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COUPLED ENZYMATIC REACTIONS MEASURED IN A SINGLE PROTEIN CRYSTAL FROM MYOGEN A

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SUMMARY

Pairwise coupled reactions of fructose-1,6-bisphosphate aldolase and sn-glycerol-3-phosphate dehydrogenase, 3-phosphoglycerate kinase and D-glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase and sn-glycerol-3-phosphate dehydrogenase have been detected by microspectrophotometry in single crystals obtained from myogen A in the presence of polyethylene glycol. Microspectrophotometric measurements with polarized light demonstrate that the protein molecules are oriented and that NADH is bound with a definite orientation to the dehydrogenases within the crystal.

INTRODUCTION

In the last 10-15 years the regulation of metabolism is in the center of scientific interest (1-4). The metabolic pathways are composed of a sequence of coupled reactions (5, 6). These coupled reactions may be catalyzed by non-interacting or interacting enzymes (7-14). To understand the structural basis of interactions the crystal structure of cocrystals of enzymes catalyzing subsequent reactions would have a primary importance. Early crystalline protein preparations from rabbit muscle (myogen A and myogen B) were found to contain several glycolytic enzymes (15), some of which were demonstrated to interact in solution (16-21). Myogen A seemed to be a uniform crystalline preparation containing both fructose-1,6-bisphosphate aldolase and sn-glycerol-3-phosphate dehydrogenase (22).

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The interaction of these latter enzymes was also detected in solution (23, 24). Thus, we were encouraged to produce myogen A macrocrystals suitable for future X-ray investigations. In addition, to relate structural and functional data we investigated the catalytic competence of myogen A single crystals: the occurrence of coupled enzymatic reactions were detected by single crystal microspectrophotometry.

MATERIALS AND METHODS

Myogen A was isolated from rabbit muscle according to Baranowski (15). After centrifugation, myogen A microcrystals were dissolved in water and the protein dialyzed against 0.1 M sodium phosphate buffer, 1 mM EDTA, pH 7.2. To the protein solution (4-6 mg/ml) polyethylene glycol (PEG) Mr 6000 was added to reach 5 w/w% concentration. The solution was transferred into capillaries and dialyzed at room temperature against 12% PEG 6000 containing 0.1 M sodium phosphate buffer, 1 mM EDTA, pH 7.2. In about two weeks crystals shaped as either rhombic plates (0.5x0.5x0.02 mm) or hexagonal bipyramids of similar size were formed, suitable for single crystal microspectrophotometry. These crystals were found to diffract better than 0.5 nm resolution. Experiments were performed as described previously (25-27) with a Zeiss microspectrophotometer on-line with a Hewlett-Packard 9816 desk-top computer and plotter.

Protein composition of single crystals was determined by separation of their components using FPLC (Pharmacia). A number of single macrocrystals were collected and washed repeatedly with 20% PEG 6000 in 50 mM Tris buffer, pH 7.3, containing 1 mM EDTA. After centrifugation the crystals were dissolved in 50 mM Tris buffer, pH 7,3, containing 1 mM EDTA. The resulted protein solution (0.6 ml of about 5 mg/ml) was loaded onto a Polyanion SI-17 column, equilibrated with the above buffer. Flow rate was 0.5 ml/min and fraction volume was 1 ml. After washing the column with buffer for 10 min a NaCl gradient elution (from 0 up to 0.5 M NaCl) was applied for 60 min. The eluted protein peaks were identified by recording the light absorbance of the fractions at 280 nm and by measuring their enzymatic activities.

The sn-glycerol-3-phosphate dehydrogenase was isolated from rabbit muscle according to Telegdi (28). Growing single macrocrystals of this enzyme was carried out in the same way as in the case of myogen A.

RESULTS AND DISCUSSION

A single crystal of myogen A was incubated for a few minutes in a solution containing 30% PEG 6000, 50 mM Tris-HCl, 1 mM EDTA, 1 mM NADH, pH 8.1, then excess coenzyme was removed

by repeated washings. The NADH bound within the crytal exhibited an absorption spectrum with the characteristically perturbed λ_{max} = 360 nm (Fig. 1) and an intensity comparable with that of pure dehydrogenases (29). Coenzyme was found not to diffuse out of the crystal into the suspending medium at least for one hour and after several washings. The crystal was resuspended in a PEG medium containing 5-10 mM fructose-1,6-bisphosphate and the disappearance of the absorption band was monitored (Fig. 1).

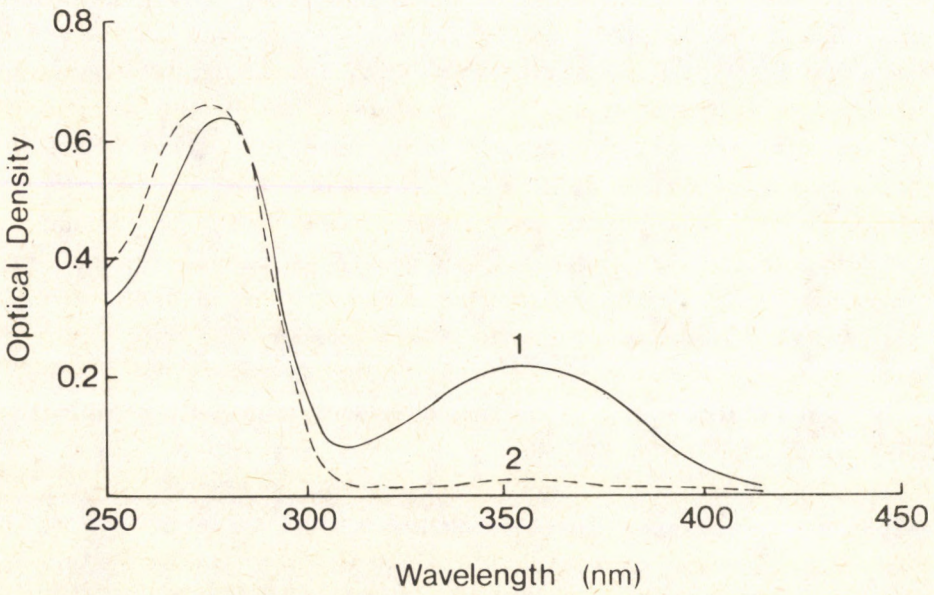

Fig. 1. Unpolarized light spectra of a single myogen A crystal. 1. After addition of 1 mM NADH in PEG solution and several washings with PEG-containing buffer; 2. The same crystal after 35 minutes of incubation in PEG solution containing 10 mM fructose-1,6-bisphosphate.

The disappearance of NADH from the crystal suggests the formation of dihydroxyacetonephosphate from fructose-1,6-bisphosphate catalyzed by fructose-1,6-bisphosphate aldolase and

the coupled redox reaction of the triosephosphate with NADH catalyzed by sn-glycerol-3-phoshate dehydrogenase. This result proves that within the single crystal both major components of myogen A are present and catalytically active. Although the crystal exhibited fine crackings following the reaction, birefringence was maintained. NAD remained bound within the crystal, as shown by the reappearance of NADH after changing the incubation mixture with a medium containing glycerol-3-phosphate.

Activities of some other glycolytic enzymes were also detected in the single crystal from myogen A. Upon diffusion of D,L-lactate (25 mM) and NAD (1 mM), appearance of NADH bound within the crystal was observed, indicating the presence of lactate dehydrogenase. When D-glyceraldehyde-3-phosphate (2 mM) was diffused into the crystal containing bound NADH, the disappearance of NADH spectrum was observed due to the presence of triosephosphate isomerase catalyzing the production of dihydroxyacetonephosphate, subsequently transferred to sn-glycerol-3-phosphate dehydrogenase to react with NADH. Furthermore, when 3-phosphoglycerate (5 mM) and Mg-ATP (5 mM) were diffused into the crystal containing bound NADH, the disappearance of its absorption band was detected, due to the consecutive reactions of 3-phosphoglycerate kinase and D-glyceraldehyde-3-phosphate dehydrogenase. The results of the various experiments are summarized in Table 1.

The question marks in Table 1 denote the cases in which enzymatic reactions possibly occur, but in lack of a spectral signal they escape detection. E.g. in the presence of fructose-1,6-bisphosphate, not only the reaction catalyzed by fructose-1,6-bisphosphate aldolase, but also the subsequent reaction of products with triosephosphate isomerase can be expected. In addition, we were unable to observe the possible occurrence of the consecutive reactions catalyzed by fructose-1,6-bisphosphate aldolase and D-glyceraldehyde-3-phosphate dehydrogenase when fructose-1,6-bisphosphate and NAD were added to the crystal. Although in this case NADH should be formed, it can further react (thus disappearing) with the concomitantly formed dihydroxyacetonephosphate through the action of sn-

Table 1. Enzymatic reactions detected in single crystals of myogen A

Reactants added to a single crystal of myogen A in 30% PEG 6000, 50 mM Tris buffer, pH 8.1, 1 mM EDTA, 20°C	NADH bound in the crystal	Enzymatic reaction	Occurrence of crystal cracking
NADH	+	-	-
NADH, incubated for 1 hour in a NADH-free medium and after several washings	+	-	-
NADH* + fructose-1,6-bisphosphate	-**	+	+
NADH* + dihydroxyacetonephosphate	-**	+	+
glycerol-3-phosphate + NAD	+	+	+
dihydroxyacetonephosphate or D-glyceraldehyde-3-phosphate	-	?	-
fructose-1,6-bisphosphate	-	?	-
dihydroxyacetonephosphate + D-glyceraldehyde-3-phosphate	-	?	-
NADH* + dihydroxyacetonephosphate and after the reaction (disappearance of NADH) + glycerol-3-phosphate	+	+	+
D,L-lactate + NAD	+	+	-
NADH* + D-glyceraldehyde-3-phosphate	-**	+	+
NADH* + 3-phosphoglycerate + MgATP	-**	+	-

*NADH bound in the crystal after washing with PEG 6000 (30%), 50 mM Tris buffer, pH 8.1, 1 mM EDTA

**A small residual amount of NADH remains bound in the crystal

?Not detectable in the absence of spectral signal

glycerol-3-phosphate dehydrogenase (both being in high excess as compared with glyceraldehyde-3-phosphate and D-glyceraldehyde-3-phosphate dehydrogenase, respectively).

Measurements with polarized light show differences in the intensity of optical absorptions depending on crystal orientation, both in the absence of NADH at λ = 280 nm and in the presence of NADH at λ = 340 nm. The maximum of absorption occurs at different crystal orientations for the two bands. When NADH was produced in the crystal via reaction of glycerol-3-phosphate and NAD, or D,L-lactate and NAD, the intensity of polarized light absorption for the resulting NADH was the same as for NADH added directly to and bound by the crystal protein. These findings suggest that protein molecules within the crystal are oriented and, similarly, that NADH is bound with a definite orientation to the dehydrogenases.

The above functional and spectral properties were equally exhibited by single crystals obtained from three different myogen A preparations. However, variable amount of their constituent enzymes were found with FPLC analysis (Table 2).

Table 2. Composition of different myogen A crystal preparations

Myogen A preparation	Percentage of components		
	sn-glycerol-3-phosphate dehydrogenase	fructose-1,6-bisphosphate aldolase	other enzymes
1 (microcrystal)	50 ± 5	20 ± 2	30 ± 3
2 (single rhombic plate crystal)	70 ± 7	10 - 30*	1 - 10
3 (single hexagonal bipyramid crystal)	5 - 10*	90 ± 9	1 - 10

FPLC analysis was described in the Materials and Methods

*The enzymes were practically inactive after elution from the column

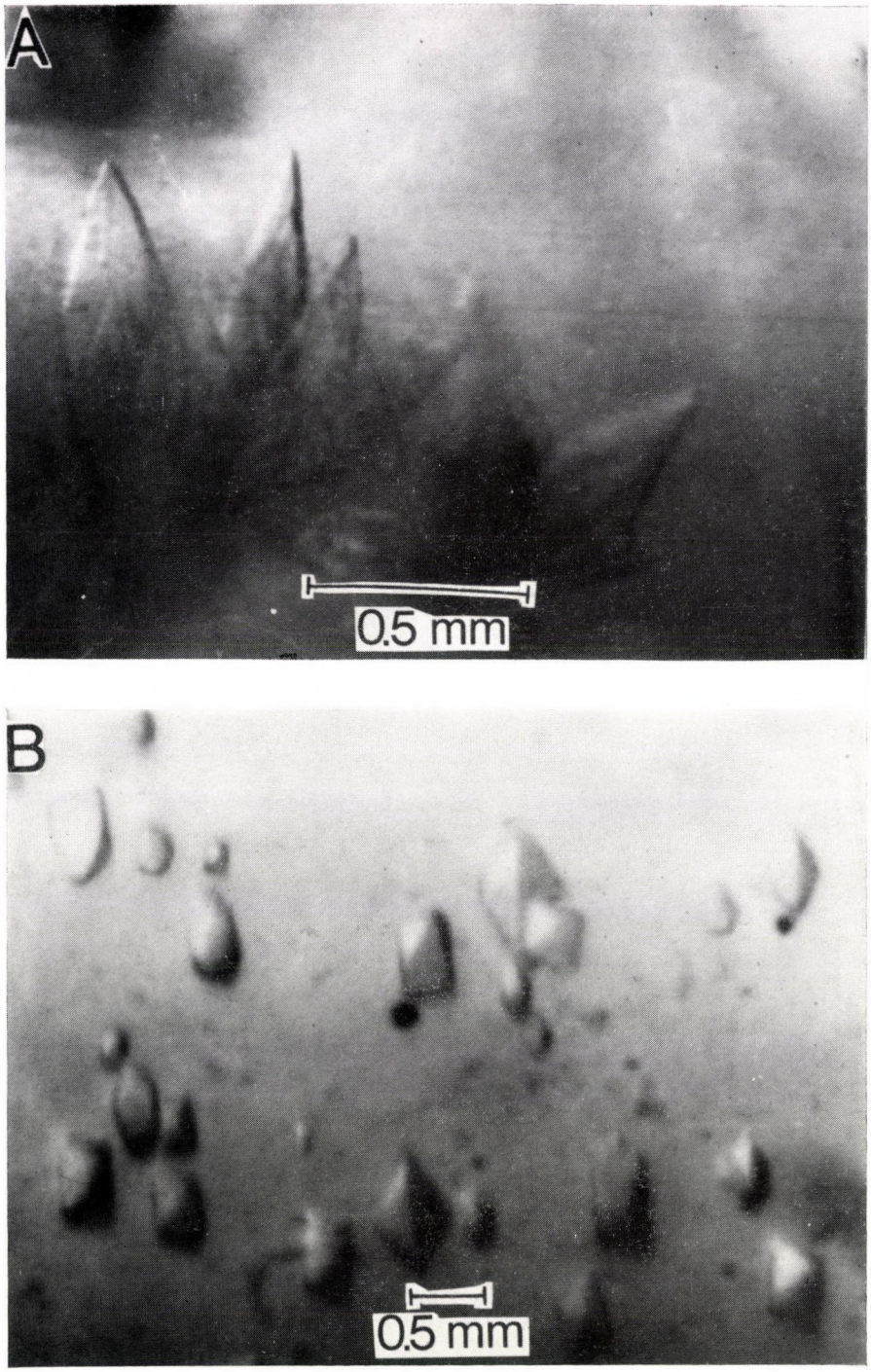

Fig. 2. Microscopic photographs of myogen A crystals. (A) Preparation 1, (B) Preparation 3. Magnification: 20×.

It is particularly interesting to relate these findings to the crystal forms of different myogen A preparations. While preparations 1 and 2 (where sn-glycerol-3-phosphate dehydrogenase was the dominating component) crystallized in rhombic plates, the crystals of the mainly fructose-1,6-bisphosphate aldolase-containing preparation 3 appeared as hexagonal bipyramids (Fig. 2). The latter form is similar to the crystals of the originally published preparation of myogen A (15).

We prepared single macrocrystals also from the isolated rabbit muscle sn-glycerol-3-phosphate dehydrogenase in the presence of PEG 6000 and found the same morphology of the crystals of myogen A preparations 1 and 2. Thus the main component of myogen A determines the mode of arrangement of protein molecules within the crystal lattice. The lattice can accomodate smaller number of other enzyme molecules, too. Crystalline sn-glycerol-3-phosphate dehydrogenase can bind NADH, form a complex with similar spectrum as the multienzyme crystal and is catalytically competent.

One might wonder whether cocrystallization of different enzymes and the occurrence of coupled reactions in a single crystal are the consequence of some specific interaction(s) among the various constituent enzymes in solution. In fact, complex formation between pairs of the enzymes present in myogen A has been previously documented in solution (16-21, 23, 24). Considering that complexes involving glycolytic enzymes are possible transient or weak (and therefore sometimes escape detection by conventional techniques; 30-32), it is possible that composition of the crystals depends strongly on the slightly different conditions of both isolation and crystallization of myogen A. However, the occurence of the pairwise coupled reactions in the single crystal of myogen A indicates that the enzymes present are catalytically competent. Thus, presumably, it will be possible to connect structural and functional data if the X-ray structure determination of the complex will be realized. Further systematic crystallization attempts are required to find conditions favouring stoichiometric complex formation within single macrocrystals.

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