PURIFICATION, CHARACTERIZATION AND CRYSTALLIZATION OF PORK MYOKINASE

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1. Introduction

Myokinase (ATP: AMP phosphotransferase, EC 2.7.4.3) from rabbit muscle is a well characterized crystalline enzyme [1–5]. Crystals of this protein, however, seem to be unfavourable for structural analysis (Holmes and Noda, unpublished results).

Pork myokinase, as described in this paper, has molecular properties which are very similar to those of the analogous rabbit protein. As suitable crystals can be readily prepared from the porcine enzyme, X-ray studies and the determination of the amino acid sequence of this protein have been started.

2. Materials and methods

2.1. *Preparation of the crystalline enzyme*

2.5 kg of shoulder muscles from freshly killed pigs were cooled in ice and processed within 2 hr. The isolation procedure reported for rabbit muscle myokinase [3] had to be modified at several steps (table 1). Step 2: the pH of the muscle extract was adjusted only to 2.5; after neutralization, the precipitate was removed by centrifugation instead of by celite. Step 3: the concentration of zinc acetate was 55 mM, not 18.5 mM. Step 4: the precipitate obtained on the addition of zinc was extracted with 0.4 M sodium-EDTA, pH 7.5, and not with ammonium citrate. The volume of the EDTA solution was equal to that of the 1 M zinc acetate used in step 3. Steps 7 and 8: after elution of the protein from the cellulose phosphate column, fractions with a specific activity higher than 1000 U. per mg were collected; the protein was precipitated with ammonium sulfate, dissolved in the smallest possible volume of 0.1 M imidazole-HCl, pH 7.0, and chromato-

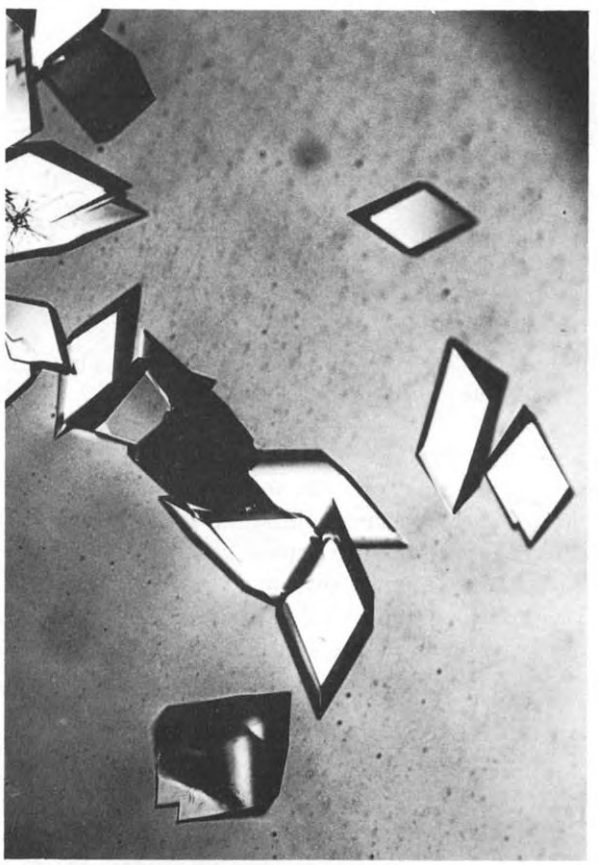

Fig. 1. Pork myokinase crystals in partially polarized light.

Table 1
Purification of pork myokinase. (Initially 2.5 kg of muscle)*

Step	Resulting fraction	Volume (ml)	Total protein (mg)	Total units $\times 10^{-3}$	Units per mg	Overall purification	Yield (%)
1 Extraction of muscle with 0.01 M KCl	I	7000	108,000	212	1.96	(1)	(100)
2. pH-Fractionation (pH 7.0 → pH 2.5 → 7.0)	II	6550	19,600	196	10.0	5.1	92.0
3 Precipitation with 0.055 M Zn-acetate at pH 7.0							
4 Extraction of the precipitate with 0.4 M sodium EDTA, pH 7.5	III	700	14,400	188	13.1	6.7	88.7
5 $(NH_4)_2SO_4$-fractionation (0% → 54%, 54% → 88% saturation)							
6 Dialysis of $(NH_4)_2SO_4$ precipitate against 0.01 M imidazole.HCl, pH 7.0	IV	170	7,200	170	23.4	11.9	80.2
7 Gradient elution from cellulose phosphate	V	400	248	150	605	309	70.8
8 Gel filtration on Sephadex G-75 in 0.1 M imidazole.HCl, pH 7.0	VI	51	85.8	144	1680	857	67.9
9 Crystallization							
10 Dialysis of crystals against water	VII	4.0	74.3	134.5	1810**	923	63.4

* If more than 2.5 kg of muscle are to be processed the capacity of the cellulose phosphate column (step 7) must be increased.
** This value corresponds to a turnover number of 38,000 per min.

graphed on a 140 cm × 2.4 cm column of Sephadex G-75 in the same buffer. In order to combine a purification step with a preliminary molecular weight determination, the G-75 column was calibrated with bovine serum albumin, ovalbumin, rabbit muscle myokinase, and myoglobin [7]. A contaminating protein of about 60,000 daltons was removed; myokinase was eluted as a separate peak at a position which corresponds to a molecular weight 22,000 daltons (fraction VI).

Crystals for X-ray analysis (fig. 1) were reproducibly prepared from fraction VII (table 1) at 4° in the following way: 10 μl of solution A (0.1 μ phosphate buffer, pH 6.0, saturated with ammonium sulfate after the pH had been adjusted) was carefully dropped into a depression slide; 20 μl of solution B (fraction VII containing 20 mg/ml of protein and 1.6 mmoles per ml of ammonium sulfate) was layered on top of the drop. Suitable crystals grew within 3 days at 4° and within 10 days at room temperature. The phosphate in solution A can be replaced by EDTA, tris-maleate, imidazole-HCl or cacodylate.

2.2. *Enzyme assay*

The enzymic activity was measured according to Mahowald et al. [4]. For determining the protein concentrations [8] in fractions I to V a biuret factor of 35 mg per absorbance unit was used. For measuring the myokinase concentration in fractions VI and VII, all procedures listed in table 2, lines 7, 8, 9, 10, 11 proved to be equally valid.

Table 2
Molecular properties of pork myokinase.

Property	Value	Method	References for methods and/or conditions
1 Sedimentation constant $S^{0\%}_{20°}$ (sec)*	2.30×10^{-13}	Sedimentation velocity (Spinco Model E, equipped with Schlieren optics)	2, 10
2 Diffusion constant $D^{0\%}_{20°}$ ($cm^2\ sec^{-1}$)*	10.2×10^{-7}	Optical analysis of diffusion column	11
	10.0×10^{-7}	Gel filtration on Sephadex G-75, G-100, G-200	12
3 Apparent specific volume $\bar{\nu}_{20°}$ ($ml\ g^{-1}$)*	0.74	Pycnometry	2
	0.736	Amino acid analysis	6, 13
4 Molecular weight (daltons)*	21,000	Gel filtration on Sephadex G-100	7, 14
	21,600 ± 700	Sedimentation equilibrium	15
	21,000 ± 700	Calculated by use of Svedberg's equation ($S = 2.30 \times 10^{-13}$ sec, $D = 10.1 \times 10^{-7}\ cm^2\ sec^{-1}$, $\nu = 0.736\ ml\ g^{-1}$)	2, 10
	21,400 ± 600	Titration of 2 SH-groups	3, 16, 17
	21,400	Amino acid analysis	6, 5
5 Frictional ratio f/f_0	1.15	Calculated from data on lines 1, 2, 3 and 4	18
6 Isoelectric point (for $\tau/2 = 0.1\ \mu$)	6.0 (?)	Electrophoresis on cellulose acetate	20
7 Refractive index increment $(\Delta n/\Delta c)_{25°}$ for $\lambda = 589$ nm (g per 100 ml)$^{-1}$*	1.78×10^{-3}	**	2
8 Nitrogen content	17.3%	**	2
9 Specific absorbance $A^{1\%}_{1\ cm}$ at $\lambda = 277$ nm*	5,38	**	2
10 Molar absorbance at $\lambda = 277$ nm*	11.500	**	2
11 Biuret factor (10 ml total volume, 1 cm light path, $\lambda = 540$ nm) (mg/absorption unit)	30.6	**	1, 8

* Measured in 0.15 M KCl-0.01 M potassium phosphate, pH 7.0, $\rho_{20°} = 1.0051\ g\ ml^{-1}$, $(\eta_{rel})_{20°} = 1.003$.
** Gravimetric standardization.

2.3. *Physical properties*

For ease of comparison, the porcine enzyme was studied under conditions similar to those used previously for rabbit muscle myokinase [2]. In additional experiments only standard methods were used; these will be reviewed elsewhere [9].

3. Results and discussion

3.1. *Enzyme*

The purification of the enzyme is summarized in table 1. The crystalline protein was found to satisfy the following criteria of homogeneity: gel electrophoresis (fig. 2), sedimentation, diffusion, and chromatography on Sephadex G-75. The physical properties of pork myokinase (table 2) are similar to those of the analogous enzyme from rabbit muscle. As both proteins could be expected to be basic from their amino acid composition [5, 6] the values found for their isoelectric points are surprisingly low and should be regarded as preliminary.

3.2. *Crystals*

Crystals of pork myokinase are very similar to those of rabbit muscle myokinase (table 3). They have the same space group, approximately the same unit cell dimensions, similar shapes and X-ray diffraction patterns. However, the parameters important for X-ray analysis, i.e. crystal size, mosaic spread, resolution, and lifetime in X-ray beam, are considerably better with pork myokinase.

As an example for the X-ray diffraction pattern of the pork myokinase crystals a precession photograph of the Okl-plane is shown in fig. 3. These crystals seem to be very suitable for X-ray analysis. Consequently, data collection and search for heavy atom derivatives have been started by use of the flow cell technique [19].

Acknowledgements

We are much indebted to Dr. R.K.Scopes; he was the first to crystallize pork myokinase and he and Dr.

Table 3
Properties of pork and rabbit muscle myokinase crystals.

Property	Pork	Rabbit muscle
Crystal shape	Pointed rhombohedron or prism	Pointed rhombohedron
Crystal length	$\leqslant 1000$ μm	300 μm
Crystal density	1.27 ± 0.01 g cm^{-3}	Nor determined
Space group	$P3_121$ or $P3_221$	$P3_121$ or $P3_221$
Crystal axes	$a = b = 48.75 \pm 0.05$ Å $c = 139.9 \pm 0.1$ Å	$a = b = 47.5 \pm 0.5$ Å $c = 142 \pm 1$ Å
Numbers of molecules in the unit cell	6	6
Number of molecules in the asymmetric unit	1	1
Volume fraction of the solution in the crystal	46%	44%
Resolution	3 Å	6 Å
Mosaic spread	$\leqslant 0.1^\circ$	1°
Lifetime in X-ray beam at room temperature	40 hr	5 hr

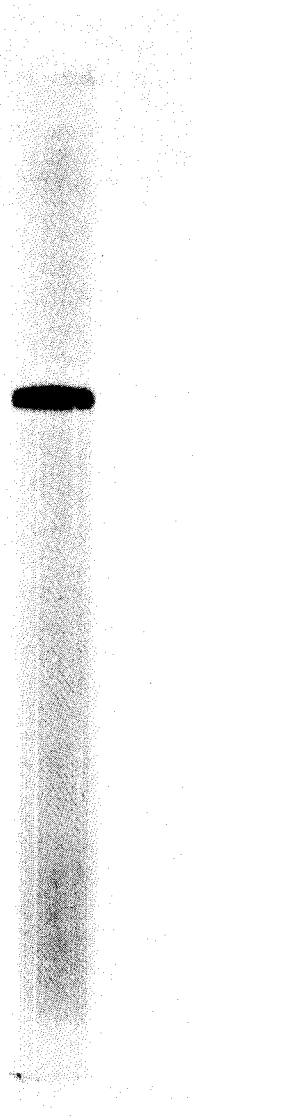

Fig. 2. Gel electrophoresis of pork myokinase. Fraction VII was dissolved in urea and submitted to electrophoresis in gels of 7.5% acrylamide and 0.2% methylene bisacrylamide containing 5 M urea at pH 4.3. Electrophoresis was carried out at room temperature for 3 hr at 4 mA/tube, origin at top, cathode at bottom. The gels were stained with Coomassie brillant blue and destained in 10% acetic acid-7% methanol.

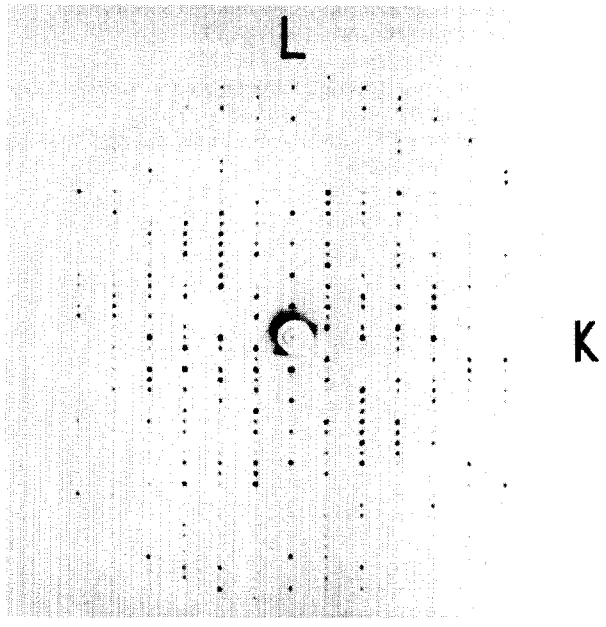

Fig. 3. X-ray precession photograph of the Okl-plane of a pork myokinase crystal, irradiated with nickel-filtered Cu Kα radiation from a rotating anode tube (GRX-6, Elliot Automation, Borehamwood, Great Britain). The precession angle was 8°.

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