Molecular Characteristics of Rat Liver Arginase*

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SUMMARY

The molecular weight of approximately 1,500-fold purified arginase from rat liver (specific activity = 19,500 μmoles of urea per min at 25° per mg of protein nitrogen) was determined by the method of sedimentation equilibrium to be 118,000. The sedimentation velocity constant is 6.1 S, the diffusion coefficient 5.2 × 10^{-7} cm^2 per sec. Alteration in pH, removal of Mn^{2+}, and replacement of Mn^{2+} by Co^{2+} did not yield evidence of a change in molecular size. The partial specific volume (ρ) was determined by a differential sedimentation equilibrium technique in heavy water. Dissociation of the enzyme in 8 M urea yielded a protein which sedimented in a single boundary and gave one protein band after acrylamide gel electrophoresis. Sedimentation equilibrium analysis of the dissociation compound gave a molecular weight of 30,800, which suggests that rat liver arginase is composed of four subunits.

Amino acid analysis has been carried out and the values compared to those of arginases from other species.

Differences in substrate inhibition, molecular weight, K_m, and antigenicity of arginases from ureotelic and uricotelic species have been found (1). The recently reported preparation of highly purified rat liver arginase (L-arginine amidinohydrolase, EC 3.5.3.1) (2, 3) is of interest because of its different electrochemical characteristics from that of purified horse (4, 5) and calf liver arginase (6). Whereas the latter are electro-negative in the physiological pH range, rat liver arginase is electro-positive.

Lack of information on the molecular characteristics of this species of arginase, as well as the lack of information on the number of polypeptide chains per molecule of any species of arginase, prompted us to undertake a study of the molecular properties of rat liver arginase.

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EXPERIMENTAL PROCEDURE

Materials and Methods

α-Isonitrosopropiophenone was obtained from Eastman Kodak Company, Rochester, New York; L-arginine (free base) from Sigma; tris(hydroxymethyl)aminomethane (A grade) from Calbiochem. Deuterium oxide, 99.9%, was purchased from Bio-Rad Laboratories, Richmond, California, and carboxymethyl cellulose from Schleicher and Schuell, Keene, New Hampshire.

Rat liver arginase was purified by a procedure reported by Schincke (3) which consists of certain steps used by Greenberg (5) for horse liver arginase and Grassmann, Hormann, and Janowsky (6) for the isolation of calf liver arginase. The purification procedure was usually carried out with the livers of 5 to 10 Sprague-Dawley rats (approximately 400 g in weight). The fresh livers were either immediately worked up or frozen at -20°.

In crude extracts the protein was measured by the biuret method (7) with crystalline bovine serum albumin (Nutritional Biochemicals Corporation) as standard. For more purified arginase preparations, the absorbance was read in a Gilford spectrophotometer at 280 and 320 μm and the protein nitrogen concentration was calculated according to the method of Bach and Killip (8). The nitrogen content of rat liver arginase was determined by micro-Kjeldahl procedure to be 14.3%.

Enzyme activity was assayed either by incubation of arginase in arginine solutions for 10 min at 25° and determination of the urea with isonitrosopropiophenone (9-12) or by following the enzymatic reaction in a Cary model 14 double-beam spectrophotometer at 2057 Å (13). Because of the high absorbance of protein in the far ultraviolet, this last method can only be used for purified preparations of arginase. By using quartz cells with 10-mm, 1-mm, and 0.5-mm path lengths the enzyme reaction can be followed at substrate concentrations of 10^{-4} to 2 × 10^{-7} M. Fig. 1 shows a substrate dependence curve, applying both methods. It should be mentioned that the spectrophotometric procedure is a very simple and rapid method, but can only be used with difficulty at concentrations of substrate saturation.

One enzyme unit is defined as the amount of enzyme that produces 1 μmole of urea per min at 25° (9). Specific activity is expressed in enzyme units per mg of protein nitrogen.

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At attainment of equilibrium was judged by following the blank-0.4 to 1.5 mg per ml and dialyzed against solvent prior to each optimal rotation rate of 20,410 rpm by using a multichannel out according to the method of Yphantis (16) at a calculated the gradient curve was measured with a compensating polar Nikon microcomparator. The gradient curve and base-line at 20°. The enzyme samples (4 to 12 mg per ml) were dialyzed a rotation speed of 12,590 rpm. The rotor temperature was kept during the run the rotor temperature was kept at 20°. The interference patterns were magnified 50-fold with a Nikon microcomparator and the fringe displacement was determined exactly after reaching full speed, then after 36 and 48 hours. Attainment of equilibrium was judged by following the blank-corrected fringe displacement until it approached a constant value. During the run the rotor temperature was kept at 20°. The interference patterns were magnified 50-fold with a Nikon microcomparator and the fringe displacement was determined at intervals of 10 to 20 μ.

Low speed sedimentation equilibrium runs were carried out at a rotor speed of 5784 rpm. Enzyme concentrations ranged from 1.5 to 3.0 mg per ml. Initial protein concentrations were determined from the number of fringes obtained in a synthetic boundary run as described by Richards and Schachman (17). The molecular weight was calculated from the slope of the loga-rithm c versus r².

The partial specific volume was determined by a differential sedimentation equilibrium technique according to the method of Edelestein and Schachman (18). The method is based upon the change produced in the equilibrium concentration distribution when the density of the solution is increased by the use of heavy water. For the experiments arginase preparations of 0.4, 0.8, and 1.2 mg per ml in either water or 99% deuterium oxide (each 0.01 m Tris-HCl buffered, pH 7.5) were dialyzed for several days against solvent.

The amount of hexoses in the enzyme preparation was assayed with anthrone according to the method of Trevelyan and Harrison (19) by using galactose as standard.

Amino acid hydrolyses were carried out with 6 × HCl as described by Moore and Stein (20). Hydrolysis temperature was 105°. The amino acids were determined with a Beckman amino acid analyzer, model 120.

For the study of the dissociation of rat liver arginase, enzyme preparations at concentrations of 0.3 to 1.0 mg per ml were dissolved in and dialyzed against 8 M deionized urea (pH 7.3) at 4°. The denaturation was achieved by shaking the urea solution with a mixed bed resin of AG 501-X8, 20 to 50 mesh, analytical grade (Bio-Rad Laboratories). In order to prevent the oxidation of sulfhydryl groups, mercaptoethanol was added to the urea solution to give a final concentration of 10⁻⁴ M.

**RESULTS**

**Enzyme Purification**

Following the isolation of the enzyme according to the pro-cedure of Schimke (3), a further purification of the arginase could be obtained by a second passage through a CM-cellulose column (10 × 1 cm). Fig. 2 shows a typical elution pattern. After the first passage through CM-cellulose (Fig. 2A), Fractions 12 to 15 (1 ml each) were pooled, lyophilized to a small volume, and dialyzed against 0.01 M Tris-HCl buffer, pH 7.5. A second CM-cellulose column was equilibrated with this buffer, and the enzyme from the first passage was introduced onto the column. The enzyme then was eluted from the column with a 0.2 M arginine-0.005 M Tris-HCl solution, pH 7.5 (Fig. 2B). The fractions which contained the enzyme activity (12 to 14) were pooled, lyophilized, dialyzed against 0.01 M Tris-HCl buffer, pH 7.5, and stored by freezing at −20°. There was only slight loss of activity over a period of several weeks of storage.

This arginase preparation gave a single protein band after acrylamide gel electrophoresis at pH 6.0 and 8.3 (Fig. 3A) and sedimented in the analytical ultracentrifuge in a single boundary (Fig. 4A).

Rat liver arginase could be chromatographed over Sephadex G-100 without loss of activity (0.01 M Tris-HCl buffer, pH 7.5). The enzyme was eluted in a symmetrical protein peak with a constant specific activity (19,500).

The preparation of highest purity obtained by the above procedure had a specific activity of 19,500 after activation in 0.05 M MnCl₂ (pH 7.5) at 55° (21). The preparation of Schimke (3) was estimated to have a specific activity of 14,800 under the

![Graph](image-url)
same conditions. This estimation was made by reducing the data of Schimke (37) to 25° by means of a temperature coefficient of 2.5, calculated from the activation energy of the arginase reaction. Under similar conditions the preparation of Kossman et al. (2) yielded a specific activity of 4,700.

**Sedimentation Velocity Coefficient**

In sedimentation experiments, carried out in a 12-mm single-sector cell and a rotor speed of 50,740, the enzyme moved in a single boundary as shown in Fig. 4. In order to determine the concentration dependence of the sedimentation velocity coefficient, experiments were performed at protein concentrations of 2 to 12.5 mg per ml in 0.01 M Tris-HCl buffer, pH 7.5. Corrections of the observed sedimentation coefficients, $s_{obs}$, to standard state (20°, water) were made (15), and no change could be observed in the sedimentation velocity coefficient of the enzyme, when the experiments were carried out at temperatures between 10 and 25°.

The $s_{20,w}$ values, calculated for various protein concentrations, are plotted in Fig. 5. For zero enzyme concentration, the sedimentation coefficient $s_{20,0}$ was estimated by extrapolation to be 6.1 S.

It was of interest to see if arginase, whose activity is reported to be rather unstable below 7.0 and above 8.0 (5), would show any dissociation or aggregation dependence on pH. By dialysis against Tris-HCl buffer solutions, enzyme samples were brought to pH 5.8, 8.3, and 9.6. Table I gives the sedimentation coefficients obtained in this series of experiments.

No change in the sedimentation behavior could be detected at various pH values. The small differences in the sedimentation coefficients given in Table I are due to differences in the protein concentration.

In another experiment arginase was dialyzed extensively against a $5 \times 10^{-3}$ M EDTA solution (pH 7.5) to remove the manganese from the enzyme complex. A sample thus treated was subsequently dialyzed against a $5 \times 10^{-3}$ M Co(NO$_3$)$_2$ solution (pH 7.5) for metal ion activation. As shown in Table I removal of Mn$^{2+}$ and subsequent replacement of Mn$^{2+}$ by Co$^{2+}$ did not change the sedimentation behavior of arginase.
Extrapolation for zero concentration yielded $D_{n,0} = 5.2 \times 10^{-2} \text{ cm}^2\text{ per sec.}$

**Partial Specific Volume ($\nu$)**

For the determination of the partial specific volume, parallel sedimentation equilibrium experiments were carried out. Parameters such as temperature, angular velocity, and protein concentration were kept constant, but the density of the solvent was varied by using either $\text{D}_2\text{O}$ or $\text{H}_2\text{O}$.

Fig. 6 shows a plot of $\log f$ versus $r^2$ for arginase in $\text{H}_2\text{O}$ or $\text{D}_2\text{O}$. From the slopes, $\nu$ was calculated by Equation 2

$$\nu = \frac{k - [(\sigma \ln c/\sigma r^2)_{\text{D}_2\text{O}}/\sigma \ln c/\sigma r^2)_{\text{H}_2\text{O}}]}{\rho_{\text{D}_2\text{O}} - \rho_{\text{H}_2\text{O}}}$$

where $c$ is the concentration, $r$ the distance from the center of rotation, $\rho$ the density of the solvent in milligrams per ml, and $k$ the measure of the exchangeability of hydrogen atoms of the protein. Experiments to determine the weight increase by deuterium exchange have been carried out with a number of proteins and gave a value of $k = 1.0155$ ($23, 24$).

This method yielded a partial specific volume of 0.75 ml per g, which is in good agreement with $\nu$ calculated from the amino acid composition as described by Cohn and Edsall (25), by which a value of 0.74 ml per g was obtained.

**Determination of Molecular Weight**

*From Sedimentation and Diffusion Coefficients*—The molecular weight ($M_w$) was calculated according to the Svedberg equation (26) and a value of 116,000 was obtained.

![Fig. 6. Sedimentation equilibrium analyses of rat liver arginase. Two separate experiments were carried out in a multichannel cell. Operational speed was 20,410 rpm. The plots represent the fringe displacement in the cell at equilibrium corrected for the blank for arginase in water ($\Delta -$) and $\text{D}_2\text{O}$ ($\Delta$). Protein concentration was 100 $\mu$g in 0.1 ml. Each solution contained 1 $\mu$ mole of Tris-HCl buffer, pH 7.5.](http://www.jbc.org/)
From Sedimentation Equilibrium—For low protein concentrations, sedimentation equilibrium experiments were carried out at a high rotor speed according to the method of Yphantis (16). When the logarithm of the fringe displacement was plotted against \( r^2 \), the points fell on a straight line.

Experiments at higher enzyme concentrations (1.5 to 3.0 mg per ml) and low rotor speed were carried out according to the method of Richards and Schachman (17).

The molecular weight \( (M_w) \) was calculated for each concentration by Equation 3

\[
M_w = \frac{2RT}{\Delta \ln c} \frac{\varphi}{\Delta r^2}
\]

where \( R \) is the gas constant, \( T \) the absolute temperature, \( c \) the concentration, \( \varphi \) the partial specific volume, \( \omega \) the angular velocity in radians per sec, and \( r \) the distance from the center of rotation. The molecular weight obtained from these two different sedimentation equilibrium techniques were plotted in a 1/M versus \( c \) diagram and fell on a straight line. Extrapolation to zero concentration (Fig. 7) yielded a molecular weight of 118,500.

To obtain further evidence of the homogeneity of the enzyme preparation \( M_r \) was calculated from these experiments according to the method of Van Holde and Baldwin (27). The weight and \( z \)-average molecular weights, extrapolated to zero concentration, are summarized in Table II. The small differences of the molecular weights obtained by these three methods as well as the close agreement of the calculated \( w \)- and \( z \)-averages can be taken as good evidence for the homogeneity of the arginase preparation.

Frictional Coefficient

From the data obtained from the diffusion experiments and the sedimentation equilibrium, the frictional coefficient was determined as described by Tanford (28) and a value of 1.26 was obtained.

Determination of Hexose Content

Greenberg, Bagot, and Roholt (4) found from the amino acid analysis of horse liver arginase that only 75\% of the dry weight was represented by amino acids, whereas 107\% of the nitrogen appeared in the form of amino nitrogen. It was concluded that this low recovery of amino acid residues per 100 g of protein could be at least partially attributable to amino acid degradation during hydrolysis. The amino acid analysis by Grassmann et al. (6) of calf liver arginase yielded 102.2\% of the nitrogen, but only 61.5\% of the dry weight represented amino acid residues. An assay with anthrone gave a hexose value of 43.7\% in the enzyme preparation. From these results Grassmann et al. concluded that arginase might be a glycoprotein.

In our experiments, samples of rat liver arginase (specific activity 19,500) were assayed with anthrone according to the method of Trevelyan and Harrison (19) and the absorption values were assayed with anthrone according to the method of Richards and Schachman (17).

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from these experiments and extrapolated to zero concentration are given in Table II.

An acrylamide gel electrophoresis run of the dissociated protein at pH 6.0 in 8 M urea solution gave a single protein band (Fig. 3B).

**DISCUSSION**

A number of criteria support the conclusion that rat liver arginase prepared by us is a single molecular species of protein. These are the nature of the ultracentrifuge sedimentation peak, the appearance of only a single protein band on acrylamide gel electrophoresis, and the good agreement of the calculated u- and z-average molecular weights. It should be noted from the recalculated values of the specific activities of the enzyme preparations of Schimke (3, 21) and Kossmann et al. (2) as well as from our findings that arginase activity is highly dependent on manganese activation.

The molecular weight $M_w$ of the enzyme, estimated by three different methods, is close to 118,000. This value is lower than the 138,000 to 142,000 reported by Schimke (21) based on sucrose gradient experiments with crystalline alcohol dehydrogenase as a standard, where no corrections for the partial specific volume or the frictional ratio have been made.

Mora, Tarrab, and Bojalil (1) reported that there is a remarkable difference between arginases from ureotelic and uricotelic species. For chicken liver arginase (30), lizard liver arginase (30), and arginase from *Neurospora crassa* (1) molecular weights of 276,000 and 278,000 were obtained. Greenberg found a molecular weight of 138,000 (5) for horse liver arginase. Our calculated molecular weight for rat liver arginase therefore fits very well into the group of the much lower molecular weights for arginases from ureotelic species.

Rat liver arginase is a comparatively stable enzyme and does not dissociate readily. Alteration in pH, removal of Mn\(^{2+}\), and replacement of Mn\(^{2+}\) by Co\(^{2+}\) did not yield evidence of a change in molecular size (Table I).

The report of Grassmann et al. (6) on the carbohydrate content of their arginase preparation, and the low values for amino acid residue recovery per unit weight of protein indicated that arginase contained a high content of bound carbohydrate. The comparatively low nitrogen content of rat liver arginase (14.3%) does suggest the possibility of a non-nitrogen containing contamination. The nitrogen value obtained from the amino acid composition is lower than the above; it does not include amide nitrogen. This tendency to support the conclusion that the low nitrogen content of the arginase is not a result of a non-nitrogen containing contamination. The analytical results with anthrone show that rat liver arginase has very low or no bound carbohydrate. Also the value obtained for the partial specific volume ($\bar{V} = 0.75$ ml per g) is characteristic of unconjugated proteins. It can be expected that a substantial amount of hexoses ($\bar{V} = 0.5$ to 0.6 ml per g) attached to the protein would substantially lower the value of $\bar{V}$ derived from sedimentation equilibrium. The fact that the partial specific volume $\bar{V}$ calculated from the amino acid composition (0.74 ml per g) is in excellent agreement with the experimentally determined value is further evidence for the absence of large amounts of hexose.

The results obtained with urea dissociated arginase lead to the reasonable conclusion that the rat liver enzyme is composed of four subunit polypeptide chains with a molecular weight of about 30,000. The linearity of the slope of the logarithm of fringe displacement (log $f$) versus the square of the distance from the center of rotation ($r^2$) indicated a comparatively homogeneous dissociation compound. Acrylamide gel electrophoresis gave a single protein band, suggesting that the polypeptide subunits are probably identical. It should be emphasized, however, that small differences in the sequence of the individual subunits would not be detected by the methods employed in this study. Studies on the identity of the subunits by peptide mapping and determination of the terminal amino acids are in progress.

Further studies on the comparative molecular properties of arginases from different genera of ureotelic vertebrates should yield interesting information of evolutionary processes and the relation of structure and enzyme activity to changing physiological requirements.

**REFERENCES**

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