Role of enzyme binding in muscle metabolism of the goldfish

JOHN A. DUNCAN AND KENNETH B. STOREY[1]

Institute of Biochemistry and Department of Biology, Carleton University, Ottawa, Ont., Canada K1S 5B6

DUNCAN, J. A., and STOREY, K. B. 1991. Role of enzyme binding in muscle metabolism of the goldfish. Can. J. Zool. **69**: 1571–1576.

Regulation of glycolytic metabolism in muscle by the reversible association of enzymes with the particulate fraction of the cell was assessed in heart and white skeletal muscle of the goldfish *Carassius auratus*. Three physiological states were compared: rested aerobic controls, 21 h of anoxia exposure in water bubbled with N_2 and CO, and exhaustive swimming. Heart muscle responded to the anoxia exposure with an increase in the percentage bound for phosphofructokinase (PFK), aldolase, and pyruvate kinase, the greatest increase being for PFK (from 35 to 48%). When fish swam to exhaustion, however, no changes in the percentage bound occurred for the eight enzymes assayed in heart. In white muscle neither anoxia nor exhaustive swimming altered the binding of the eight enzymes to the particulate fraction of the cell, except for a significant decrease in the percentage of alcohol dehydrogenase bound in anoxic muscle. PFK binding was particularly high in white muscle (63–72% bound, assessed by two methods). PFK binding was sensitive to pH in both organs, with binding increasing as pH decreased. This suggests that binding of PFK could be enhanced when metabolism is highly dependent on glycolytic ATP production due to the fall in cellular pH as a result of metabolic acidosis.

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Le contrôle du métabolisme glycolytique dans le muscle par association réversible entre les enzymes et la fraction particulaire de la cellule a été étudié dans le muscle cardiaque et le muscle squelettique blanc du poisson rouge *Carassius auratus*. Trois états physiologiques ont été comparés : le repos dans des conditions aérobiques (témoins), une exposition de 21 h à l'anoxie dans de l'eau barbotée au N_2 et CO, enfin l'épuisement par la nage. Le muscle cardiaque réagit à l'anoxie par une augmentation de la fraction pourcent destinée à être liée à la phosphofructokinase, à l'aldolase et à la pyruvate kinase, mais surtout de la fraction liée à la phosphofructokinase (de 35 à 48%). Lorsque les poissons nagent jusqu'à l'épuisement, cependant, il ne se produit pas de changements dans la fraction pourcent destinée à être liée aux huit enzymes étudiés. Dans le muscle blanc, ni l'anoxie, ni l'épuisement par la nage n'entraînent de modifications des liaisons entre les huit enzymes et la fraction particulaire de la cellule, à l'exception d'une réduction importante du pourcentage d'alcool déshydrogénase liée dans le muscle anoxique. La liaison à la phosphofructokinase est particulièrement forte dans le muscle blanc (63–72% liée, pourcentage estimé selon deux méthodes). La liaison à la phosphofructokinase s'est avérée sensible au pH dans les deux organes et la liaison est plus forte aux pH plus faibles, ce qui permet de croire que la liaison de la phosphofructokinase pourrait être favorisée lorsque le métabolisme est fortement lié à la production d'ATP glycolytique à la suite d'une chute du pH cellulaire produite par acidose métabolique.

[Traduit par la rédaction]

Introduction

The goldfish *Carassius auratus* L. has an impressive capacity for survival without oxygen. Though a number of species of fish can deal effectively with hypoxic conditions, the goldfish and the crucian carp (*Carassius carassius*) are two of the few species that can withstand long-term anoxia. LT_{50} values for goldfish were 22, 65, and 45 h at 20, 10 and 5°C, respectively (van den Thillart et al. 1983), and carp can apparently endure several months of anoxia while overwintering in ice-covered ponds at 0°C (Blazka 1958).

Several metabolic strategies underlie anaerobic survival of goldfish. Firstly, these fish have a high capacity for glycolytic ATP production based upon high levels of glycogen in all organs and high activities of glycolytic enzymes (van den Thillart et al. 1980; van den Thillart and Smit 1984). Secondly, goldfish have evolved an effective method of dealing with the acidosis that accompanies lactate accumulation as a result of anaerobic glycolysis. Lactate produced by goldfish organs is transported to red or white skeletal muscle where it is converted to ethanol and CO_2, two products that can then be excreted (Shoubridge and Hochachka 1981; van den Thillart et al. 1983). High activities of a cytosolic alcohol dehydrogenase have been found in both types of muscle (Mourik et al. 1982). Thirdly, and probably most

importantly for long-term survival, goldfish respond to anoxia by strongly reducing their metabolic rate to about 30% of the comparable resting, normoxic rate (Anderson 1975; van den Thillart et al. 1976; van Waversveld et al. 1989*a*, 1989*b*). By using a metabolic arrest strategy, goldfish can greatly prolong the time that fixed organ reserves of fermentable fuels (carbohydrate and amino acids) can sustain survival.

Anoxia-induced metabolic rate depression is, in fact, a common survival strategy used by many facultative anaerobes and has been particularly well documented for freshwater turtles and marine molluscs (for reviews see Storey 1988; Storey and Storey 1990). The biochemical regulation of metabolic rate depression has received much attention in recent years. Because the glycolytic pathway is central to anaerobic survival, most of these studies have focused on the control of glycolytic rate depression (Storey 1988; Storey and Storey 1990). To date, three mechanisms of glycolytic rate depression have been identified in anoxia-tolerant animals. These are (*i*) post-translational modifi-cation of regulatory enzymes via protein phosphorylation or dephosphorylation reactions, (*ii*) fructose-2,6-bisphosphate ($F2,6P_2$) regulation of phosphofructokinase (PFK) for the control of carbohydrate use by biosynthetic reactions, and (*iii*) enzyme or pathway regulation by the association or dissociation of enzymes bound to the subcellular particulate fraction.

Previous studies have examined the first two mechanisms as they apply to the anoxic goldfish. Changes in the properties of

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[1]Author to whom all correspondence should be addressed.

glycogen phosphorylase, PFK, and pyruvate kinase (PK), indicative of covalent modification of these enzymes, occur in several organs of the anoxic goldfish, and in most cases these were consistent with the conversion of the enzymes to less active forms in the anoxic state (Storey 1987; Rahman and Storey 1988). Interconversion of the aerobic and anoxic forms of liver PFK and PK was mimicked by the action of protein kinases and phosphatases on the enzymes in vitro (Rahman and Storey 1988). Regulation of glycolysis by changes in F2,6P$_2$ levels was also apparent in anoxic goldfish (Storey 1987). Levels of this potent activator of PFK decreased in liver, gill, and spleen, during anoxia; in particular, a 10-fold decrease in F2,6P$_2$ in liver appeared key to arresting glycolysis and promoting, instead, glucose output from liver during anoxia.

The present study examines the role of enzyme binding to the particulate fraction of the cell in the regulation of muscle metabolism in goldfish. Previous studies have noted a positive correlation between glycolytic rate and the percentage of glycolytic enzymes associated with the particulate (F-actin containing) fraction of the cell. Conditions that increased glycolytic rate (ischemia in mammalian heart, electrical stimulation of mammalian skeletal muscle, burst swimming for trout white muscle) increased the percentage of enzymes bound, whereas conditions that decreased glycolytic rate (anaerobiosis for whelk muscle; starvation for fish white muscle) decreased enzyme binding (Clarke et al. 1980, 1984; Plaxton and Storey 1986; Lowery et al. 1987; Brooks and Storey 1988a). The present paper analyzes the relevance of binding of glycolytic enzymes to the particulate fraction to the regulation of goldfish muscle metabolism by comparing the response to anoxia (which induces a metabolic rate depression) with the response to exhaustive swimming (metabolic activation).

Materials and methods

Chemicals and animals

All biochemicals and coupling enzymes were purchased from Sigma Chemical Co., St. Louis, MO, or Boehringer Mannheim, Montreal, Que. Goldfish (Carassius auratus), 15–20 cm, were obtained locally and maintained in circulating, dechlorinated, aerated water at 7°C for 4–6 weeks before use. Animals were fed ad libitum on commercial goldfish pellets and were fasted overnight prior to use. Control fish were gently netted from the holding tank and quickly killed by a blow to the head. Tissues were dissected out within 20 s and processed immediately. For anoxia experiments, fish were transferred to sealed aquaria (maximum of three fish per tank) containing 18 L water that had been bubbled with nitrogen gas for 9 h previously. Fish were then given anoxia exposure for 21 h, during which time the water continued to be bubbled with nitrogen gas, and bubbling with carbon monoxide was added (Rahman and Storey 1988); the tank was bathed in 7°C water throughout. To exercise fish, they were transferred from the holding tank to a second aquarium containing water at 7°C. Swimming was induced by tail pinching and continued until the fish were exhausted, about 30 min on average. Fish were killed and tissues were sampled as described above for controls. Control and experimental fish exhibited little or no struggling upon removal from the tanks.

Measurement of enzyme binding to the particulate fraction

Fresh tissues were immediately processed for the "dilution" method, using the procedure of Clarke et al. (1984). Tissue samples were rapidly weighed and then homogenized (1:4 w/v) in ice-cold homogenization solution (HS) (250 mM sucrose with 10 mM 2-mercaptoethanol and 0.1 mM phenylmethylsulfonyl fluoride) for 2 × 15 s, using an Ultraturrax homogenizer at 80% of full speed. A 0.3-mL aliquot of this homogenate was removed and diluted (1:4 v/v) with ice-cold stabilization buffer (SB) containing 100 mM potassium phosphate (pH 7.5), 1 mM EGTA, 1 mM EDTA, 25 mM sodium fluoride, 10 mM 2-mer-

captoethanol, 0.1 mM fructose-1,6-bisphosphate, and 0.1 mM ATP (the latter two components being added to stabilize the PFK). This fraction was used to measure the total activity of enzymes to check the recovery of enzymes in the soluble and particulate fractions. A second aliquot (0.3 mL) of the crude homogenate was centrifuged at 12 000 × g for 5 min at 20°C in an IEC microcapillary centrifuge (model MB) with an 837 rotor. The supernatant was removed and diluted (1:4 v/v) in SB; this represents the free or soluble fraction. The pellet was then extracted twice to release bound enzyme, each extraction consisting of resuspension in 0.75 mL of SB and centrifugation as above. The supernatants from these two extractions were combined to represent the bound fraction of enzymes. Enzyme fractions were stored on ice and assayed for enzyme activities. Less than 2% of the total activity remained in the final pellet, owing to nonspecific trapping; the results were not corrected for this amount. The percentage of total enzyme bound to the particulate fraction was calculated as follows: units bound /(units free + bound) × 100.

For the "pressed" method, samples of white skeletal muscle (0.5–1.0 g) from control fish were placed in a French pressure cell (Carleton University Science Technology Centre) which had a small-diameter hole in the bottom of the cylinder. Two layers of 200-mesh nylon screening were placed between the tissue sample and the hole to prevent extrusion of tissue. The tissue was then pressed at 264 tonnes/sq. in. (1 sq. in. = 6.45 cm²) for 15 s at 20°C. The fluid that passed through the hole at the bottom was collected and centrifuged at 12 000 × g for 5 min at 20°C to remove any cell debris. Less than 2% of the total activity was associated with the 12 000 × g pellet. The supernatant was then diluted (1:24 v/v) in SB (to give an identical dilution factor to the dilution method) and stored on ice until it was assayed; this represents free enzyme activities. Since the remaining tissue could not be easily retrieved from the pressure cell (and would not be completely free from cellular fluid), it was not processed for the measurement of bound enzyme activities. Instead, samples for the pressed method were taken from individual fish that were also sampled for the dilution method, and measurements of total enzyme activities were made as described above for that method. The percent free values were determined from the ratio of enzyme activities in the juice versus whole tissue, corrected for the percentage of tissue that is water, using the equation

$$\% \text{ free} = \frac{\text{IU}/\text{mL}_{\text{free}} \times 0.80}{\text{IU}/\text{mL}_{\text{total}}}$$

Enzyme assays

All enzymes were assayed at 22°C by means of a Gilford recording spectrophotometer. Optimal assay conditions for creatine kinase (CK), glyceraldehyde-3-P dehydrogenase (GAPDH), and phosphoglycerate kinase (PGK) were as in Brooks and Storey (1988a); hexokinase (HK), phosphofructokinase (PFK), aldolase (ALD), pyruvate kinase (PK), and lactate dehyrogenase (LDH) were assayed as in Plaxton and Storey (1986). The assay for alcohol dehydrogenase (ADH) contained 50 mM Tris–HCl (pH 8.5), 100 mM ethanol, and 0.2 mM NAD$^+$. One unit of enzyme activity is defined as the amount of enzyme utilizing 1 μmol of substrate per in at 22°C.

pH titration of PFK binding

Homogenates of fresh tissues were prepared in HS as above. Aliquots were then diluted 1:1 v/v with solutions containing 250 mM sucrose and 40 mM imidazole, with or without KCl, and adjusted to pH values ranging from 5.5 to 8.0. After mixing, samples were incubated for 2 min and then 300 μL of homogenate was removed and centrifuged at 12 000 × g for 5 min at 20°C. The supernatant was removed and the pH was measured. A 100-μL aliquot of supernatant was then diluted with 400 μL SB; the remaining supernatant was discarded. The pellet was resuspended in 1500 μL of SB. Both fractions were then stored on ice until PFK activity was measured using the standard assay conditions.

Data calculations and statistics

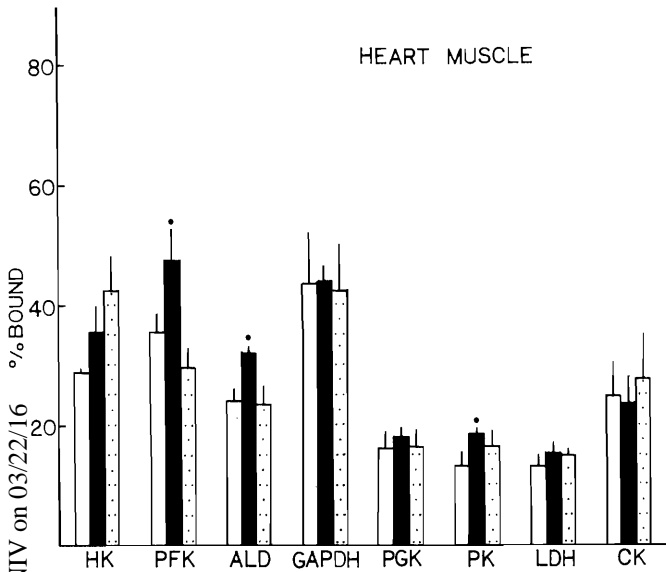Data are given as the mean ± SE, with n representing determinations

FIG. 1. Percentage of recovered enzyme activity associated with the particulate fraction of the cell in goldfish heart under three metabolic states: aerobic, control (□), 21 h of anoxia (■), and exercised to exhaustion (▧). Data are given as the mean ± SE, (n = 5 or 6 individual fish. •, significantly different from the corresponding control aerobic value, $P < 0. 05$, using Student's t-test. For explanation of abbreviations see text.
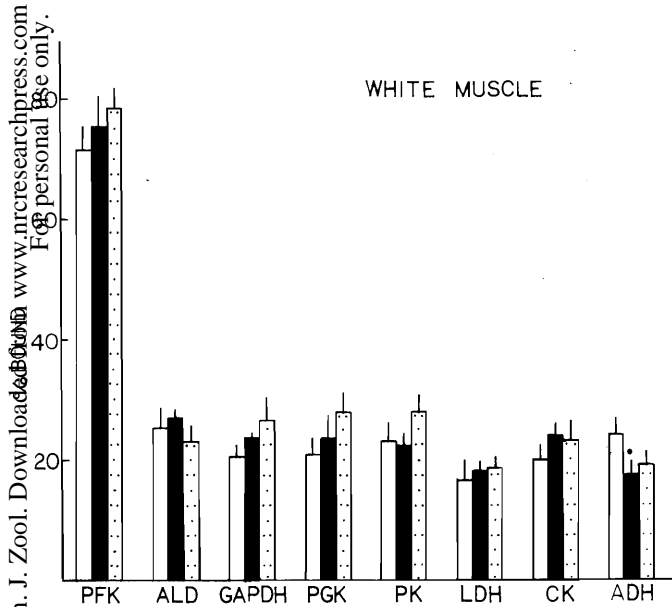

FIG. 2. Percentage of recovered enzyme activity associated with the particulate fraction of the cell in goldfish white muscle under three metabolic states: aerobic, control (□), 21 h of anoxia (■), and exercised to exhaustion (▧). For other information see Fig. 1.

on separate individual animals. Statistical significance was assessed by Student's t-test.

Results

Figures 1 and 2 show the percentages of several glycolytic and other enzymes associated with the particulate fraction of goldfish heart and white skeletal muscle as assessed by the dilution method. Three physiological states are compared: control (resting, aerobic), 21 h of anoxia in water bubbled with N_2 and CO, and swimming to exhaustion. Figure 1 shows that a con-

TABLE 1. Maximal activities (units/g wet weight) of nine enzymes in goldfish heart and white muscle

	Heart	White muscle
Hexokinase	6.6±0.5	nd
Phosphofructokinase	19±3	16±1
Aldolase	41±6	56±6
Glyceraldehyde-3-P dehydrogenase	374±52	327±31
Phosphoglycerate kinase	234±26	118±15
Pyruvate kinase	172±17	169±14
Lactate dehydrogenase	415±45	312±26
Alcohol dehydrogenase	nd	7.4±1.3
Creatine kinase	94±9	353±40

NOTES: Values are given as the mean ± SE (n = 14 or 15 fish), determined in fractions saved for measuring total enzyme activity as described for the dilution method in Materials and methods. nd, activity not detected.

siderable fraction of glycolytic enzymes was associated with the particulate fraction of the cell in heart; the percentage bound in control, aerobic heart ranged from 13 ± 2.2 for LDH to 43.4 ± 8.0 for GAPDH. In anoxia, binding by three enzymes (PFK, ALD, PK) increased significantly in heart. PFK showed the greatest redistribution of enzyme from soluble to particle-bound, an increase from 35% bound in control fish to 48% bound in anoxic fish. The percentage of PK bound increased from 13 to 18%, whereas bound ALD increased from 24 to 32%. Anoxia exposure had no effect on binding by the five other enzymes in heart. In contrast to the effect of anoxia, when fish were exercised to exhaustion by continuous swimming no significant changes in enzyme binding in heart were observed for any of the eight enzymes. Recovery of enzyme activity in the free versus bound fractions was calculated and compared with total activity measured separately. Recovery of activity in free + bound fractions ranged from 95 ± 8% for PFK to 134 ± 8% for CK relative to total activity, with an average value for all enzymes of 116 ± 6%.

Figure 2 shows enzyme association with the particulate fraction in goldfish white muscle. In the control, aerobic situation, the percentage bound was relatively low for seven enzymes, ranging from 17 ± 3 for LDH to 25 ± 3 for ALD. However, PFK was remarkably different, with 72 ± 4% of the enzyme bound. In white muscle, exposure to anoxia resulted in a small decrease in the percentage of ADH bound to the particulate fraction but had no effect on the percentage bound for any other enzyme. Exhaustive exercise, as in heart, resulted in no significant changes in the percentage bound for any of the enzymes. For white muscle, recovery of activity in bound + free fractions, relative total activity measured separately, ranged from 96 ± 3% for PFK to 163 ± 8% for CK, the average for all enzymes being 145 ± 7%.

Table 1 shows the maximal activies, measured in units per gram wet wet weight, of the nine enzymes in goldfish heart and white muscle. Neither anoxia nor exercise had any effect on the activity measured for any of the enzymes, so the values for all treatments were combined. Some distinct differences between the two tissues were found. ADH was not found in heart but occurred in white muscle, as has been shown previously (Mourik et al. 1982),whereas HK was detected in heart but not in white muscle. Low activities of an unstable HK have been previously
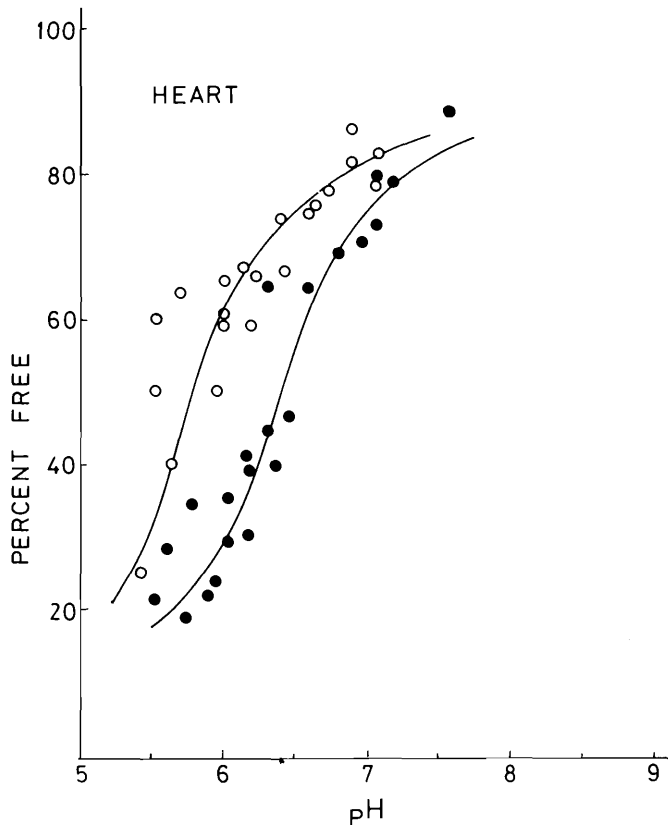
FIG. 3. The pH titration profile of goldfish heart muscle phosphofructokinase binding in the absence of KCl (●) and the presence of 35 mM KCl (○). Each data point represents a single determination.
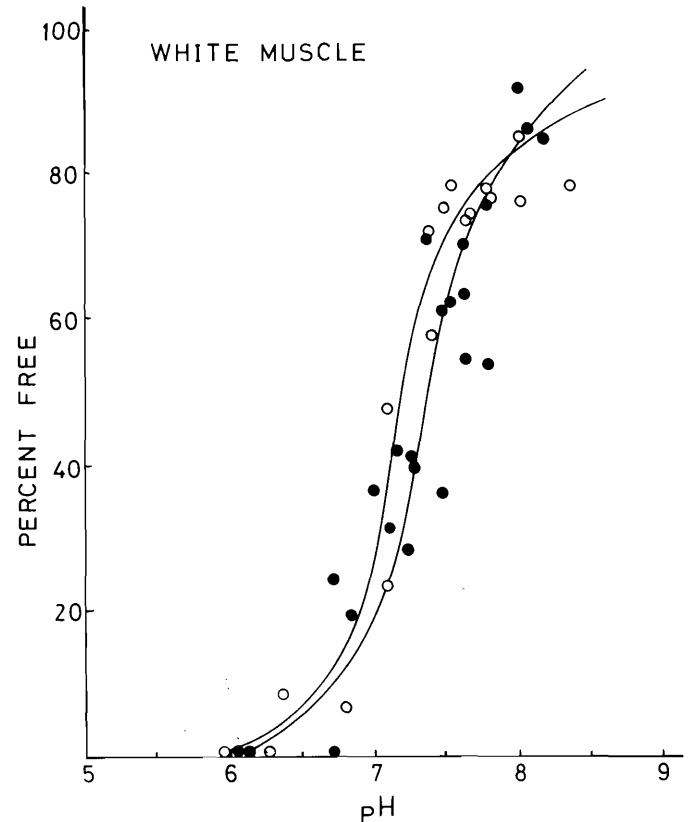

FIG. 4. The pH titration profile of goldfish white muscle phosphofructokinase binding in the absence of KCl (●) and the presence of 100 mM KCl (○). Each data point represents a single determination.

reported for goldfish white muscle (van den Thillart and Smit 1984), but we were unable to detect significant activity above a high blank activity. PGK and LDH levels in skeletal muscle were substantially lower than in heart but CK activity in skeletal muscle was 3.5-fold that in heart, in line with the role of phosphagen in burst swimming.

To further analyze enzyme binding in white muscle we compared a second method of measuring enzyme binding with the dilution method. The pressed method extrudes soluble enzymes from the tissue by using high pressure (264 tonnes/ sq. in.) and separates bound from free fractions without the need to dilute the cytosol during a homogenization step. Perhaps not unexpectedly because of the method used, we found greater variability in the percentages of enzymes bound when the pressed method was used (SEMs of 6–20% for the eight enzymes in muscle from control aerobic fish, $n = 3$), but there were no significant differences among the mean percentages bound, as determined by the pressed method versus the dilution method, for any of the enzymes tested. Like the dilution method, the pressed method showed high levels of PFK binding (63 ± 6%), but uniformly lower binding for all other enzymes (ranging from 17 ± 15% for ALD to 38 ± 9% for ADH).

The effects of pH and salt on binding by PFK are shown in Figs. 3 and 4 for heart and white muscle, respectively. Binding of PFK to subcellular particulate matter was pH sensitive; in both organs binding increased (percent free decreased) as pH decreased. Parenthetically, recovery of PFK activity in the bound + free fractions averaged 96 ± 5% relative to total activity in unfractionated samples, indicating that though pH treatments

altered PFK binding, they did not change total enzyme activity. In the absence of salt, pK_a values (midpoint of the titration curves) were approximately 6.4 for heart PFK and 7.4 for white muscle PFK. Both figures also show the effect of added KCl (35 mM for heart, 100 mM for white muscle) on the pH titrations. In the presence of KCl the percentage of PFK bound in heart extracts decreased at all pH values tested, and the pK_a values shifted by 0.6 units to pH 5.8. In skeletal muscle extracts, the addition of KCl, even at a level 3-fold that in heart, had little effect on the relationship between pH and PFK binding, the pK_a value shifting only slightly to 7.2. These differences in pH sensitivity of binding and salt effects on binding for PFK from the two organs suggest that goldfish heart and white muscle contain different isozymic forms of PFK, as do mammalian heart and skeletal muscle (Pilkis et al. 1987).

Discussion

In recent years a number of authors have demonstrated that various cellular enzymes can bind reversibly to other enzymes or to structural elements of the cell (e.g., membranes, F-actin) (for reviews see Srivastava and Bernhard 1986; Srere 1987; Keleti and Ovadi 1988; Brooks and Storey 1988b). The physiological relevance of such binding for individual glycolytic enzymes, as well as for the formation of a glycolytic complex, has been supported by various studies that have shown a positive correlation between glycolytic rate and the percentage of glycolytic enzymes bound to the particulate fraction of the cell (Clarke et al. 1980, 1984; Plaxton and Storey 1986; Brooks and Storey 1988a). Studies to date with animal systems have focused on an

individual transition: resting versus active muscle metabolism or resting versus metabolic arrest. We reasoned that the goldfish was a good animal model with which to assess the effects of both metabolic activation (vigorous swimming) and metabolic depression (anoxia-induced) on glycolytic enzyme binding.

In anoxia, goldfish sustain an overall metabolic arrest that reduces the metabolic rate to only about 30% of the resting aerobic rate (van Waversveld et al. 1989a, 1989b). However, this undoubtedly reflects varying degrees of metabolic depression by individual organs. As a further complication, the glycolytic rate may increase during anoxia in some organs despite a decrease in the overall metabolic rate of the organ because of a change in fuel use from aerobic lipid-based oxidative phosphorylation to anoxic fermentation of glucose. Thus, despite a bradycardia during anoxia, the glycolytic rate in heart increased, undoubtedly as a result of the lipid to carbohydrate transition (Shoubridge and Hochachka 1983). However, white skeletal muscle, which may rely upon carbohydrate fuels at all times, showed a very distinct glycolytic rate depression, and an analysis of changes in glyco-lytic intermediates revealed strong inhibitory control at the PFK reaction (Shoubridge and Hochachka 1983).

In light of the above information, the effect of anoxia on glycolytic enzyme binding in goldfish heart can be explained. Binding of PFK, ALD, and PK increased significantly and increased enzyme binding has previously been correlated with increased glycolytic rate. Indeed, this is one of the responses to ischemia by mammalian heart (Clarke et al. 1984), and Choate et al. (1985) have shown that PFK binding in sheep heart increases with the length of ischemia. Enhanced binding by glycolytic enzymes could help to localize ATP production by anaerobic glycolysis into close physical proximity with the myofibrils to support anaerobic muscle work. The largest increase in binding was for PFK and, as this is a primary regulatory enzyme of glycolysis and one of the glycolytic enzymes in lowest concentration in the cell (Brooks and Storey 1988c), the central role of PFK binding in enhancing the function of a "glycolytic complex" can be appreciated.

Among the factors involved in increasing PFK binding to particulate matter during anoxia might be changes in cellular pH. As Figs. 3 and 4 show, PFK binding in vitro to particulate matter in heart or muscle extracts increased as pH decreased. Changes in cellular pH frequently occur as a result of anoxia or exercise. Heart lactate concentration rose 20-fold within 3 h of anoxia exposure in goldfish (Shoubridge and Hochachka 1983), indicating that intracellular pH undoubtedly dropped consider-ably in anoxia. Indeed, a fall in skeletal muscle pH during anoxia (from 7.36 for aerobic controls to 6.88 after 3.5 h of anoxia) has been clearly demonstrated in goldfish by means of ^{31}P NMR (van den Thillart et al. 1989), and a decrease in cellular pH typically occurs during anoxia in all facultative anaerobes (for a review see Storey and Storey 1990). Decreased cellular pH, by changing the charge state on enzymes, could therefore enhance enzyme binding to particulate matter during anoxia. However, the role of pH is still far from understood, and indeed, the opposite correlation of enzyme binding and pH occurs in anoxia-tolerant marine molluscs. Here, all organs display a strong glycolytic rate depression that is supported by a strong decrease in the percentage of glycolytic enzymes bound to particulate matter, and yet cellular pH also decreases during anoxia (Plaxton and Storey 1986; Brooks and Storey 1989). Furthermore, despite a decrease of nearly 0.5 pH unit in anoxic goldfish skeletal muscle (van den Thillart et al. 1989) and a clear dependence of the binding of skeletal muscle PFK on pH (Fig. 4), there was no

change in the percentage of skeletal muscle PFK bound during anoxia in vivo (Fig. 2). Obviously, then, pH may be only one of several factors that influence enzyme binding in vivo.

The kinetic properties of PFK and PK isolated from anoxic goldfish heart showed stable changes that gave evidence of anoxia-induced covalent modifications producing less active enzyme forms (Rahman and Storey 1988). Thus, PFK from anoxic goldfish heart showed decreased sensitivity to activators (K_a values for F2,6P$_2$ and AMP increased 2-fold), whereas PK showed increased sensitivity to L-alanine inhibition (I$_{50}$ decreased 3-fold) (Rahman and Storey 1988). At first glance, then, the binding data and kinetic data appear to be at odds but this might be resolved if the two phenomena have somewhat different functions. As noted above, the glycolytic enzyme binding may serve to localize glycolytic ATP output when glycolysis, rather than lipid oxidation, must fuel myfibrillar contraction. However, the purpose of anoxia-induced covalent modification, in creating an enzyme that is less sensitive to activators, may be to alter enzyme function for operation under a state of overall reduced organ metabolic rate during anoxia.

In white skeletal muscle, anoxia exposure had no effect on the binding of the six glycolytic enzymes assayed, or on CK binding, and resulted in only a small decrease in the percentage of alcohol dehydrogenase bound. Despite this, strong inhibitory control of glycolysis, focused on the PFK reaction, has been indicated from the analysis of changes in glycolytic intermediates during anoxia (Shoubridge and Hochachka 1983). The present data indicate that the mechanism of such PFK inhibition is not changes in enzyme binding with the myofibrillar fraction (the high level of sucrose used in the dilution method sediments myofibrils) of white muscle. Furthermore, the kinetic constants of PFK were unaltered in anoxia (Rahman and Storey 1988), and levels of the PFK activator F2,6P$_2$ in white muscle were not affected by anoxia (Storey 1987). Clearly, the factors regulating PFK inhibition and overall glycolytic rate depression in white muscle during anoxia remain elusive. Since levels of glycogen phosphorylase a and kinetic properties of PK were also unaltered in anoxia in goldfish white muscle (Storey 1987; Rahman and Storey 1988), the mechanics of glycolytic rate depression might lie elsewhere, such as in the regulation of glucose uptake and (or) phosphorylation (the primary fuel for muscle in anoxia being blood glucose).

Though anoxia changed the binding of glycolytic enzymes in heart, swimming exercise did not alter the percentages of bound enzymes in either heart or white muscle. For heart, the lack of change in glycolytic enzyme binding during exercise suggests that any increase in heart activity during the exercise period did not require an increase in the glycolytic rate and may have been supported fully by lipid-based aerobic metabolism. The same lack of change in the percentages of bound glycolytic enzymes was also found in trout heart during either brief (30 s) or exhaustive bouts of swimming (Brooks and Storey 1988a). The absence of an effect of exercise on enzyme binding in white muscle is more surprising, however, since an activation of glycolysis would be expected during such swimming. Indeed, burst swimming in trout, when analyzed by the same dilution method, was accompanied by rapid (within 30 s) and large increases in the amounts of PFK, ALD, GAPDH, and PGK bound to particulate matter (Brooks and Storey 1988a). For PFK, the increase was from about 65 to 95% bound, and persisted when fish were swum to exhaustion. However, the percentage of PFK bound was high (72–79%) in goldfish muscle under all physiological states and binding was not appreciably affected by

high KCl levels (Fig. 4), whereas PFK binding in heart (Fig. 3) and trout muscle (Brooks and Storey 1988c) was disrupted by increasing ionic strength within the physiological range. The differences in enzyme binding in white muscles between the two species are not readily explainable but provide intriguing questions for further research. In both systems, however, the high percentage of PFK bound, coupled with the fact that the total number of molecules of PFK is typically the lowest of all glycolytic enzymes (Brooks and Storey 1988c), suggests that PFK binding is key to the action of any functional glycolytic complex bound to subcellular structural elements.

The dilution method of measuring enzyme binding to the particulate fraction has received some criticism because, by diluting the tissue 4-fold with sucrose homogenization solution, binding may be artificially increased by the resulting low ionic strength of the medium. Indeed, studies have shown that enzyme binding to nonspecific particulate matter or to F-actin can be titrated by salt; increasing ionic strength decreases enzyme binding (Brooks and Storey 1988c). This effect is also obvious from Fig. 3; at any given pH the percentage of free PFK in heart homogenates is higher in the presence than in the absence of 35 mM KCl. However, when the pressed method, which causes no dilution of the cytoplasm, was compared with the dilution method there were no significant differences in the measured binding of any of the enzymes in white muscle from control fish. These results indicate, therefore, that neither the dilution of the cytoplasm nor the physical forces of homogenization used in the dilution method significantly changed the distribution of bound versus free enzymes in white muscle. This contrasts somewhat with equivalent measurements on trout white muscle, which showed that the percentages of PFK and GAPDH bound were substantially higher when binding was assessed by the dilution method than by the pressed method (Brooks and Storey 1988c).

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