachandran and Colman (4) except that the initial extraction of heart tissue was carried out in Tris, 0.01 M citrate buffer, pH 7.2, containing 10% glycerol and 2 mM MnSO\(_4\). Improved yields were obtained when this extraction procedure is used (4). The final enzyme preparation is comparable to specific activity to that described by Ramachandran and Colman (4, 5) and gives the same amino acid composition as well as band pattern upon electrophoresis in polyacrylamide gels containing sodium dodecyl sulfate and under isoelectric focusing in the presence of urea.

**Light-scattering Velocity Measurements**—Sedimentation velocities were measured in a Beckman model E analytical ultracentrifuge. Two samples in 0.05 M PIPES, pH 6.1, containing 0.3 mM MnSO\(_4\), 0.2 mM dithiothreitol, and 2% glycerol were sedimented simultaneously in double-sector cells using an AN-D rotor at 55,000 rpm and maintained at 20 °C. Photographs taken with schlieren optics using plane and 1° wedge windows were measured on a Nikon comparator. For measurements made using 2,5 mg/ml of isocitrate dehydrogenase, the boundary point was determined from second moments giving a weight average sedimentation coefficient (6). Corrections were made for base-line distortion due to the presence of glycerol. A partial specific volume, \( \varepsilon \), of 0.737 was calculated from the amino acid composition (4) and partial specific volumes for the amino acids given by Lee and Timasheff (7). Sedimentation coefficients were calculated for water at 20 °C using this calculated partial specific volume and correcting for solvent density, \( \rho \) (1.046 g/cm\(^3\)), and viscosity, \( \eta \) (1.704 c.p.) (8).

**Molecular Weight Determination by Sedimentation Equilibrium**—The molecular weight was determined using the meniscus-depletion method (9). Measurements were made at 12,000 and 18,000 rpm in a Beckman model E analytical ultracentrifuge with interference optics. Three sample/buffer pairs were centrifuged in a six-channel cell. Photographs were taken at several time intervals starting at 18 h. The identity of fringe displacements at 18 and 24 h indicate that, at 20 °C, equilibrium was achieved in this time period. At 4 °C, the photographs were taken at 64, 72, and 88 h; within this time period, the fringe patterns were approximately the same. After measurements were taken to ascertain that equilibrium was reached, the speed of the centrifuge was changed and photographs were taken at intervals until equilibrium was again reached. Control samples of enzyme which were not centrifuged retained approximately 70% of their initial activity after 42 h at 20 °C.

Measurements of the photographic plates were made using a Nikon comparator. Near-the-meniscus measurements were made at 500-μm intervals. When the fringe displacements were about 40 μm, the intervals were 200 μm or 100 μm. Near the base of the cell an interval of 50 μm was used. The measurements of fringe displacements were made on five fringes and the average value was used in subsequent calculations. Point-by-point weight and number average molecular weights were calculated using procedures outlined by Teller (10) (equations 8, 54, 25, 27). The slopes of ln \( C \) versus \( r^2 \) curves (where \( C \) is measured by fringe displacement and \( r \) is the radial distance in the rotor) were obtained from least square fits to five points.

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* This work was supported by United States Public Health Service Grant AM 17552. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 E. V. Stevens and R. F. Colman, manuscript in preparation.

2 The abbreviation used is: PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid).
Molecular Weight Determination by Light Scattering—The light-scattering measurements were made at 90° at a wavelength of 436 nm in a Phoenix-Brice Universal light-scattering photometer. The experiments were conducted at room temperature in 0.05 M PIPES buffer, pH 6.1, containing 1 mM MnSO$_4$, 0.2 mM diithiothreitol, and 20% glycerol, which exhibited a refractive index of 1.374. The enzyme solutions were cleaned by centrifugation for 20 min at 15,000 rpm, followed by filtration through a fine sintered glass filter. Buffer and substrate solutions were filtered separately. The protein concentration in milligrams per ml was determined after filtration by multiplying the absorbance at 280 nm by 1.55 (11). The molecular weight was determined from the following equation (12):

\[
\frac{Hc}{\tau} = \frac{1}{M_w}
\]

and the possibility that \( Hc/\tau \) varies with protein concentration was tested.

Gel Filtration—Sepharose 6B and Bio-Gel P-300 were packed in 1.5-cm diameter columns to a depth of approximately 30 cm. The sample volumes applied were 0.5 ml. Filtration was done at 4 °C. Protein concentrations were determined either by absorbance at 280 nm or by use of the Bio-Rad Protein Assay which is based on the method of Bradford (13). The elution behavior of isocitrate dehydrogenase was monitored from its catalytic activity and sometimes also from protein determinations. The Stokes radii (\( R_s \)) of porcine muscle lactate dehydrogenase and thyroglobulin were calculated from published diffusion coefficients (14); the \( R_s \) values for the other standard proteins were those listed by Ackers (15).

RESULTS

Sedimentation Velocity—Sedimentation coefficients (\( s_{20,W} \)) determined by using the peak of the schlieren patterns were 9.1, 9.0, and 8.8 S at enzyme concentrations of 0.4, 1.0, and 2.4 mg/ml, respectively. At 2.4 mg/ml, a sedimentation coefficient of 9.1 S was determined by using second moments (6). In all cases, a single, apparently symmetrical schierien peak was observed. From the small concentration dependence of the sedimentation coefficient, the association of the subunits did not appear to be dependent on protein concentration in the range of 0.4 to 2.4 mg/ml. Isocitrate (1 mM) caused a small change in the sedimentation coefficient at either 2.4 mg/ml of enzyme (\( s_{20,W} = 9.5 \) S) or at 1 mg/ml of enzyme (\( s_{20,W} = 10.3 \) S).

Sedimentation Equilibrium—A typical plot of \( \ln C \) versus \( r^2 \) is shown in Fig. 1A. A small but definite curvature may be seen, indicative of heterogeneity. Point-by-point number (\( M_n \)) and weight average (\( M_w \)) molecular weights were calculated and are shown in Fig. 1B. These values were extrapolated to zero concentration (zero fringe displacement) to obtain the minimum molecular weight. Number average molecular weights obtained at points within approximately 0.6 mm of the base of the cell were extrapolated to the base of the cell to obtain the weight average molecular weight of the sample (\( M_w \)) (9). Values of minimum molecular weights and weight average molecular weights are given in Table I for a variety of conditions.

At pH 6.1 the weight average molecular weight (experiments 1 to 6, Table I) shows little dependence upon protein concentration. The results are also independent of rotor speed, indicating that errors due to a finite concentration at the meniscus are not appreciable. Since measurements at different rotor speeds were taken on the sample after different times, the identity of results at different speeds also indicates that the heterogeneity is not a consequence of denaturation during the time course of the centrifugation. The dependence of the weight average molecular weight on fringe displacement is not a function of initial protein concentration. This is evidence that the heterogeneity is not primarily the result of irreversible enzyme dissociation (10). The mean weight average molecular weight from experiments 1 to 6 is 224,000 ± 8,000 (standard deviation). The results at pH 7.0 are similar (experiments 7 to 10) with a slight decrease in the weight average molecular weight.

Extrapolations of point molecular weights to zero concentration generally give values in the range 160,000 to 180,000. In some cases a break is observed at small fringe displacements, and the initial part of the curve extrapolates to a minimum molecular weight of approximately 125,000. Since relative errors are large at low displacements these extrapolated values must be interpreted cautiously.

Addition of 1 mM isocitrate results in a small increase in the weight average molecular weight at either pH 6.1 (experiments...
Molecular Weight of NAD-specific Isocitrate Dehydrogenase

Table I
Molecular weights from sedimentation equilibrium

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Protein concentration (mg/ml)</th>
<th>pH</th>
<th>Temperature (°C)</th>
<th>Rotor speed (rpm)</th>
<th>Ligand</th>
<th>M, min* (× 10^6)</th>
<th>M, (× 10^6)</th>
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<tr>
<td>1</td>
<td>1.0</td>
<td>6.1</td>
<td>20</td>
<td>12,000</td>
<td>Isocitrate</td>
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<td>229</td>
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<td>2</td>
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<td>20</td>
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<td>Isocitrate</td>
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<td>226</td>
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<tr>
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<td>160</td>
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</tr>
<tr>
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<tr>
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<td>224</td>
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<tr>
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<tr>
<td>11</td>
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<td>270</td>
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<tr>
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</tr>
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<tr>
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<td>239</td>
</tr>
<tr>
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<td>6.1</td>
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<td>Isocitrate</td>
<td>137</td>
<td>219</td>
</tr>
<tr>
<td>16</td>
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<td>6.1</td>
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<td>Isocitrate</td>
<td>134</td>
<td>228</td>
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<td>Isocitrate</td>
<td>166</td>
<td>226</td>
</tr>
<tr>
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<td>0.6</td>
<td>7.0</td>
<td>20</td>
<td>18,000</td>
<td>Isocitrate</td>
<td>164</td>
<td>228</td>
</tr>
<tr>
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<td>0.5</td>
<td>7.0</td>
<td>4</td>
<td>12,000</td>
<td>Isocitrate</td>
<td>177</td>
<td>217</td>
</tr>
<tr>
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<td>0.5</td>
<td>7.0</td>
<td>4</td>
<td>12,000</td>
<td>Isocitrate</td>
<td>160</td>
<td>241</td>
</tr>
</tbody>
</table>

* Minimum molecular weight and weight average molecular weight were determined from data such as are shown in Fig. 1B as described in the text.

** Protein with molecular weight below 160,000 is more readily detectable in samples with or without isocitrate at 1 mg/ml of initial enzyme concentration than at lower initial concentrations, implying that a small amount of non-equilibrium dissociation may occur.

***The buffer was 0.05 M Tris/citrate.

11 to 16) or pH 7.0 (experiment 17). The mean value is 238,000 ± 17,000. A minimum weight molecular weight of about 160,000 is observed.

At 4 °C (experiments 18 to 20), the weight average molecular weights and minimum molecular weights do not differ appreciably from the values obtained at 20 °C. However, at 4 °C, the weight average molecular weight declined after 88 h, indicating that irreversible dissociation was probably occurring at this long time period under these conditions. When the temperature is raised to 25°C (experiments 13 and 14), the weight average molecular weight is not changed significantly.

The point values of M, and M, may be used to calculate a possible stoichiometry of association by means of the two species equation (10):

\[ n = \frac{M_r(M_s - M_t)}{M_t(M_s - M_r)} \]  

where \( n \) is the stoichiometry of association and \( M_t \) is the lowest molecular weight of the associating species. The value of \( n \) is found to be in the range 1.9 to 2.4 for several of the experiments summarized in Table I, provided only data with \( M_s > 160,000 \) are used. However, the agreement of these values of \( n \) with 2.0 does not exclude models which include more complex equilibria with more than two species.

From the sedimentation coefficient, \( s \), and mean molecular weight, \( M_r \), a frictional coefficient may be calculated (6, 16):

\[ f = \frac{M_r(1 - \omega^2)}{N s} \]  

where \( N \) is Avogadro's number. This may be compared with the theoretical frictional coefficient for a sphere of equivalent molecular weight:

\[ f = 6\pi n \frac{(3M_r^2)}{4nN} \]  

where \( f \) is the frictional coefficient, \( M_r \) is the molecular weight of the associating species, and \( n \) is Avogadro's number. This may be compared with the theoretical frictional coefficient for a sphere of equivalent molecular weight.

In the absence of isocitrate, \( f = 1.04 \times 10^{-7} \) g/s and \( f/f_0 = 1.39 \), indicating significant departure from sphericity and/or hydration. The diffusion coefficient, \( D = kT/f \), can be obtained from the experimentally derived frictional coefficient to yield a value of \( 3.9 \times 10^{-7} \) cm²/s at 20 °C. The ratio \( f/f_0 \) is the same in the presence of isocitrate, indicating no observable changes in gross structure. The somewhat higher molecular weights found in the presence of isocitrate probably result from a stabilization of more aggregated species.

Light Scattering—The weight average molecular weights of the NAD-dependent isocitrate dehydrogenase as determined by light scattering are illustrated in Table II. The weight molecular weight appeared to be independent of the protein concentration from 0.17 to 1.3 mg/ml, yielding an average value of 192,000. The measured average molecular weight of the enzyme varied somewhat with the preparation, although most preparations exhibited values under 220,000. These results are in general agreement with those obtained by the method of sedimentation equilibrium. The addition of 1 mM isocitrate to enzyme present at 0.4 mg/ml or 0.6 mg/ml led to no appreciable change in the molecular weight; and the molecular weight of enzyme maintained in the presence of constant concentrations of 1 mM isocitrate and 1 mM MnSO₄ did not change significantly as the protein concentration was varied from 2.1 to 0.4 mg/ml.

Gel Filtration—It has previously been reported (2) that filtration of partially purified pig heart NAD-specific isocitrate dehydrogenase through Sepharose 6B resulted in a single peak of enzymatic activity that eluted at a position corresponding to a molecular weight of 340,000 on a plot of log molecular weight versus \( K_D \). The same apparent molecular weight was obtained in the present study of highly purified enzyme. However, Ackers (15) has pointed out that this plot is not suitable for loosely cross-linked gels because the elution of proteins from these gels is dependent on ionic strength and frictional interactions as well as simply on exclusion. If the protein of interest differs significantly from the standards in shape and/or hydration, its behavior on gel filtration can best be described in terms of its Stokes radius. Therefore, the data were analyzed by the statistical treatment developed by Ackers (17) and modified by Nozaki et al. (18), in which the Stokes radii of standard proteins are plotted against the inverse error function of \( 1 - K_D \). Although the rigorous treatment uses \( K_D \), the ratio of penetrable volume to total interior solvent volume, the high water regain of Sepharose 6B causes the interior solvent volume to be nearly equivalent to the total column volume \( V_c \). Therefore, the data have been plotted using \( erf^{-1}(1 - K_{AV}) \) where \( K_{AV} = (V_s - V_D)/(V_s - V_c) \), and where \( V_s \) is the elution volume and \( V_D \) is the void volume.

The position of isocitrate dehydrogenase on the standard curve is indicated by the arrow in Fig. 2 and corresponds to a

Table II
Molecular weights determined by light scattering

<table>
<thead>
<tr>
<th>Protein concentration (mg/ml)</th>
<th>Molecular weight (× 10^6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.30</td>
<td>187,000</td>
</tr>
<tr>
<td>0.91</td>
<td>212,000</td>
</tr>
<tr>
<td>0.79</td>
<td>180,000</td>
</tr>
<tr>
<td>0.54</td>
<td>193,000</td>
</tr>
<tr>
<td>0.42</td>
<td>192,000</td>
</tr>
<tr>
<td>0.26</td>
<td>195,000</td>
</tr>
<tr>
<td>0.17</td>
<td>188,000</td>
</tr>
</tbody>
</table>
Dehydrogenase (1 mg/ml) was filtered, 2 mM MnSO₄ and 0.1 mM dithiothreitol were also present. The standard proteins were: thyroglobulin (molecular weight 669,000, function complements of 1 – Xₜ), and pig heart muscle lactate dehydrogenase (molecular weight 142,000, function 2). When measured by sedimentation equilibrium, the average molecular weight of the pig heart isocitrate dehydrogenase is about 224,000 and the sedimentation equilibrium results (Fig. 1) indicate some degree of heterogeneity, the enzyme in solution must, rather than consisting of either pure tetramer (160,000) or of pure octamer (320,000), be composed of a mixture of oligomeric species. The failure to observe more than one discrete peak in the sedimentation velocity or gel filtration experiments suggests that equilibrium among the predominant enzyme species occurs rapidly. Although in the present studies no significant dependence of average molecular weight upon protein concentration has been observed with protein concentrations ranging from 0.17 to 2.4 mg/ml, these results must be interpreted cautiously. An associating-dissociating system with each species having different concentration-dependent terms could explain the apparent lack of sensitivity of the light scattering and sedimentation velocity measurements to changes in protein concentration over the range tested.

The NAD-dependent isocitrate dehydrogenase has been shown to be composed of three types of subunits: α exhibiting a molecular weight of 39,000, and β and γ each with molecular weights of 41,000 (4). Since the α, β, and γ subunits occur in the ratio of 2:1:1, respectively, it might be expected that 160,000 would be the minimum molecular weight for an intact enzyme molecule. In fact, the present physical studies indicate that, when maintained in solution in an environment in which it is reasonably stable, isocitrate dehydrogenase generally exhibits a minimum molecular weight of 160,000. However, it is not known whether all three types of subunits are absolutely required for enzymatic activity. Recent studies of Ehrlch and Colman demonstrate that the enzyme has only 2 mol of binding sites/160,000 g of protein or one binding site per two subunits on average for a variety of ligands, e.g., isocitrate, Mn, NAD, and ADP (20). These results may be interpreted to indicate that only half of the subunits are catalytic; but, until these components have been studied in isolation, their function, whether catalytic, regulatory, or structural, cannot be defined conclusively. Experiments are in progress which are aimed at separating the three types of subunits under conditions which preserve their native structure.

**DISCUSSION**

Although the results of early studies (1, 2) of mammalian NAD-specific isocitrate dehydrogenase suggested that the usual form of the enzyme was an octamer of about 340,000 molecular weight, it has more recently become apparent that the enzyme might dissociate into smaller active species (3, 19). In the present study, both ultracentrifugation and light scattering have yielded an average value of 245,000. This value is in reasonable agreement with that of 224,000 determined by equilibrium ultracentrifugation.

**REFERENCES**


![Graph: Standard curve of Stokes radii (Rₛ) versus the error function complements of (1 – Xₜ) for Sepharose 6B. The column dimensions were 1.5 x 27 cm, and the buffer was Tris/citrate (50 mM in citrate), pH 7.0, containing 20% glycerol. When isocitrate dehydrogenase (1 mg/ml) was filtered, 2 mM MnSO₄ and 0.1 mM dithiothreitol were also present. The standard proteins were: thyroglobulin (molecular weight 669,000, function complements of 1 – Xₜ), and pig heart muscle lactate dehydrogenase (molecular weight 142,000, Rₛ 4.2 nm). The position of the enzyme is indicated by the arrow labeled ICDH.](image-url)
Molecular Weight of NAD-specific Isocitrate Dehydrogenase