PURIFICATION AND SOME OPTICAL CHARACTERISTICS OF FLAGELLAR ATPase DYNEIN I FROM SEA URCHIN SPERM

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1. Introduction

According to [1], the term 'dynein' denotes a class of proteins associated with a system of microtubules of flagella and cilia possessing ATPase activity and high molecular weight of their polypeptide subunits ($3-6 \times 10^5$). Flagella of sea urchin sperm contain at least two forms of ATPase (dynein I and dynein II) varying in their electrophoretic and immunochemical characteristics [2]. Localization and function of the minor ATPase (dynein II) has not been clarified yet. Dynein I is a better studied protein. It was shown that dynein I is located in the external arms of outer doublet microtubules [3]. It was reported [1] enzymatic and structural evidence in favour of an active role of dynein I in the mechanochemical conjugation at flagellar motility analogous to the role of myosin in muscular contraction.

Here we describe the modified method of purification of dynein I from flagella of sea urchin sperm, which allowed us to obtain a homogeneous preparation of purity >90%, we give optical characteristics (absorption spectra at 240–360 nm and circular dichroism spectra at 186–310 nm) of the purified preparation and estimate the secondary structure of dynein I from circular dichroism spectra.

Abbreviations: DTT, dithiothreitol; EDTA, ethylendiaminetetraacetic acid; SDS, sodium dodecyl sulfate; CD, circular dichroism

2. Materials and methods

2.1. *Preparation of glycerinated flagella*

Spermatozoa were collected from sea urchin *Strongylocentrotus intermedius* after introduction of 0.5 M KCl into perivisceral cavity. Flagella were separated from the heads by Triton X-100 treatment and purified by differential centrifugation as in [4]. The purified flagella were suspended in a medium consisting of 60% glycerol, 20 mM $MgCl_2$, 20 mM Tris–HCl (pH 8.4), 1 mM DTT at 4°C and stored at −20°C.

2.2. *Purification of flagellar ATPase*

The subsequent procedures were carried out at −4°C. The glycerinated flagellar suspension (50 ml) was diluted to 200 ml with 25 mM Tris–HCl buffer (pH 8.3) containing 1 mM $MgCl_2$ (buffer A), flagella were pelleted by centrifugation at 15 000 × *g* for 15 min, and thrice washed with buffer A under the same conditions. Extraction of ATPase from the flagella was done by dialysis against the Tris–EDTA solution and the subsequent purification of the crude extract was carried out by chromatography on Sepharose 4B and hydroxylapatite as in [5] with some modification. The crude extract of flagellar ATPase (50 ml) was concentrated with a Diaflo membrane filter (XM-300) to 3 ml and clarified by centrifugation (50 000 × *g*, 20 min) before gel filtration. Gel filtration was done on a 1.6 × 83 cm column with Sepharose 4B, the elution rate was 13 ml/h and the fraction volume 3 ml. The fractions containing ATPase

activity from a Sepharose 4B column were pooled and dialyzed against 3 l of 1 mM Tris–HCl buffer (pH 8.3) containing 0.05% β-mercaptoethanol for 18 h to remove EDTA. 1 M potassium phosphate buffer (pH 7.6) was added to the dialyzed fractions to 0.1 M final conc. and layered on a hydroxylapatite column (1.6 × 15 cm) pre-equilibrated with 0.1 M potassium phosphate buffer (pH 7.6) containing 0.05% β-mercaptoethanol. The adsorbed proteins were eluted from the hydroxylapatite column by 300 ml of a linear gradient of 0.1–1.0 M potassium phosphate buffer (pH 7.6) in 0.05% β-mercaptoethanol. The elution rate was 17 ml/h and the fraction volume 2.5 ml.

2.3. *Determination of ATPase activity*

For determination of ATPase activity the samples in buffer A were incubated with 1 mM ATP for 60 min at 25°C in 0.5 ml final vol. Under these conditions the dependence between the incubation time and the amount of split ATP remained linear. The reaction was stopped by addition of an equal volume of 3% $HClO_4$ and the amount of P_i was measured as in [6].

2.4. *Sodium dodecylsulfate electrophoresis*

Samples for SDS–polyacrylamide gel electrophoresis were prepared as in [7]. SDS gel electrophoresis was performed on linear 4–10% polyacrylamide gel gradient in the Weber and Osborn system [8].

2.5. *Determination of protein concentration*

Protein concentration was determined by the method in [9], assuming the nitrogen content to be 16%. To determine the extinction coefficient, the absorption spectra of protein solutions were registered by a Hitachi EPS-3T instrument. The spectra were registered in the range of 360–230 nm with correction for turbidity of solutions according to [10].

2.6. *CD spectra measurement*

For measuring CD spectra we used the ATPase preparation after chromatography on hydroxylapatite (peak III fractions, fig.1) preliminarily dialyzed against 10 mM potassium phosphate buffer (pH 7.6) containing 1 mM DTT. CD spectra were measured directly after the dialysis using a JASCO J41A instrument.

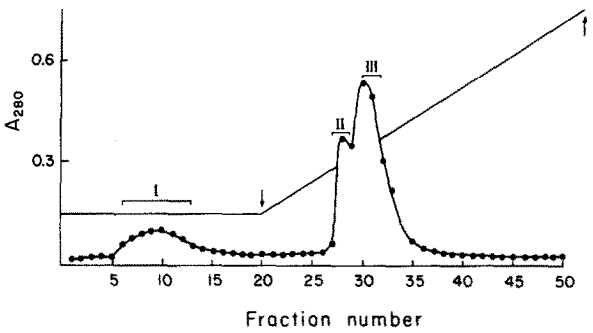

Fig.1. Column chromatography of the purified flagellar ATPase on hydroxylapatite. 20 ml of the active fractions eluted from the Sepharose 4B column in 0.1 M potassium phosphate buffer (pH 7.6) containing 0.05% β-mercaptoethanol were applied to the hydroxylapatite column and the column was washed with 50 ml of the same buffer. Arrows indicate concentrations of potassium phosphate buffer; (↓) 0.1 M; (↑) 0.5 M.

The instrument was calibrated according to [11]. The thickness of the cell was 0.186, 0.5 and 1.0 mm for the far and 10 mm for the near ultraviolet regions. All the measurements were made at 12°C. In calculating the ellipticity the mean residue weight was taken as 115. The secondary structure content was estimated as in [12] using reference spectra [13].

3. Results

3.1. *Characteristics of the dynein preparation*

A satisfactory purification level of flagellar ATPase of dynein I was achieved after chromatography on hydroxylapatite. The chromatographic pattern is shown in fig.1. At least two protein peaks (II, III) were usually observed. Each of the protein peaks showed ATPase activity; at the same time, the protein (peak I) that was not adsorbed on the column had no activity. Protein composition of peaks II and III was analyzed by SDS electrophoresis on gradient polyacrylamide gel. Peak II contained a mixture of two high-molecular-weight proteins (fig.2a) corresponding to A-band (dynein I) and D-band (dynein II) according to [1]. Peak III consisted of almost pure high-molecular-weight A-band protein (dynein I) (fig.2b). The admixture of minor components was <10%. It should be noted that peak III was in some cases asymmetrical,

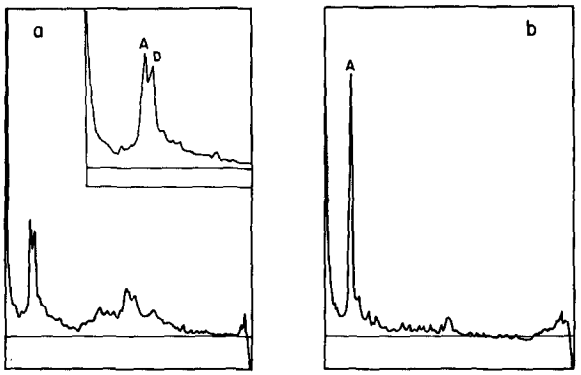

Fig.2. Densitometric scans of SDS-gradient gels of peaks II (a) and III (b) from the hydroxylapatite column. The gels loaded with 10 μg protein were stained with Coomassie brilliant blue R-250 and scanned at 630 nm. The insert in (a) shows the upper region of the gel scanned at a 2.5-fold expansion along the abscissa.

however analysis of its fractions by gel electrophoresis demonstrated homogeneity of its composition.

The material of protein peak III from the hydroxylapatite column was used as purified dynein I. Starting from 50 ml glycerinated flagellar suspension we usually obtained 2–3 mg purified dynein I. The purified enzyme showed spec. act. 0.5–0.7 μmol P_i .min^{-1} .mg $protein^{-1}$ under the conditions in section 2. The specific activity and electrophoretic characteristics did not change after measuring CD spectra.

3.2. *Determination of extinction coefficient*

The dynein I preparation has a maximum at 281 nm in the near ultraviolet region of absorption spectrum, typical of proteins. Figure 3 shows that

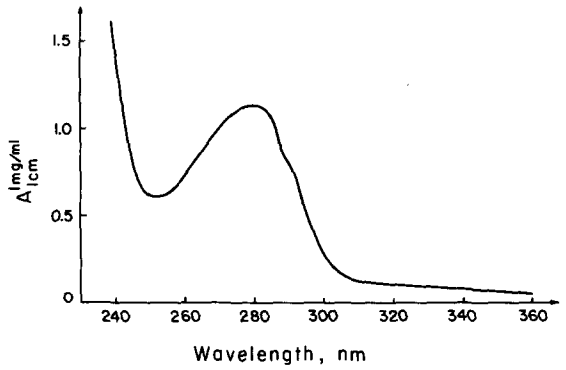

Fig.3. Dynein I absorption spectrum in 10 mM potassium phosphate (pH 7.6), 1 mM DTT.

dynein I solution is characterized by noticeable turbidity; the contribution of turbidity to absorption is 15–20% of the apparent A_{281}. The extinction coefficient corrected for turbidity is $A^{1\,mg/ml}_{1\,cm} = 0.95 \pm 0.04$ at 281 nm. This error corresponds to the scatter of values obtained from 6 measurements of nitrogen content for two different protein preparations.

3.3. *CD spectra*

Figure 4 shows CD spectra of dynein I at 186–310 nm. A positive maximum in the spectrum emerges at 191 nm and is ~24 000 deg .cm^2 .$dmol^{-1}$; the crossover is at 200.3 nm. Negative minima appear at 208 nm and 220 nm and their values are −13 300 and −12 300 deg .cm^2 .$dmol^{-1}$. Ellipticity values at 256–310 nm pertaining to side groups of aromatic amino acids are positive and have slightly prominent maxima at 258 nm, 265 nm, 272.2 nm, 285 nm and 291 nm. To determine the secondary structure content of dynein I, we chose the ellipticity values at 210 nm, 219 nm and 225 nm. Estimations made as in [12] gave 40% for the content of α-helices and 20% for the β-form content. Using two ellipticity values

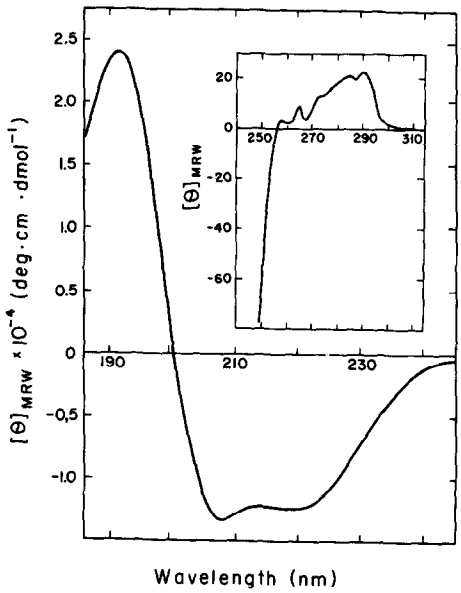

Fig.4. Dynein I CD spectrum in the far ultraviolet region. The insert shows the CD spectrum in the near ultraviolet region. 10 mM potassium phosphate (pH 7.6), 1 mM DTT, t = 12°C, MRW mean residue weight.

(at 210 nm and 225 nm) instead of the three above, we obtained practically the same results for the content of α-helices and the β-form.

4. Discussion

Preparation of dynein I in a homogeneous state is impeded by the capability of this protein to form non-specific aggregates in the process of isolation and storage [5] and by the existence of the other form of flagellar ATPase (dynein II) [1]. Both the forms pass into the Tris–EDTA extract. These proteins are very similar in molecular weight and thus on gel-chromatography on Sepharose 4B they are eluted practically together. The results of this work (fig.1, 2) allow one to conclude that chromatography on hydroxylapatite enables the separation of two forms of dynein. This conclusion indirectly follows from the method of isolating dynein II in [2].

The specific activity value of our dynein I preparation is 3–4-times less than that in [3,4]. The difference is, in our opinion, a consequence of different conditions of measuring ATPase activity and different methods of determining the protein concentration. A higher Mg^{2+} concentration in the incubation mixture, a higher incubation temperature and a higher extinction coefficient calculated by the Lowry method of determining protein concentration explain the apparent increase in the specific activity values in [3,7] when compared with our specific activity value.

If CD curves obtained by us for dynein I are compared with those published for myosin subfragment S1 [14,15], it is safe to say that the shape, position and values of the extremes on the curves of dynein I and myosin fragment S1 are significantly similar.

Such similarity of CD spectra of these proteins might reflect certain likeness in their secondary structure.

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