Adenosine 5'-Triphosphate-Arginine Phosphotransferase from Lobster Muscle

MOLECULAR WEIGHT

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(Received 14 September 1965)

1. The molecular weight of arginine kinase from lobster muscle has been determined by three procedures: ultracentrifuge analysis, gel filtration and density-gradient centrifugation. 2. The three methods give similar results and the best estimate of the molecular weight is 37000. 3. The enzyme does not readily show association-dissociation phenomena. 4. The usefulness of density-gradient centrifugation for determinations of molecular weight is briefly discussed.

The molecular weight of arginine kinase (EC 2.7.3.3) from *Potamobius astacus* was determined by Elodi & Szorenyi (1956) using sedimentation and diffusion measurements giving a value of 43000. Pradel, Kassab, Regnouf & Thoai (1964) determined the sedimentation coefficient for the enzyme from *Homarus vulgaris* (lobster) and inferred a similar molecular weight. Our investigations suggested a rather lower molecular weight for the lobster enzyme. Determinations by several methods are presented and explanations are suggested for the differences that were found.

MATERIALS AND METHODS

Arginine kinase. This was prepared by the method of Virden, Watts & Baldwin (1965). Several preparations were used and their specific activities are given in the Tables and Figures. Protein determinations were made by the biuret method (Gornall, Bardawill & David, 1949) calibrated with a nitrogen determination of arginine kinase by the micro-Kjeldahl procedure and assuming a 16.7% nitrogen content (Virden & Watts, 1966).

Buffers. All pH measurements were made with an E.I.L. model 23a pH-meter fitted with a temperature compensator. Values of pH given are those for the temperature of the experiments unless otherwise stated. Phosphate buffer, pH 6.6 and *I* 0.1, was prepared from NaH_2PO_4 and Na_2HPO_4 by using the amounts given in the Tables of Datta & Grzybowski (1961). Glycylglycine-NaCl-NaOH buffer, pH 8.3 and *I* 0.1, was prepared by dissolving sufficient NaCl to achieve *I* 0.1 in glycylglycine buffer, pH 7.8 and *I* 0.001, prepared as described by Virden *et al.* (1965). Glycylglycine-NaOH buffer, pH 8.2 and *I* 0.001, was prepared by diluting a stock solution of glycylglycine-NaOH buffer, pH 7.8 (20°) and *I* 0.05 (Virden & Watts,

1964). Glycylglycine-NaOH buffer, pH 8.5 (20°) and *I* 0.06, was prepared as described by Virden *et al.* (1965). Tris-KCl-HCl buffer, pH 7.5 (tris, recrystallized from EDTA, 50 mm; HCl, AnalaR, 0.1N), was prepared as described by Andrews (1964).

Sedimentation analysis. Sedimentation and diffusion experiments were performed in a Spinco model E analytical ultracentrifuge. All experiments were carried out at 4–6° with the rotor temperature controlled to $\pm 0.1^\circ$. Diffusion coefficients were determined in a capillary-type synthetic boundary cell with a rotor speed of 9945 rev./min.: the same runs yielded the area of the schlieren peak required for the calculations in sedimentation equilibrium experiments. Sedimentation coefficients were determined in sedimentation velocity experiments performed at a rotor speed 59780 rev./min. Sedimentation and diffusion coefficients were measured on the same enzyme solution and corrected to a standard state of water at 20° by the usual procedures. The enzyme solution examined had a protein concentration 6.7 mg./ml. It was dialysed against glycylglycine buffer, pH 8.3 and *I* 0.1.

Apparent molecular weights ($M_{a,D}$) were computed from sedimentation and diffusion coefficients by means of the Svedberg equation (Svedberg & Pedersen, 1940) and by using a partial specific volume of 0.735 ml./g. This value was determined from the amino acid composition (Virden & Watts, 1966) by the procedure of Cohn & Edsall (1943). Sedimentation equilibrium experiments were carried out in a 12 mm. cell with a standard Kel-F double-sector centre-piece into which 3 mm. columns of enzyme solution were layered over perfluorotributylamine (Fluorochemical FC-43; Minnesota Mining and Manufacturing Co. Ltd., London). The analysis and computation of molecular weight averages M_w and M_z were carried out according to the methods described by Van Holde & Baldwin (1958). The rotor speeds were 11272 or 12590 rev./min. and equilibrium was attained in about 24 hr.

Sephadex gel filtration. Columns (50 cm. $\times$ 2.4 cm.) of

Sephadex G-100 [Pharmacia (Great Britain) Ltd., London] were prepared and used in the manner described by Andrews (1964). The effluent from the column was monitored for protein with an LKB 4700 (LKB Instruments Ltd., London) Uvicord ultraviolet absorptiometer in conjunction with the LKB 6520A recorder and LKB 3400 RadiRac fraction collector. The position of the protein peak was checked by measuring arginine-kinase activity by the method of Virden *et al.* (1965). To measure myokinase activity the following mixture was incubated for 10 min. at 30°: arginine, 25 mM; $MgSO_4$, 6 mM; ADP, 1.25 mM; arginine kinase (specific activity 224), 22 $\mu g./ml.$; glycylglycine buffer, pH 8.5 (20°) and *I* 0.024; myokinase. The reaction was stopped, and arginine phosphate was assayed, as described by Virden *et al.* (1965). A calibration curve was prepared which related myokinase activity to arginine phosphate produced in 10 min. The proteins used to calibrate the Sephadex column were: ribonuclease A (a gift from Dr A. Deavin), assumed mol.wt. 12700 (Hirs, Moore & Stein, 1956);

myoglobin (a gift from Dr R. Naftalin), assumed mol.wt. 17800 (Edmundson & Hirs, 1962); myokinase (Boehringer und Soehne G.m.b.H., Mannheim, Germany), assumed mol.wt. 21300 (Mahowald, Noltmann & Kuby, 1962); ovalbumin (Armour Laboratories), assumed mol.wt. 45000 (Warner, 1954); bovine plasma albumin fraction V (Armour Laboratories), assumed mol.wt. 67000 (Phelps & Putnam, 1960); rabbit-muscle creatine kinase (prepared by method B of Kuby, Noda & Lardy, 1954), assumed mol.wt. 81000 (Noda, Kuby & Lardy, 1954).

Sucrose density-gradient centrifugation. This was carried out with the SW 39 rotor and a Spinco model L2 preparative ultracentrifuge. The gradients, in a volume of 4.4 ml., were made with a device similar to that described by Britten & Roberts (1960) and were linear, between 5 and 20% (w/v), in sucrose. The gradients also contained glycylglycine buffer, pH 8.2 and *I* 0.001. After centrifugation for 18 hr. the sedimentation pattern was recorded by passing the contents of the Spinco tube through a flow cell in the Cary model 14 recording spectrophotometer set at 280 m μ (Edwards & Mathias, 1963). Sedimentation distance was measured from the start of the recorded trace of the gradient (see Fig. 1) to the apex of the protein peak.

RESULTS

Analysis in the ultracentrifuge. The results of experiments on sedimentation velocity, sedimentation equilibrium and diffusion are summarized in Table 1. In preliminary experiments carried out at pH 6.6 to minimize secondary charge effects, the enzyme recovered from the ultracentrifuge cell had lost 50% of the original activity. For this reason buffer at pH 8.3, where the enzyme is stable, was used.

Sephadex gel filtration. In agreement with the results of Andrews (1964), the elution volumes of the standard proteins bore a linear inverse relationship to log mol.wt. Table 2 gives the results for three determinations of the molecular weight of arginine kinase and shows that a tenfold change in protein concentration had little effect on the determined molecular weight. The alternative approach of relating the observed exclusion coefficient with the Stokes' radius of the protein

Fig. 1. Sedimentation profiles of proteins in linear density gradients (sucrose, 5–20%, w/v, in glycylglycine buffer, pH 8.2). Sedimentation was carried out for 18 hr. at 5° in a SW 39 rotor at 37000 rev./min. in a Spinco model L2 centrifuge. After sedimentation the gradients were extruded through a flow cell as described in the text and the profiles were automatically recorded. The chart distance is measured from the top of the gradient and is directly proportional to the distance along the gradient. The proteins, added in 0.1 ml. of glycylglycine buffer, were: A, bovine plasma albumin, 1 mg.; B, arginine kinase, 0.6 mg.; C, ribonuclease, 0.4 mg.

Table 1. *Sedimentation analysis of lobster arginine kinase*

Constant	Specific activity 224 (6.7 mg./ml. in glycylglycine–NaCl buffer, pH 8.3, <i>I</i> 0.1)
$10^{13}S_{20,w}$ (sec.)	3.18
$10^7D_{20,w}$ (cm. ² /sec.)	7.66
$\bar{v}$ (ml./g.)	0.735
$M_{s,D}$	38100
M_w	37300
M_z	40100
M_z/M_w	1.08

Table 2. Elution volumes for arginine kinase on a Sephadex G-100 column

Measurement of the elution volume was started when half the sample (2 ml.) had entered the column. The elution volume is that volume at which the middle of the peak of enzyme activity emerges from the column. The molecular weight was estimated by using a standard curve as described in the text. The specific enzyme activity was 224.

Protein applied (mg.)	Elution volume (ml.)	Mol.wt.
6.50	119.2	37 800
6.50	119.8	37 200
0.65	121.0	36 000

(Ackers, 1964) gave essentially similar results. The value for Stokes' radius, calculated from the average elution volume, was 27.0 Å. This gives a diffusion coefficient 7.9×10^{-7} cm.²/sec., in reasonable agreement with the value obtained by ultracentrifugation. Inserting this value in the Svedberg equation, and using the $S_{20,w}$ value of 3.18×10^{-13} sec. from ultracentrifuge analysis (Table 1), gives mol.wt. 36 900.

Sucrose density-gradient analysis. Fig. 1 compares the protein concentration profile of arginine kinase with those of ribonuclease and bovine plasma albumin after 18 hr. centrifugation. Other proteins gave similar traces and the sedimentation distance showed a high degree of reproducibility for ribonuclease, arginine kinase and creatine kinase. Fig. 2 shows a plot of protein molecular weight against a function of the sedimentation distance. If a linear relationship is assumed this plot gives a value for the molecular weight of arginine kinase between 37 000 and 42 000, and a sedimentation coefficient (obtained by plotting sedimentation distance against sedimentation coefficient for standard proteins) between 3.0 and 3.2. The sedimentation distances for ovalbumin and bovine plasma albumin showed considerable scatter and were generally lower than would be expected from the linear relationship. Thus the points included in Fig. 2 are for fresh solutions, but storage of the proteins in the refrigerator for a few days resulted in the value of (sedimentation distance)^{3/2} decreasing by nearly 50%.

DISCUSSION

The most precise results are probably those obtained by ultracentrifugation analysis (Table 1). Good agreement between M_w and $M_{s,D}$ was obtained. The Z-average molecular weight (M_z) was greater than the weight-average molecular weight, the difference being less than 10% at pH 8.3 but larger at pH 6.6. However, the experiment

Fig. 2. Determination of the molecular weight of arginine kinase by sedimentation in linear density gradients (sucrose 5–20%, w/v, in glycylglycine buffer, pH 8.3). Sedimentation was carried out as described in Fig. 1, by using as standard proteins: ○, ribonuclease; ●, ovalbumin; ▲, bovine plasma albumin; △ creatine kinase. The range of seven determinations of arginine kinase is indicated by the double broken line.

at pH 6.6 was accompanied by considerable loss of enzyme activity. As measurements can be made with a precision of about 10% the preparation investigated at pH 8.3 could be homogeneous with respect to molecular weight. On this basis the best estimate of the molecular weight of arginine kinase by ultracentrifuge analysis is 37 000. In contrast with the present data (Table 1), Pradel *et al.* (1964) obtained a value for $S_{20,w}$ of 2.69. Because of the similarity of this value to that obtained by Elodi & Szorenyi (1956) for arginine kinase from *Potamobius astacus*, the molecular weight was inferred to be the same, namely 43 000. However, Elodi & Szorenyi observed considerable denaturation during ultracentrifuge runs and Pradel, Kassab, Terrossian & Thoai (1965) have recently reported an almost twofold increase in the specific activity of their enzyme preparation.

The mean value for the molecular weight of arginine kinase from gel filtration on Sephadex (Table 2) is also 37 000. Since the effect of protein concentration on elution volume was negligible it must be assumed that if enzyme sub-units exist they are not readily dissociable. When a diffusion coefficient was calculated from the gel-filtration data and this value was used for calculating the molecular weight a value of 36 900 was obtained. The best estimate of the molecular weight by gel filtration is therefore concluded to be 37 000.

Sucrose density-gradient analysis was investigated to find out whether the procedure of Martin & Ames (1961) might be adapted to more accurate determination of molecular weight by means of a calibration curve plotted for standard proteins

spanning a relatively small range of molecular weights around that of arginine kinase. As with the fractions obtained by gel filtration, the protein peak was coincident with the peak of arginine-kinase activity, which provided an additional criterion of purity. The time of centrifugation, 18 hr., was longer than any of those of Martin & Ames (1961). For this reason, and also because larger protein loadings were used in this work so that E_{280} readings could be made, a greater diffusion of the protein peaks occurred and it was not possible to apply more than one standard protein to each gradient. Thus a disadvantage of this modification of the method of Martin & Ames (1961) is that, for low-molecular-weight proteins, a large number of time-consuming centrifuge runs have to be made. Even with a sedimentation time of 18 hr., creatine kinase, the heaviest standard protein, reached only the middle of the gradient. There is more scatter in the values for the molecular weight than in those obtained by gel filtration. However, the range 37 000–42 000 (Fig. 2) is in reasonable agreement with the molecular weight obtained by the other two methods and is consistent with the conclusion that 37 000 is the best minimum molecular weight for arginine kinase from lobster muscle.

We are grateful to the Medical Research Council who provided the analytical ultracentrifuge and a grant for special research to D. B. G. A grant from the Central Research Fund for the effluent analyser and a personal grant to R. L. W. from the Department of Scientific and Industrial Research are also gratefully acknowledged.

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