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Role of covalent modification in the control of glycolytic enzymes in response to environmental anoxia in goldfish

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Summary. The effects of environmental anoxia (24 h at 7 °C in N_2/CO bubbled water) on the maximal activities, selected kinetic properties, and isoelectric points of phosphofructokinase and pyruvate kinase were measured in eight tissues of the goldfish, *Carassius auratus*, in order to evaluate the role of possible covalent modification of enzymes in glycolytic rate control and metabolic depression during facultative anaerobiosis. Both enzymes showed modified kinetic properties as a result of anoxia in liver, kidney, brain, spleen, gill, and heart. Effects of anoxia on properties of pyruvate kinase included reduced V_{max}, increased $S_{0.5}$ for phosphoenolpyruvate, increased K_a for fructose-1,6-bisphosphate, and strongly reduced I_{50} for alanine; all these effects are consistent with an anoxia-induced phosphorylation of pyruvate kinase to produce a less active enzyme form. Anoxia-induced alterations in phosphofructokinase kinetics included tissue-specific changes in $S_{0.5}$ for fructose-6-phosphate, Hill coefficient, K_a values for fructose-2,6-bisphosphate, AMP, and NH_4^+, and I_{50} values for ATP and citrate, the direction of changes being generally consistent with the production of a less active enzyme form in the anoxic tissue. Enzymes from aerobic versus anoxic skeletal muscle (both red and white) did not differ in kinetic properties but anoxic enzyme forms had significantly different pI values than the corresponding aerobic forms. Enzyme phosphorylation-dephosphorylation as the basis of the anoxia-induced changes in the kinetic properties of PFK and PK was further tested in liver: treatment of the aerobic forms of both enzymes with cAMP dependent protein kinase altered enzyme kinetic properties to those typical of the anoxic enzymes while alkaline phosphatase treatment of the anoxic enzyme forms had the opposite effect. The data provide strong evidence that coordinated glycolytic rate control, as part of an overall metabolic rate depression during anoxia, is mediated via anoxia-induced covalent modification of regulatory enzymes.

Introduction

A well-developed tolerance of long-term anoxia occurs among various animal species that naturally experience periods of oxygen absence from the environment. The common goldfish, *Carassius auratus*, is one such species, its tolerance for hypoxia and/or anoxia allowing survival in stagnant waters or in ice-covered small ponds during overwintering. Anoxia tolerance can extend to several weeks at 5 °C (Walker and Johansen 1977).

The primary adaptive strategy supporting facultative anaerobiosis in animals is a profound depression of metabolic rate, lowering ATP requirements to a level that can be supported over the long term by less efficient fermentative pathways of catabolism (Storey 1985, 1987a). As a result of metabolic depression no Pasteur effect is seen; indeed, in some cases, a depression of glycolytic rate occurs. In addition, some species employ alternative pathways of fermentative catabolism to supplement ATP production.

Goldfish respond to anoxia with a drop in metabolic rate to a level 1/3 to 1/5 that of normoxic

Abbreviations: *cAMP* cyclic 3′5′ adenosine monophosphate; *$F16P_2$* fructose-1,6-bisphosphate; *$F26P_2$* fructose-2,6-bisphosphate; *F6P* fructose-6-phosphate; *PEP* phosphoenolpyruvate; *PFK* phosphofructokinase (E.C. 2.7.1.11); *PK* pyruvate kinase (E.C. 2.7.1.40); *PMSF* phenylmethylsulfonyl fluoride

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basal rate (Andersen 1975; van den Thillart et al. 1976). Metabolism is reorganized in a tissue specific manner. Liver glycogen, supplying blood glucose, becomes the primary fuel reserve supporting metabolism in all organs [a low level of amino acid fermentation may also occur (van den Thillart et al. 1983)]; glycogen reserves of brain and heart are depleted early in anoxia whereas muscle glycogen is retained to fuel burst swimming as needed (Shoubridge and Hochachka 1983). Relative rates of glycolysis increase in heart and brain but decrease in other organs, in line with the overall metabolic depression. Lactate is produced as the metabolic end product in all organs but, when anoxia is extended, metabolic acidosis is limited by the conversion of lactate to ethanol plus CO_2 in skeletal muscles and the subsequent excretion of these compounds across the gills (Shoubridge and Hochachka 1981, 1983; van den Thillart and Verbeek 1982).

Control of glycolysis is obviously the key to anaerobic survival. Glycolytic rate in each organ must be adjusted to match the requirements for anoxic survival: a) the dependence on carbohydrate fermentation for ATP synthesis, b) the overall reduction in ATP utilization due to metabolic depression, and c) changes in fuel use with the aerobic-anaerobic transition (e.g. organs such as heart switch from lipid to carbohydrate catabolism). Control over glycolytic rate is exerted at key regulatory enzymes, phosphofructokinase (PFK) being the primary site and pyruvate kinase (PK) an additional site of flux control. Recent studies of anoxia tolerance in marine molluscs have shown the importance of covalent modification, via phosphorylation-dephosphorylation reactions, in producing large changes in the activity states of these enzymes during anaerobiosis (Storey 1984, 1985, 1987a; Plaxton and Storey 1984a, b). Both glycolytic rate control and coordinated control over other enzymes/proteins in the cell appear to be achieved via covalent modification with the aerobic-anaerobic transition. The present study seeks to determine whether covalent modification of regulatory enzymes is also a mechanism of glycolytic rate control employed by anoxia-tolerant goldfish.

Materials and methods

Animals. Goldfish (*Carassius auratus*) of 15–20 cm length were purchased from Grassy Forks Fishery Co., Indiana. Animals were held in running, dechlorinated water at 7 °C for 2–3 weeks before use and fed commercial goldfish pellets. Fish were fasted overnight before use.

Control goldfish were gently netted from the holding tank, killed by decapitation, and organs were quickly dissected out and frozen in liquid nitrogen. For anoxia experiments, a tank (18 l) of dechlorinated water was first bubbled with nitrogen gas for 9 h. Two fish were then added to the tank and bubbling with N_2 gas was resumed for 15 h followed by bubbling with carbon monoxide for an additional 9 h. The tank was bathed in 7 °C water throughout. Following 24 h of anoxia, fish were killed and organs sampled as described above. Frozen tissues were stored at −80 °C until further use.

Chemicals. Coupling enzymes and biochemicals were purchased from Sigma Chemical Co., St. Louis, MO, or Boehringer Mannheim Corp., Montreal. Ampholines were from LKB Bromma, Sweden. All other chemicals were of reagent grade and distilled, deionized water was used throughout.

Enzyme preparations. Frozen tissue samples were weighed and homogenized (3 × 20 sec using a Polytron PT10 homogenizer) in ice-cold 50 mM imidazole-HCl buffer, pH 7.0 containing 10 mM EDTA, 10 mM EGTA, 100 mM NaF, and 30 mM 2-mercaptoethanol with 0.1 mM PMSF added just prior to homogenization. Homogenates were centrifuged at 27000 *g* for 30 min at 4 °C. Supernatants were removed and dialyzed for 2–4 h at 4 °C against homogenization buffer minus PMSF.

Enzyme assay. Activities in dialyzed supernatants were measured at 22 °C using a Pye Unicam SP 1800 recording spectrophotometer. For PFK, optimal assay conditions were 50 mM imidazole-HCl buffer, pH 7.0, 10 mM F6P, 1 mM ATP, 50 mM KCl, 5 mM $MgCl_2$, 0.15 mM NADH, and dialyzed coupling enzymes (0.2 U glycerol-3-phosphate dehydrogenase, 1.2 U triosephosphate isomerase, 0.1 U aldolase). Due to the presence of phosphoglucoisomerase in enzyme preparations, a 1:3 mixture of F6P:glucose-6-phosphate was used as the substrate in all instances. Inhibitor effects were tested with a 1:1 mixture Mg:ATP and 2:1 Mg:citrate. For PK, conditions for maximal enzyme activity were 30 mM imidazole-HCl buffer, pH 7.0, 5 mM PEP, 2 mM ADP, 50 mM KCl, 5 mM $MgSO_4$, 0.20 mM NADH, 1 U lactate dehydrogenase. One unit of enzyme activity is defined as the amount utilizing 1 μmol F6P or PEP per min at 22 °C. Kinetic constants were determined as described by Plaxton and Storey (1984a).

Isoelectrofocusing. Isoelectrofocusing was performed as described by Plaxton and Storey (1985) using an LKB 8101 column (110 ml) and an ampholine-sucrose gradient of pH 3.5–10 for PFK and 4–6 for PK. After running at 300 V for 20 h at 4 °C, 2 ml fractions were collected and assayed.

In vitro phosphorylation. Liver tissue from aerobic goldfish was homogenized in 50 mM imidazole-HCl buffer, pH 7.0, containing 100 mM NaF, 30 mM 2-mercaptoethanol, and 0.1 mM PMSF. Centrifugation and dialysis were as above. Aliquots of dialyzed supernatant were incubated in imidazole buffer, pH 7.0 with or without 900 U rabbit muscle cAMP-dependent protein kinase, 5 mM ATP, 10 mM $MgCl_2$, and 0.1 mM cAMP at 22 °C and sampled at timed intervals.

In vitro dephosphorylation. Liver tissue from anoxic goldfish was homogenized in 50 mM Tris-HCl buffer, pH 8.3 containing 10 mM $MgCl_2$, 25 mM KCl, 10 mM 2-mercaptoethanol, 0.75 M sucrose, and 0.1 mM PMSF. Centrifugation and dialysis were as above. Dialysis was against homogenizing buffer plus 2 mM EDTA but minus PMSF. Dialyzed enzyme preparations were incubated in the Tris-sucrose buffer with or without 20 U *E. coli* alkaline phosphatase for 4–5 h at 30 °C and then assayed.

Results

Isoelectrofocusing

Enzyme modification via the incorporation of covalently bound phosphate typically alters the net charge on an enzyme and shifts the isoelectric point. Isoelectric points of PFK and PK from liver, kidney, and red and white skeletal muscle of aerobic and anoxic goldfish are shown in Table 1. Anoxia shifted the pI of both enzymes towards alkalinity in liver and skeletal muscles but did not affect the enzymes in kidney. The increase in pI was about 0.4 pH units for PK in all instances, 0.44 for PFK in liver, and a lesser amount, 0.16–0.19 pH unit for PFK in skeletal muscles.

Kinetic properties of phosphofructokinase

Kinetic properties of PFK from aerobic versus 24 h anoxic goldfish are shown in Table 2 for liver, kidney, brain, and spleen and Table 3 for heart, gill, and red and white skeletal muscle. PFK in five tissues (liver, kidney, brain, spleen, heart) showed significant alterations in three or more of the parameters measured as a result of anoxia. For the soft tissues (liver, kidney, brain, spleen) a significant decrease in the I_{50}(ATP) was a consistent

Table 1. Isoelectric points (pI) of phosphofructokinase and pyruvate kinase from tissues of aerobic versus 24 h anoxic goldfish

	Phosphofructokinase		Pyruvate kinase	
	Aerobic	Anoxic	Aerobic	Anoxic
Liver	3.86±0.04	4.30±0.01*	3.87±0.07	4.30±0.01*
Kidney	4.00±0.04	4.04±0.07	4.05±0.06	4.09±0.09
Red muscle	4.20±0.02	4.39±0.02*	4.47±0.02	4.84±0.02*
White muscle	3.32±0.03	3.48±0.09*	3.79±0.03	4.23±0.03*

Values are means ± SEM for $n=3$ determinations on separate preparations. * Significantly different from corresponding aerobic values by Student's t-test, $P<0.05$

Table 2. Kinetic properties of phosphofructokinase in liver, kidney, brain, and spleen of aerobic versus 24 h anoxic goldfish

	Liver		Kidney		Brain		Spleen	
	Aerobic	Anoxic	Aerobic	Anoxic	Aerobic	Anoxic	Aerobic	Anoxic
V_{max} (U/g wet wt)	4.6 ±0.76	4.3 ±0.90	7.6 ±1.32	6.8 ±1.09	7.1 ±0.45	7.7 ±0.49	2.6 ±0.60	1.9 ±0.47
$S_{0.5}$(F6P) (mM)	1.56 ±0.26	2.33* ±0.23	1.43 ±0.18	1.41 ±0.10	2.20 ±0.10	2.80* ±0.10	1.74 ±0.07	1.84 ±0.14
n_H	2.64 ±0.14	3.28* ±0.26	2.35 ±0.16	2.55 ±0.27	2.42 ±0.10	3.20* ±0.22	2.22 ±0.08	2.70* ±0.15
K_a(fructose-2,6-P_2) (μM)	0.17 ±0.05 (37)	0.08* ±0.02 (30)	0.11 ±0.05 (20)	0.16 ±0.05 (22)	0.31 ±0.05 (30)	0.28 ±0.06 (18)*	0.08 ±0.01 (19)	0.13* ±0.03 (11)*
K_a(AMP) (mM)	0.29 ±0.02 (17)	0.37* ±0.04 (12)*	0.14 ±0.02 (13)	0.07* ±0.02 (17)*	0.26 ±0.04 (26)	0.47 ±0.08 (17)*	0.24 ±0.03 (11)	0.17* ±0.03 (5)*
$K_a(NH_4^+)$ (mM)	4.3 ±0.91 (4)	1.7* ±0.32 (10)*	1.5 ±0.50 (3)	1.4 ±0.50 (2)	4.5 ±0.50 (5)	4.1 ±0.32 (5)	1.7 ±0.50 (4)	3.1* ±0.35 (3)
I_{50}(ATP) (mM)	1.43 ±0.10	1.25* ±0.05	1.53 ±0.10	1.35* ±0.04	1.47 ±0.12	1.08* ±0.16	1.62 ±0.10	1.30* ±0.10
I_{50}(citrate) (mM)	0.38 ±0.10	0.38 ±0.10	1.53 ±0.07	0.80* ±0.18	0.29 ±0.03	0.21* ±0.01	0.92 ±0.15	0.80 ±0.10

Values are means ± SEM, $n=3$ determinations on separate preparations of enzyme. n_H is the Hill coefficient. Effector constants were determined at subsaturating F6P concentrations, 0.3, 0.5, and 1.0 mM for K_a(fructose-2,6-P_2), K_a(AMP), and $K_a(NH_4^+)$, respectively, and 2.0 mM for I_{50}(ATP) and I_{50}(citrate). Numbers in parentheses refer to the fold activation by optimal levels of the activator. * Significantly different from corresponding aerobic value as determined by the Student's t-test, $P<0.05$

Table 3. Kinetic constants for phosphofructokinase from heart, gill, and red and white skeletal muscle of aerobic versus 24 h anoxic goldfish

	Heart		Gill		Red muscle		White muscle	
	Aerobic	Anoxic	Aerobic	Anoxic	Aerobic	Anoxic	Aerobic	Anoxic
V_{max} (U/g wet wt)	5.8 ±0.97	3.5* ±0.38	2.0 ±0.30	1.9 ±0.20	11.6 ±0.63	11.0 ±0.33	19.3 ±2.44	12.7* ±1.76
$S_{0.5}$(F6P) (mM)	1.26 ±0.19	1.42 ±0.11	1.43 ±0.21	1.63 ±0.32	1.26 ±0.12	1.34 ±0.08	1.14 ±0.15	1.34 ±0.05
n_H	1.53 ±0.22	2.08* ±0.25	1.10 ±0.11	1.18 ±0.03	1.15 ±0.10	1.16 ±0.10	1.10 ±0.04	1.11 ±0.10
K_a(fructose-2,6-P_2) (μM)	0.19 ±0.07 (23)	0.32* ±0.03 (20)	0.13 ±0.02 (26)	0.26* ±0.04 (18)	0.21 ±0.06 (12)	0.22 ±0.02 (13)	0.20 ±0.02 (8)	0.18 ±0.03 (9)
K_a(AMP) (mM)	0.20 ±0.04 (11)	0.46* ±0.06 (8)	0.21 ±0.04 (7)	0.27 ±0.06 (10)	0.29 ±0.05 (5)	0.29 ±0.04 (5)	0.22 ±0.04 (3)	0.23 ±0.07 (3)
$K_a(NH_4^+)$	1.7 ±0.31 (2)	2.3 ±0.46 (2)	2.0 ±0.40 (2)	2.0 ±0.32 (2)	1.7 ±0.15 (2)	2.1 ±0.32 (2)	2.0 ±0.15 (2)	2.0 ±0.16 (2)
I_{50}(ATP) (mM)	1.41 ±0.14	1.50 ±0.16	1.59 ±0.05	1.54 ±0.05	1.83 ±0.12	1.90 ±0.18	1.70 ±0.05	1.78 ±0.05
I_{50}(citrate) (mM)	0.56 ±0.07	0.50 ±0.09	0.74 ±0.15	0.81 ±0.06	0.55 ±0.13	0.69 ±0.17	1.00 ±0.18	1.11 ±0.22

Conditions are as in Table 2 legend except that K_a(fructose-2,6-P_2) was determined at 0.2 mM F6P for red and white skeletal muscle enzymes

Table 4. Kinetic properties of pyruvate kinase from liver, kidney, brain, and spleen of aerobic versus 24 h anoxic goldfish

	Liver		Kidney		Brain		Spleen	
	Aerobic	Anoxic	Aerobic	Anoxic	Aerobic	Anoxic	Aerobic	Anoxic
V_{max} (U/g wet wt)	15.7 ±1.8	15.7 ±1.7	154 ±11	109* ±12	119 ±14	121 ±18	52 ±4.6	45* ±3.2
$S_{0.5}$(PEP) (mM)	0.45 ±0.10	0.80* ±0.04	0.36 ±0.10	0.59* ±0.09	0.26 ±0.03	0.36* ±0.05	0.30 ±0.03	0.55* ±0.10
K_m(ADP) (mM)	0.22 ±0.04	0.21 ±0.06	0.23 ±0.05	0.21 ±0.07	0.19 ±0.03	0.24 ±0.08	0.17 ±0.04	0.22 ±0.05
K_a(fructose-1,6-P_2) (mM)	0.20 ±0.05 (2.4)	0.30* ±0.05 (2.6)	0.21 ±0.04 (2)	0.32* ±0.03 (2)	0.29 ±0.06 (2)	0.43* ±0.06 (2)	0.45 ±0.05 (2)	0.47 ±0.08 (2)
I_{50}(L-alanine) (mM)	25.1 ±3.6	11.6* ±1.3	17.5 ±1.6	9.7* ±2.6	21.0 ±7.3	11.4* ±2.4	30.6 ±6.6	20.7* ±2.7

Values are means ± SEM, $n=3$ or 4 determinations on separate preparations of enzyme. Effector constants were determined at subsaturating, near K_m levels of PEP: for K_a(fructose-1,6-P_2), values were 0.2 mM PEP for brain and 0.3 mM for other tissues; for I_{50}(alanine), values were 0.5 mM PEP for all tissues. Values in parentheses refer to the-fold activation by optimal levels of fructose-1,6-P_2. * Significantly different from corresponding aerobic value by the Student's t-test, $P<0.05$

response of the anoxic enzyme form; for kidney and brain, I_{50}(citrate) was also reduced for the anoxic enzyme form. Enzyme affinity for F6P decreased in anoxic liver and brain and the activating effects of AMP were also reduced by anoxia for these enzyme forms (and for the heart enzyme). The activating effects of $F26P_2$ were also reduced for the anoxic enzyme forms in brain, spleen, and heart but liver responded oppositely [$K_a(F26P_2)$ dropped by 50%]. PFK in gill showed only a single kinetic change [a doubling of $K_a(F26P_2)$] as a result of anoxia. Kinetics of PFK from red and white

Table 5. Kinetic properties of pyruvate kinase from heart, gill, and red and white skeletal muscles of aerobic versus 24 h anoxic goldfish

	Heart		Gill		Red muscle		White muscle	
	Aerobic	Anoxic	Aerobic	Anoxic	Aerobic	Anoxic	Aerobic	Anoxic
V_{max} (U/g wet wt)	170 ±17	176 ±9	53 ±6.5	40* ±2.1	247 ±30	281 ±12	141 ±14	157 ±14
$S_{0.5}$(PEP) (mM)	0.25 ±0.04	0.27 ±0.04	0.25 ±0.06	0.39* ±0.07	0.14 ±0.03	0.09 ±0.02	0.09 ±0.01	0.09 ±0.01
K_m(ADP) (mM)	0.25 ±0.04	0.25 ±0.02	0.20 ±0.05	0.23 ±0.04	0.36 ±0.11	0.26 ±0.05	0.32 ±0.09	0.37 ±0.06
K_a(fructose-1,6-P_2) (mM)	0.34 ±0.06 (1.8)	0.42 ±0.06 (1.6)	0.29 ±0.04 (1.8)	0.29 ±0.03 (1.7)	0.28 ±0.05 (1.4)	0.27 ±0.05 (1.5)	0.27 ±0.04 (1.5)	0.31 ±0.06 (1.7)
I_{50}(L-alanine) (mM)	11.6 ±2.7	3.4* ±0.6	20.2 ±3.8	13.1* ±2.7	n.e.	n.e.	n.e.	n.e.

Conditions are as in Table 4 legend except that PEP concentrations for K_a determinations were 0.1 mM for skeletal muscles and 0.2 mM for gill heart. *n.e.* no effect of alanine, in amounts up to 30 mM, on skeletal muscle PK

Table 6. Effects of in vitro phosphorylation and dephosphorylation on the kinetic properties of goldfish liver phosphofructokinase and pyruvate kinase

	Protein kinase treatment of aerobic form		Alkaline phosphatase treatment of anoxic form	
	Control	Treated	Control	Treated
Phosphofructokinase:				
V_{max} (U/g)	4.27±0.89	3.12±0.84	2.64±0.15	3.90±0.20*
$S_{0.5}$(F6P) (mM)	1.44±0.04	2.11±0.21*	2.01±0.22	1.52±0.14*
n_H	2.71±0.21	3.36±0.22*	2.48±0.01	1.65±0.01*
K_a(AMP) (mM)	0.29±0.04	0.40±0.03*	n.d.	n.d.
I_{50}(citrate) (mM)	2.10±0.23	1.22±0.22*	0.84±0.14	2.76±0.36*
Pyruvate kinase:				
$S_{0.5}$(PEP) (mM)	0.38±0.04	0.43±0.04	0.54±0.11	0.34±0.05*
n_H	1.31±0.10	1.72±0.23*	1.63±0.12	1.33±0.10*
I_{50}(L-alanine) (mM)	18.5±2.0	14.0±1.4*	11.8±1.7	18.9±1.2*

Values are means ± SEM, $n=3$ determinations on separate preparations. Conditions for protein kinase or alkaline phosphatase treatment of liver extracts are described in Materials and Methods. For PFK, V_{max} was measured at 10 mM F6P and 1 mM ATP, and effector constants, K_a(AMP) and I_{50}(citrate) were determined at 0.5 mM and 2 mM F6P, respectively. I_{50}(alanine) for PK was determined at 0.5 mM PEP. * Values significantly different from corresponding controls, $P<0.05$; *n.d.* not determined

muscles were unaffected by anoxia although the enzyme from anoxic white muscle showed a reduced V_{max}.

Kinetic properties of pyruvate kinase

Kinetic properties of PK from aerobic versus 24 h anoxic goldfish are shown in Table 4 for liver, kidney, brain, and spleen and in Table 5 for heart, gill, red muscle, and white muscle. The kinetic properties of PK were substantially altered as a result of anoxia in five tissues: liver, kidney, brain, spleen, and gill. In all cases, affinity for substrate PEP was reduced [$S_{0.5}$(PEP) was increased by 38–83%] for the anoxic, compared to the aerobic, enzyme form whereas inhibition by L-alanine increased [I_{50}(alanine) decreased to 46–68% of the aerobic value]. V_{max} of the enzyme decreased significantly in anoxic kidney, gill, and spleen and activation by $F16P_2$ was reduced in liver, kidney, and brain [$K_a(F16P_2)$ values increased by 48–52%]. PK in heart showed only a single kinetic difference between the aerobic and anoxic enzyme forms, a sharp reduction in the I_{50} for L-alanine, to 29%

of the aerobic value, as a result of anoxia. Kinetic properties of PK in red and white skeletal muscle were not altered as a result of anoxia.

Enzyme modification in vitro

Dialyzed extracts from liver were treated in vitro with cAMP-dependent protein kinase (plus ATP, cAMP, Mg^{2+}) or alkaline phosphatase to determine whether anoxia-induced changes in PFK and PK kinetics could be mimicked by subjecting the enzymes to phosphorylating versus dephosphorylating conditions, respectively. The data in Table 6 show that treatment of liver extracts from aerobic goldfish with protein kinase produced kinetic changes in both enzymes very similar to those seen with the aerobic-anoxic transition (Tables 2, 4). For PFK, protein kinase treatment produced an enzyme with significantly higher $S_{0.5}$(F6P), n_H, and K_a(AMP) values and a significantly lower I_{50}(citrate). For PK, I_{50}(alanine) was significantly reduced and n_H significantly increased after protein kinase treatment for 24 h. When liver extracts were exposed to protein kinase for 48 h, both $S_{0.5}$(PEP), n_H, and I_{50}(alanine) were affected, $S_{0.5}$ rising by 33% and I_{50} decreasing to 9.6 ± 1.2 mM.

Exposure of liver extracts from anoxic fish to alkaline phosphatase produced the opposite results, converting the kinetics of both enzymes back to those of the aerobic enzyme forms. For PFK, alkaline phosphatase treatment resulted in a decrease in $S_{0.5}$(F6P) and n_H and an increase in I_{50}(citrate); V_{max} of the enzyme also increased. For PK, alkaline phosphatase treatment produced an enzyme form with a reduced $S_{0.5}$(PEP) and n_H and an increased I_{50}(alanine).

Discussion

Anoxia tolerance in the goldfish involves a tissue specific reorganization of metabolism and an overall metabolic rate depression (Shoubridge and Hochachka 1983). Glycolysis is the primary pathway of ATP synthesis during anoxia and, as such, control of glycolytic rate, through controls over the activities of regulatory enzymes, is key to matching ATP synthesis to ATP utilization to establish and maintain a new homeostasis in the anaerobic state. The data in the present study provide strong evidence that both PFK and PK are modified in most organs of the goldfish in response to anoxia stress, the probable mechanism of this modification being a change in the amount of covalently bound phosphate. Although phosphate contents of the aerobic and anoxic enzyme forms have not been directly measured in the present study, anoxia-induced enzyme phosphorylation is strongly indicated by several factors: 1) the anoxia-induced changes in the kinetic properties of both enzymes are qualitatively similar to the effects of phosphorylation on the kinetic properties of the corresponding enzymes in mammalian organs, 2) in vitro treatment of the aerobic forms of liver PFK and PK with cAMP-dependent protein kinase mimicked the effect of anoxia on the enzymes whereas treatment of the anoxic enzyme forms with alkaline phosphatase reproduced the kinetic properties typical of the aerobic enzyme forms, and 3) isoelectric points of both enzymes in several organs shifted as a result of anoxia, a typical effect when covalently-bound phosphate is incorporated into an enzyme. With respect to this last point, it is interesting to note that the pI values of both enzymes were altered in anoxic skeletal muscles although kinetic properties of these enzymes do not change. Similarly, Foe and Kemp (1984) have shown that rabbit brain PFK was also readily phosphorylated in vitro without alteration of enzyme kinetic properties. New studies of the function of phosphorylation of rabbit skeletal muscle PFK are instructive in this regard. Luther and Lee (1986) have shown that phosphorylation state influences the binding of the enzyme to F-actin and provides a means to alter the microcompartmentation of PFK in the cell. The implication, therefore, is that enzyme phosphorylation can have major consequences for enzyme function in vivo, altering enzyme binding to active subcellular glycolytic complexes and/or altering the kinetics of the bound enzyme within the complex, without necessarily changing the kinetics of the soluble enzyme assayed in dilute solution in vitro.

Tissue specific isozymes of PFK in mammals are susceptible to covalent modification via phosphorylation-dephosphorylation reactions although the effects of phosphorylation differ radically between tissues and serve highly specific functions in each organ. That this is so should not be unexpected for the rate-limiting enzyme of perhaps the most important metabolic pathway in the cell. Present data for mammalian systems suggest that phosphorylation results in: a) PFK inactivation in liver (Sakakibara and Uyeda 1983), b) PFK activation in heart (epinephrine-mediated) (Narabayashi et al. 1985), c) PFK activation in skeletal muscle through enhanced binding to F-actin (despite minimal kinetic changes to the soluble enzyme assayed in vitro) (Foe and Kemp 1982; Luther and Lee 1986), and d) effects on the brain enzyme that are unidentified to date but do not include changes

in kinetic constants (Foe and Kemp 1984). Not unexpectedly, therefore, the effects of anoxia on the kinetic properties of PFK in goldfish tissues are very diverse. Strong kinetic evidence of anoxia-induced modification by probable phosphorylation-dephosphorylation was found for several tissues. For four soft tissues a common alteration was the increased susceptibility to inhibition by ATP [reduced I_{50}(ATP)] of the anoxic enzyme form. This was coupled in liver and brain to a decreased sensitivity to AMP activation producing an anoxic enzyme form that is possibly less responsive to allosteric control by adenylates; this could permit the lower and more variable energy charge state of anoxia without stimulating a glycolytic activation. In liver, glycolytic depression during anoxia would also be facilitated by a reduced enzyme affinity for F6P and by reduced enzyme activation by $F26P_2$ [$K_a(F26P_2)$ drops 2-fold in anoxia but measured levels of $F26P_2$ drop 10-fold (Storey 1987b)]. These kinetic changes parallel the known effects of phosphorylation on mammalian liver PFK and suggest that the anoxia-induced modification of goldfish liver PFK is due to enzyme phosphorylation. Anoxia-induced modification of goldfish heart PFK, however, may well be due to a dephosphorylation of the enzyme. Epinephrine stimulation of cardiac muscle leads to the phosphorylation and activation of PFK with kinetic changes including a drop in the K_a for activators (Narabayashi et al. 1985). The opposite responses, a rise in the K_a values for $F26P_2$ and AMP, result from anoxia suggesting a dephosphorylation to reduce enzyme activity in the anoxic state.

The present study also firmly indicates anoxia-induced phosphorylation of PK in the five soft tissues (liver, kidney, brain, spleen, gill) of the goldfish; PK in these tissues showed significant changes in three or four kinetic properties [reduced V_{max}, increased $S_{0.5}$(PEP), increased $K_a(F16P_2)$, reduced I_{50}(alanine)] as a result of environmental anoxia. These anoxia-induced in vivo modifications of PK parallel the known effects of protein phosphorylation on the kinetic properties of PK from a number of animal sources (Engstrom 1978; Marie et al. 1980; Plaxton and Storey 1984a). In all studies to date the net result of phosphorylation of PK is a reduction in the activity of the enzyme expressed in vivo. In mammalian liver phosphorylation inactivation of PK (glucagon-stimulated) permits gluconeogenic flux (Engstrom 1978) while in tissues of marine molluscs (both muscle and non-muscle) anoxia-induced phosphorylation of PK contributes to glycolytic rate depression and permits PEP catabolism via phosphoenolpyruvate carboxykinase to produce the alternative end product succinate (Storey 1985; Plaxton and Storey 1984a, 1985). Similar anoxia-induced phosphorylation inactivation of PK in soft tissues of the goldfish would contribute to the overall metabolic rate depression by contributing to glycolytic rate depression and the depression of carbohydrate use for anabolic purposes. Small differences between the tissues in the relative effect of anoxia on PK V_{max} and kinetic parameters [$S_{0.5}$(PEP), $K_a(F16P_2)$, $S_{0.5}$(alanine)] are found; these may suggest differences between tissue specific enzyme forms in both the absolute and relative amounts of phosphate in aerobic and anoxic enzyme forms and the resulting effects on enzyme properties.

Covalent modification controls over PFK and PK are only part of the regulation of glycolysis during anoxia in goldfish. Tissue specific metabolism is also regulated by covalent modification regulation of glycogen phosphorylase (the percent phosphorylase *a* drops in five tissues) and by changes in the content of $F26P_2$, a potent activator of PFK that potentiates of use carbohydrate for anabolic purposes ($F26P_2$ content drops in three tissues) (Storey 1987b). $F26P_2$ content, itself, is regulated by phosphorylation-dephosphorylation controls on the activity of 6-phosphofructo-2-kinase bringing the control of glycolysis during anoxia under the influence of covalent modification at four separate loci. For example, anoxia-induced depression of glycolytic rate in goldfish liver results from (a) a drop in phosphorylase *a* expressed to 48% of control values, (b) a 10-fold drop in $F26P_2$ content, (c) changes in PFK kinetics, due to probable covalent modification, which would reduce enzyme activity, and (d) reduced activity of PK due to similar effects of covalent modification on enzyme kinetic properties.

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