Interaction of Fish Muscle Glycolytic Enzymes with F-Actin and Actomyosin

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The binding of fish muscle glycolytic enzymes with F-actin and actomyosin was studied *in vitro* under various conditions. The binding of glucosephosphate isomerase, phosphoglycerate kinase and enolase with the myofibrillar proteins was weak, while that of aldolase and glyceraldehyde-3-phosphate dehydrogenase was strong. The latter enzymes showed the ratio of the unbound to total enzyme ranging from 9–50%, when mixed with F-actin or actomyosin. The recovery of bound enzyme ranged from 61 to 86% for aldolase and from 60 to 92% for glyceraldehyde-3-phosphate dehydrogenase.

The enzyme-myofibrillar protein binding was depressed by the increase in reaction pH, KCl or Ca^{2+} concentration. In the presence of 150 mM KCl or 50 mM Ca^{2+} , the binding was inhibited almost completely. Glycolytic metabolites also influenced the binding. Fructose-1,6-bisphosphate and 2,3-bisphosphoglycerate caused a marked adsorption-hindrance for aldolase at a concentration of 1 mM. The results obtained could account for the enzyme-specific extractability from fish muscle.

Several glycolytic enzymes such as aldolase and glyceraldehyde-3-phosphate dehydrogenase from mammalian muscle have been shown to bind reversibly to the structural muscle proteins, especially to F-actin.^{1–4)} This binding was confirmed by histochemical observations of rabbit muscle, which revealed the binding site of glycolytic enzymes to be the I band.⁵⁾ The binding of aldolase and glyceraldehyde-3-phosphate dehydrogenase to F-actin is influenced not only by pH and ionic strength but also by the specific action of certain metabolites and metal ions.^{2–4)}

The extractability of sarcoplasmic proteins from fish muscle at low-ionic strengths was species-specific,⁶⁾ with a low extractability in marine pelagic (red-fleshed) fish probably due to the interaction of sarcoplasmic proteins with myofibrils.⁷⁾ In a previous paper,⁸⁾ we examined extractability of several glycolytic enzymes from the ordinary muscle of red sea bream, Pacific mackerel and carp and found the dependency on KCl concentration of the extractability of aldolase, glyceraldehyde-3-phosphate dehydrogenase and glucosephosphate isomerase, suggesting an interaction between glycolytic enzymes and myofibrillar proteins at low-ionic strengths.

To characterize further the interaction between glycolytic enzymes and myofibrillar proteins, we carried out this time a model experiment in which some glycolytic enzymes and F-actin (or acto-

myosin) were reacted under various conditions.

Materials and Methods

Fishes

The ordinary muscle from frozen specimens of red sea bream *Pagrus major*, Pacific mackerel *Scomber japonicus* and carp *Cyprinus carpio* was used as the material.

Protein Preparations

Sarcoplasmic proteins containing glycolytic enzymes were prepared by the method of Clarke and Masters⁹⁾ with slight modifications as follows. The ordinary muscle of each species was minced and stirred vigorously with 3 volumes of 0.1 M phosphate buffer (pH 7.5) containing 2 mM dithiothreitol (DTT) and centrifuged at $15,000 \times g$ for 20 min. The supernatant was subjected to ammonium sulfate fractionation between 35% and 90% saturations. The resulting precipitate was collected and dissolved in 0.05 M Tris-HCl buffer (pH 7.5) containing 5 mM EDTA and 2 mM DTT. The sarcoplasmic proteins thus obtained was suspended in a saturated ammonium sulfate solution, and kept at 4°C until use.

F-actin and actomyosin were prepared from rabbit fast muscle by the method of Spudich and Watt¹⁰⁾ and Ebashi,¹¹⁾ respectively.

Sodium dodecylsulfate (SDS)-gel electrophore-

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sis was carried out using 10% polyacrylamide gels containing 0.1% SDS.¹²⁾

Binding Studies

Binding studies were performed according to the method of Clarke and Masters⁴⁾ with some modifications as follows. The stock sarcoplasmic protein suspension was exhaustively dialyzed against 5 mM imidazole-HCl buffer (pH 6.8) containing 1 mM MgCl₂ and 2 mM DTT, and centrifuged at $10,000 \times g$ for 20 min to remove insoluble substances. The supernatant was diluted with the same buffer so that the sarcoplasmic protein concentration was 0.5 mg/ml. In a centrifugal tube, F-actin or actomyosin pellet (containing 5–10 mg protein) obtained by the dilution-precipitation procedure¹¹⁾ was prepared, and to it was added 2 ml of the above sarcoplasmic protein solution. The mixture thus provided was incubated at 25°C. After 20 min, 0.2 ml of the mixture was taken out and immediately cooled in ice for determination of the total enzymatic activity (A) by the method described below. The remainder was centrifuged at $156,000 \times g$ for 60 min at 4°C, and the supernatant was similarly assayed for enzymatic activity (B).

The bound enzyme (%) was defined as the ratio of (A-B)/A.

The precipitate obtained by the above centrifugation was suspended in 2 ml of 0.1 M phosphate buffer (pH 7.6) containing 0.15 M KCl, and

centrifuged in the same manner. The activity in the supernatant obtained was measured.

The same procedure in the absence of F-actin or actomyosin was also performed to estimate the activity decreased by precipitation of sarcoplasmic proteins.

Assays

Activities of glucosephosphate isomerase (GPI; EC 5.3.1.9), aldolase (ALD; EC 4.1.2.13), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; EC 1.2.1.12), phosphoglycerate kinase (PGK; EC 2.7.2.3) and enolase (ENO; EC 4.2.1.11) and protein concentration were measured by the same methods as described in the previous paper.⁸⁾

Results and Discussion

The electrophoretic patterns of sarcoplasmic proteins from the ordinary muscle of red sea bream, Pacific mackerel and carp were species-specific as reported previously¹³⁾ (Fig. 1). The composition in sarcoplasmic proteins was, however, not taken into consideration in the following experiments. The F-actin preparation from rabbit fast muscle showed a single band in SDS-gel electrophoresis, and the actomyosin preparation was almost free from glycolytic enzymes such as ALD and GAPDH.

The binding of several glycolytic enzymes to these myofibrillar proteins was measured at dif-

Fig. 1. SDS-gel electrophoretic patterns of sarcoplasmic proteins from the ordinary muscle of three fish species, and of actin, actomyosin and sarcoplasmic proteins from rabbit fast muscle. Sarcoplasmic proteins from rabbit were extracted with 5 volumes of 50 mM phosphate buffer (pH 7.5).

Abbreviations used: MHC, myosin heavy chain; A, actin; Sp-P, sarcoplasmic proteins; ALD, aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; PGK, phosphoglycerate kinase; GPI, glucosephosphate isomerase; ENO, enolase; and CK, creatine kinase.

Fig. 2. Effect on the binding of fish glycolytic enzymes to F-actin (or actomyosin) of their relative proportions. In a final volume of 2 ml of 5 mM imidazole-HCl buffer (pH 6.8) containing 1 mM $MgCl_2$ and 2 mM DTT.

□, 5 mg F-actin and 2 mg sarcoplasmic proteins; ■, 5 mg F-actin and 1 mg sarcoplasmic proteins; ▤, 10 mg F-actin and 1 mg sarcoplasmic proteins; ▨, 5 mg actomyosin and 1 mg sarcoplasmic proteins; ●●, 10 mg actomyosin and 1 mg sarcoplasmic proteins.

Fig. 3. Effect of pH on the binding of fish glycolytic enzymes to F-actin or actomyosin. Five mg of F-actin and 1 mg of sarcoplasmic proteins were reacted in 5 mM imidazole-HCl buffer containing 1 mM $MgCl_2$ and 2 mM DTT at pH 6.0 (□), pH 6.8 (■) and pH 7.4 (▤). Ten mg of actomyosin and 1 mg of sarcoplasmic proteins were reacted in 5 mM imidazole-HCl buffer containing 1 mM $MgCl_2$ and 2 mM DTT at pH 6.0 (▤), pH 6.8 (●●) and pH 7.4 (▨).

ferent protein concentrations, and the results obtained are shown in Fig. 2. Within the range of protein concentrations examined, there was no binding of carp PGK to F-actin, whereas other enzymes were more or less bound to F-actin or actomyosin under the same conditions. During the binding experiments, the activity decreased by precipitation of sarcoplasmic proteins in the absence of F-actin or actomyosin was around 2% of the original activity. The bound enzyme (%) generally increased by the decrease of the weight ratio of sarcoplasmic proteins to F-actin or actomyosin. ALD and GAPDH of the three species, especially of Pacific mackerel, showed a high bound ratio to F-actin or actomyosin, while GPI, PGK and ENO showed low ratios.

The influence of pH on the binding of fish enzymes to F-actin or actomyosin is shown in Fig. 3.

Fig. 4. SDS-gel electrophoretic patterns of F-actin (or actomyosin)-bound and unbound fractions. In binding experiments, 5 mg of F-actin (or 10 mg of actomyosin) and 1 mg of sarcoplasmic proteins were reacted at pH 6.8. After binding, bound enzymes were extracted from the complex formed with 0.1 M phosphate buffer (pH 7.6) containing 0.15 M KCl, and subjected to electrophoresis as "bound fraction".

o, original sarcoplasmic proteins; u, unbound fraction; and b, bound fraction.

In a pH range between 6.0 and 7.4, the amount of bound enzyme decreased by the increase of pH, especially in the case of GAPDH. On the other hand, the binding of those enzymes to actomyosin was not so much influenced by pH. The binding of Pacific mackerel ALD to myofibrillar proteins was not so markedly affected by pH. Arnold *et al.*³⁾ reported that rabbit muscle ALD was mostly bound to F-actin in a pH range from 6.5 to 7.5.

As reported previously,⁸⁾ ALD and GAPDH from Pacific mackerel were extracted more easily with 0.3% NaHCO₃ containing 0.15% NaCl (pH 8.6) than with water. As is widely accepted, the muscle of red-fleshed fish such as mackerel shows a good gel-forming ability when washed with the above solution to remove sarcoplasmic proteins which inhibit gel formation.¹⁴⁾ The present results demonstrated that the interaction of glycolytic

enzymes with myofibrillar proteins containing F-actin was weakened under the mild alkaline conditions. Some part of Pacific mackerel ALD was, however, still retained in the muscle fibers.

SDS-gel electrophoretic patterns of the unbound and bound fractions of sarcoplasmic proteins to F-actin or actomyosin are shown in Fig. 4. ALD and GAPDH bands detected in the bound fraction from the three fish species were dense, while the other components of sarcoplasmic proteins were mainly detected in the unbound fraction, showing their little interaction with F-actin or actomyosin under the conditions applied.

The distribution of enzymes between the unbound and bound fractions is shown in Fig. 5, together with the total recovery of their activities. The total activities of GPI, PGK and ENO recovered were 99.6–118.5% for F-actin and 92.4–109.1% for actomyosin, whereas those of ALD

Fig. 5. Activity distribution of glycolytic enzymes between unbound (u, □) and bound (b, ▨) fractions to F-actin or actomyosin. In binding experiments, 5 mg of F-actin (or 10 mg of actomyosin) and 1 mg of sarcoplasmic proteins were reacted at pH 6.8. Numbers in parenthesis represent the total recovery of enzymatic activity. Bars represent the standard deviations. Refer to the legend in Fig. 4 for the determination of "bound fraction."

Table 1. Effect of metabolites on the binding of Pacific mackerel aldolase (ALD) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to F-actin.

The binding experiments were performed in a final volume of 2 ml of 5 mM imidazole-HCl buffer (pH 6.8) containing 1 mM $MgCl_2$ and 2 mM DTT, 5 mg F-actin, 1 mg sarcoplasmic proteins, and each metabolite at 0.1 and 1.0 mM

Metabolite	Binding to F-actin (%)*			
	ALD		GAPDH	
	(0.1 mM)	(1.0 mM)	(0.1 mM)	(1.0 mM)
Fructose-1,6-bisphosphate	45	34	100	86
Glucose-1-phosphate	89	94	100	100
Glucose-6-phosphate	89	84	94	98
Fructose-6-phosphate	96	97	100	100
3-Phosphoglycerate	91	35	100	78
2,3-Bisphosphoglycerate	33	30	66	45
2-Phosphoglycerate	74	23	95	74
Phosphoenolpyruvate	88	62	100	100
Adenosine-5'-triphosphate	85	72	100	95
Adenosine-5'-diphosphate	81	75	100	94
Adenosine-5'-monophosphate	94	72	100	95

* The activity value obtained without any of these metabolites was taken as 100%. Refer to the text for further details.

and GAPDH, 63.8–95.3% for F-actin and 62.7–79.9% for actomyosin. Judging from SDS-gel electrophoretic patterns (Fig. 4), the low recovery of ALD and GAPDH may be due to the insufficient solubilization of enzyme with 0.1 M phosphate buffer (pH 7.6) containing 0.15 M KCl. It has been reported that ALD and GAPDH were directly bound to the filaments, while other enzymes were bound over the above enzymes.¹⁵⁾

As shown in Table 1, the binding of ALD and GAPDH to F-actin was also influenced by several metabolites. Fructose-1,6-bisphosphate (FBP) and 2,3-bisphosphoglycerate (2,3-BPG) had a significant effect on the binding of ALD at a concentration of 0.1 mM. Moreover, 3-phosphoglycerate and 2-phosphoglycerate caused a marked effect on binding at a concentration of 1 mM as did FBP and 2,3-BPG. Hexosemonophosphates such as glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate had little effect on the enzyme binding. Only 2,3-BPG depressed the binding of GAPDH at both concentrations. Inhibitory effect of FBP on ALD binding occurred at physiological FBP levels.^{19–19)} It is, however, not clear whether this effect resulted from its specificity as the substrate for ALD or only from FBP concentration.

The ionic strength is one of the most important factors in the interaction between sarcoplasmic and myofibrillar proteins.^{2–4)} The enzyme binding to actomyosin was examined in various KCl concentrations covering a range from 0 to 150 mM. As KCl concentration was increased, the binding

Fig. 6. Effect of KCl concentration on the binding to actomyosin of muscle glycolytic enzymes from red sea bream ($\square$), Pacific mackerel ($\circ$) and carp ($\triangle$). Ten mg of actomyosin and 1 mg of sarcoplasmic proteins were reacted at pH 6.8. Refer to the legend in Fig. 4 for the determination of "bound fraction".

amount of ALD, GAPDH and GPI was decreased (Fig. 6). In the presence of 150 mM KCl, 80–100% of enzyme was not bound to actomyosin.

Fig. 7. Effect of Ca^{2+} concentration on the binding to F-actin or actomyosin of aldolase from the ordinary muscle of red sea bream (■), Pacific mackerel (●) and carp (▲).

Five mg of F-actin (or 10 mg of actomyosin) and 1 mg of sarcoplasmic proteins were reacted at pH 6.8. Refer to the legend in Fig. 4 for the determination of "bound aldolase".

SDS-gel electrophoretic patterns also revealed that these enzymes tended not to bind with myofibrillar proteins as KCl concentration was increased (data not shown). On the other hand, the intensities of PGK and ENO bands were almost the same, irrespective of KCl concentration. Arnold and Pette²⁾ reported that the KCl concentration required for desorption of enzyme was higher than that for the adsorption-hindrance of enzyme. In the present study, similar results were obtained for ALD and GAPDH; that is, the binding of these enzymes almost completely depressed in the presence of 150 mM KCl, while the release of the bound enzyme at the same KCl concentration was 61–78% for ALD and 60–73% for GAPDH. It is therefore recommended that during "kamaboko" processing, minced fish meat must be washed as soon as possible, because otherwise interactions between these glycolytic

enzymes and myofibrillar proteins may proceed further.

The effect of Ca^{2+} concentration on the binding of fish muscle ALD to F-actin and actomyosin was examined (Fig. 7). The amount of bound ALD, irrespective of fish species, decreased with the increase of Ca^{2+} concentration. In the presence of 10 mM Ca^{2+} , the amount of bound ALD of Pacific mackerel to actomyosin decreased to one-third of that in the absence of Ca^{2+} .

The major glycolytic enzymes are located at the I-band of muscle fiber, and *in vitro* binding studies using fish sarcoplasmic proteins have confirmed this.⁵⁾ Moreover, the results of the present binding study correlated well with the extractability of glycolytic enzymes from the fish muscles.⁸⁾ Further experiments are necessary to examine the binding of glycolytic enzymes to F-actin or actomyosin using purified enzyme preparations.

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