Molecular Physiology, 8 (1985) 439–461
Elsevier

MPH 00342

A8 Review

A RE-EVALUATION OF THE PASTEUR EFFECT: NEW MECHANISMS IN ANAEROBIC METABOLISM

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(Received September 1985)

Key words: Pasteur effect; Anaerobiosis; Enzyme phosphorylation; Fructose-2,6-P_2; Phosphofructokinase; Glycolysis control; Glycolytic particle

Summary

In 1861 Pasteur reported that yeasts consume much more glucose in the absence of oxygen than in its presence. In modern terms the Pasteur effect amounts to an activation of anaerobic glycolysis in the absence of oxygen in order to meet cellular ATP utilization requirements from the much lower efficiency of ATP production by fermentation compared to respiration. However, many facultative anaerobes which face natural exposures to environmental anoxia do not show a Pasteur effect and it is increasingly apparent that a key survival strategy of these animals is the ability to depress metabolic rate during anoxia. The absence of the Pasteur effect is explained if the reduced energy requirements of the anoxic animal can be met without an increase, and sometimes with a real decrease, in glycolytic rate. The molecular mechanisms responsible for the reduction of metabolic rate during environmental anaerobiosis are the most important new area of research in understanding facultative anoxia tolerance. Three new concepts in metabolic regulation are discussed in this regard using the control of glycolysis as a model. (1) The covalent modification of enzymes by phosphorylation produces less active forms of the two major regulatory enzymes of glycolysis, phosphofructokinase and pyruvate kinase, during anoxia; indeed, phosphorylation appears to be a widespread phenomenon applying to many cellular proteins during anoxia. (2) Association of enzymes with the particulate fraction of the cell appears to increase the efficiency of metabolic pathways. Environmental anoxia in facultative anaerobes results in a decrease in the proportion of glycolytic enzymes associating with the particulate, versus soluble, fractions of the cell, indicating that reduced enzyme binding is a key factor in achieving a reduced glycolytic rate. (3) The enzymatic basis of the Pasteur effect rests largely with control of phosphofructokinase. The newly discovered allosteric activator, fructose-2,6-P_2, is a potent regulator of the enzyme; levels of the compound are strongly reduced during anaerobiosis.

Introduction

With some exceptions most life forms on this planet make use of the availability of oxygen to extract maximal metabolic energy from the catabolism of organic substrates. Complete oxidation of glucose to CO_2 and H_2O produces 36 mol ATP per mol glucose versus a yield of only 2 mol ATP per mol glucose fermented to 2 mol lactate or ethanol. Respiration is also the only way to make use of lipids and many amino acids as fuels. However, organisms retain fermentative pathways of energy production which, despite their low energy yields, can be effectively utilized for survival of periods of anoxia or hypoxia stress or to augment energy production when demand outstrips aerobic capacity (e.g., during bursts of muscular work). The basic fermentative pathway is glycolysis which converts carbohydrate (glycogen, glucose) to lactate or ethanol with additional glycolytic end products and/or additional fermentative pathways coupled to glycolysis in some cases (Fig. 1).

The Pasteur effect

In 1861 Louis Pasteur published the results of experiments on yeast which showed that under anaerobic conditions the organisms consumed more sugar per unit weight than under aerobic conditions. 'Oxygen inhibits fermentation' is the common

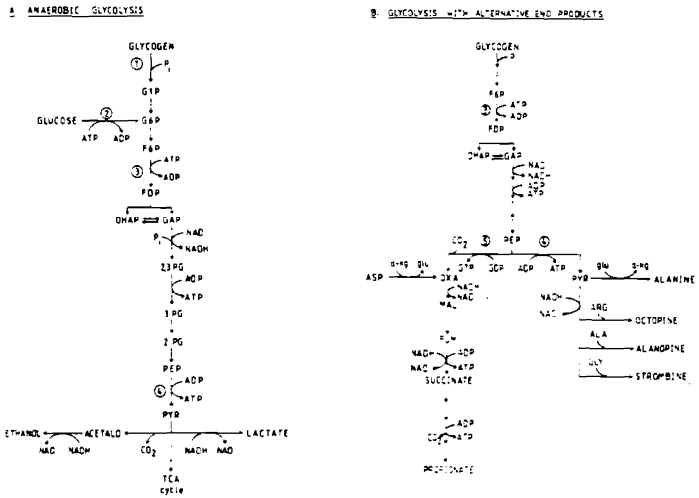

Fig. 1. Glycolysis (A) and glycolysis modified with alternative end products as found in many anoxia tolerant invertebrates (B). Enzymes are: 1, glycogen phosphorylase; 2, hexokinase; 3, phosphofructokinase; 4, pyruvate kinase; 5, *P*-enolpyruvate carboxykinase.

definition of the Pasteur effect (Krebs, 1972; Ramaiah, 1974; Tejwani, 1978) but because my concern here is the regulation of glycolysis with the transition from aerobic to anoxic conditions I will define the Pasteur effect for subsequent use as 'the effect of oxygen deprivation in increasing the rate of carbohydrate uptake and catabolism'. The basis of the phenomenon is the differential amount of ATP synthesis possible from fermentation versus respiration (see above). The existence of the Pasteur effect means that, in most instances, when oxygen is available respiration is used as the more economical method of cellular energy production: when oxygen is lacking the rate of fermentative metabolism rises to meet cellular energy demands by the less efficient pathway.

The Pasteur effect is correctly measured by quantitating changes in the rate of substrate utilization under aerobic versus anaerobic conditions. However, many studies have instead assessed the Pasteur effect in terms of end-product accumulation (lactate, ethanol, CO_2) following the development of excellent manometric methods by Warburg (1926). Measurements of end products have distorted the quantitation of the Pasteur effect as well as early studies of the molecular mechanism of the effect (Racker, 1974), and have sometimes led to the mistaken impression, not uncommon in the comparative literature, that anaerobic end-product accumulation during anoxia necessarily means an activation of glycolysis.

The Pasteur effect is a widely seen mechanism for dealing with anoxic stress. An often cited study by Lowry et al. (1964) documents a 4–7-fold increase in glycolytic flux in mouse brain as the result of ischaemia. Similar results are available for other mammalian tissues (see Ramaiah, 1974). Many types of bacteria and, of course, yeast show a Pasteur effect. The Pasteur effect has also been demonstrated in locust flight muscle (Ford and Candy, 1972) and in aerobic larval stages of the parasitic helminth *Ascaris* (Saz, 1981).

Control of the Pasteur effect

The earliest proposals for the control of the Pasteur effect centered on the competition between fermentation and respiration for ADP and P_i (see Krebs, 1972; Racker, 1974). Both could be shown to be rate-limiting in glycolysis under certain circumstances. While this competition is still valid, additional regulatory features were required to explain key observations: (1) although lactate formation was reduced when ADP and P_i were limiting, utilization of glucose was not, and (2) fructose-1,6-P_2 fermentation was not inhibited by oxygen (Racker, 1974). This led to the eventual identification of hexokinase and phosphofructokinase (PFK) as the regulatory sites in glycolysis responsible for the Pasteur effect. Regulation at the hexokinase locus is largely operative only when exogenous sugars are used as the substrate of glycolysis and results from allosteric effects, including glucose-6-*P* inhibition and P_i activation, as well as from a reversible partitioning of hexokinase between membrane-bound and soluble states (Krebs, 1972; Racker, 1974). Numerous studies (summarized by Krebs, 1972, Ramaiah, 1974; Racker, 1974; Tejwani, 1978) have led to the conclusion that regulatory control of phosphofructokinase is primarily responsible for the Pasteur effect. Anoxia or ischaemia in most mammalian tissues results in a decrease in fructose-6-*P* and an increase in fructose-1,6-P_2 content (Table 1) (Lowry et al., 1964) which, by the cross-over analysis of Williamson (1970), is indicative of an activation of PFK.

TABLE 1

Concentrations of some metabolites in mouse brain during ischaemia and *Mytilus edulis* adductor muscle during environmental anoxia

Values are nmol/g wet weight.

	Mouse brain		Mussel adductor	
	Control	25 s ischaemic	Control	45 min anoxia
Glucose-6-*P*	224	91	110	170
Fructose-6-*P*	50	27	23	35
Fructose-1,6-P_2	27	153	42	30
Dihydroxyacetone-*P*	13	39	35	30
*P-enol*pyruvate	3.5	8.5	13	10
Pyruvate	39	72	10	4
Lactate	770	1820	90	175
ATP	2580	2410	4400	3500
ADP	690	730	650	1050
AMP	130	130	50	200
Phosphagen	3740	2000	2400	2000
P_i	3350	4410	850	1650

Phosphagen is creatine-*P* in mouse brain and arginine-*P* in *M. edulis* adductor. Data are taken from Lowry et al. (1964) for mouse brain and Ebberink and De Zwaan (1980) for mussel adductor.

Kinetic properties of PFK

PFK is the first committed step in the catabolism of hexose phosphates by glycolysis and regulates the utilization of hexose phosphates from a variety of sources: glycogen mobilization, uptake of exogenous sugars, pentose phosphate pathway. The enzyme has complex regulatory properties which allow response to the varying demands placed on glycolysis: carbohydrate catabolism for energy producing purposes versus carbohydrate utilization for anabolic pathways. A wide variety of allosteric effectors occur including ATP as a substrate inhibitor and ADP, AMP and P_i as activators; these make the enzyme very responsive to adenylate energy charge (Atkinson, 1977) and phosphate potential (Erecinska et al., 1977) and are probably the key elements in determining enzyme response to the energy status of the cell. Other activators of the mammalian enzyme include NH_4^+, K^+, 3',5'-cyclic AMP and fructose-1,6-P_2; while citrate, Mg^{2+}, Ca^{2+}, creatine-*P*, *P-enol*pyruvate, 2-*P*-glycerate, 3-*P*-glycerate, 2,3-diphosphoglycerate and 6-*P*-gluconate can be inhibitors (Ramaiah, 1974; Tejwani, 1978). With respect to the Pasteur effect is has been clearly demonstrated that activators of PFK (AMP, ADP, P_i, fructose-1,6-P_2) tend to accumulate under anoxic conditions while inhibitors (ATP, citrate) increase with the return to aerobic conditions (Table 1) (Lowry et al., 1964; Ramaiah, 1974). Various

authors (Krebs, 1972; Racker, 1974) have concluded that allosteric effects by inhibitors and activators appear to be largely adequate to account for the activation of PFK and of glycolytic rate occurring at the aerobic/anaerobic transition and resulting in the Pasteur effect. Recent work has identified another potent activator of PFK, fructose-2,6-P_2 (Hers and Van Schaftingen, 1982); it and AMP appear to be the most important effectors of the enzyme. Fructose-2,6-P_2 is synthesized by an enzyme termed PFK II. Activity of PFK II can be modulated by cAMP-dependent protein kinase or by Ca^{2+}/calmodulin stimulated enzyme phosphorylation, making fructose-2,6-P_2 an effective messenger of hormone action at the PFK locus (Hers and Van Schaftingen, 1982; Hue, 1983; Furuya et al., 1982a). In general, fructose-2,6-P_2 signals the abundance of glucose and potentiates the use of carbohydrate for anabolic purposes (Hue, 1983). The role of fructose-2,6-P_2 in regulating the Pasteur effect seems variable in different systems: anoxia results in a rapid decrease in fructose-2,6-P_2 content in mammalian tissues (Hue, 1982) but elevates fructose-2,6-P_2 content in yeast (Furuya et al., 1982b).

Animal adaptation for anaerobiosis

The capacity for life without oxygen varies greatly reflecting animal adaptation for lifestyle and habitat. The range extends from highly aerobic forms (e.g., most flying insects and homeothermic vertebrates) with little or no tolerance for oxygen deprivation, through many types of facultative anaerobes (many yeasts and bacteria, many intertidal invertebrates, diving turtles) which survive days, weeks or months without oxygen, to animals which have virtually anaerobic lives (many parasitic helminths). Life stages of animals can vary in oxygen dependency; eggs and third stage larvae of *Ascaris* are aerobes, while adults require no oxygen for energy production and survival (Saz, 1981). Individual tissues within an animal can also vary greatly in oxygen dependency. Thus, while irreversible brain damage will occur within about 3–4 min in many mammals when deprived of oxygen, tissues such as retina, erythrocytes, intestinal mucosa and renal medulla normally derive energy almost exclusively from glycolytic fermentations (Krebs, 1972).

The prominence of the Pasteur effect as the response to anoxia in the major model systems used in metabolic biochemistry (laboratory mammals, yeast, bacteria) has led to an expectation that this is the normal response of cells to anoxia, i.e., when oxidative phosphorylation is shut off cells automatically raise the rate of fermentative ATP production in an attempt to compensate and meet, as much as possible, the continuing demands of ATP-utilizing processes. Thus, hundreds of studies have examined the phenomenon and its regulation and have extensively worried over the absence of the Pasteur effect in selected mammalian tissues (e.g., retina, erythrocytes) (Krebs, 1972; Ramaiah, 1974; Racker, 1974). Viewed oppositely, however, an equally valid method of dealing with oxygen deprivation would be to greatly reduce the energy demands of the cell to a level which can be easily matched by a low rate of fermentative metabolism, conserving energy for key metabolic processes until such time as oxygen is restored. This is indeed the strategy taken by most multicellular organisms with a true facility for long-term anaerobiosis.

Compensation for anoxia with a Pasteur effect

The Pasteur effect is the normal response to anoxia in many systems but when viewed closely it is apparent that organisms expressing a Pasteur effect fall into one of two categories.

(1) The first category comprises highly aerobic organisms (often homeotherms) whose only tolerance for anoxia is for very short term, transient oxygen deprivation. The primary (and perhaps only) response in these systems is an activation of fermentative metabolism in an effort to maintain high ATP production over the short term in anticipation of a very quick return to normal aerobic conditions. Endogenous carbohydrate reserves can be rapidly catabolized and high concentrations of end products (lactate) can be tolerated in the short term. Accumulated data suggest however that the rates of glycolytic energy production in these systems (glycolytic flux measured from substrate utilization or product accumulation (see Krebs, 1972; Ramaiah, 1974)) never fully compensate for aerobic rates of ATP synthesis. Therefore without mechanisms to reduce energy expenditures during anoxia, ATP availability soon becomes limiting, leading to irreversible damage to the most sensitive cellular processes and resulting in a very low tolerance for oxygen deprivation.

(2) The second category in which the Pasteur effect is the normal response to anoxia includes certain facultative anaerobes, e.g., yeast and bacteria. Here, long-term compensation for oxygen lack can be achieved by increasing glycolytic ATP production. However, this depends entirely upon unlimited supplies of substrate from the growth medium and upon the ability to excrete accumulated end products (ethanol) back into the medium. For the microenvironments in which these organisms often live, the Pasteur effect is an appropriate strategy for continued growth under anaerobic conditions.

Overall, then, organisms which display a Pasteur effect use as their primary strategy of anoxia tolerance an increase in fermentation rate as an attempt to compensate for the normal cellular demands of ATP-utilizing processes. The strategy relies on a 'reckless' catabolism of substrate and on an ability to tolerate or excrete accumulated fermentation end products.

Adaptation to anoxia by conservation of energy

As a strategy for long-term anaerobiosis in most multicellular organisms, the Pasteur effect is maladaptive. These are generally self-contained organisms which must rely throughout anoxia on endogenous carbohydrate supplies and tolerate the accumulation of toxic end products. High rates of consumption of limited endogenous carbohydrate supplies is not conducive to survival of long and indeterminate bouts of anoxia. Adaptation for long-term anaerobiosis, therefore, involves a conservation of energy expenditures by a depression of metabolic rate and a selective inactivation of non-essential physiological and biochemical processes during anoxia. This reduces energy requirements to the point where no increase in fermentative energy production is required; indeed, many organisms show not only an absence of a Pasteur effect (De Zwaan and Wijsman, 1976; Gade, 1983; Robin et al., 1979) but also an inhibition of glycolytic rate during anoxia (Ebberink and De Zwaan, 1980;

see following sections). In addition supplementary routes of fermentation are often brought into play to increase the yield of ATP from the substrate catabolized (Fig. 1) (Hochachka and Somero, 1984). At a biochemical level an energy conservation strategy is a much more complicated adaptation than is energy compensation via the Pasteur effect. The Pasteur effect requires only a localized activation of glycolysis in cells; conservation via metabolic rate reduction requires a coordinated inactivation of a vast array of physiological and biochemical activities. Because of this, energy conservation adaptations largely appear only when animals face frequent and long bouts of low oxygen stress.

Metabolic rate reduction during anoxia

Probably the most accurate estimates of whole animal metabolic rate come from calorimetry, measurements of total heat output from exothermic reactions occurring in cells. Estimates can also be derived from oxygen consumption rates in the aerobic state or, in the anoxic state, from determinations of total end product accumulation plus energy reserve (ATP, phosphagen) depletion during anoxia, or from oxygen debt analysis during aerobic recovery. Methods other than calorimetry suffer if a

TABLE 2

Some properties of aerobic (dephosphorylated) and anoxic (phosphorylated) forms of phosphofructokinase from whelk, *B. canaliculatum* foot muscle

	Aerobic PFK	Anoxic PFK
$S_{0.5}$ ATP (mM)	0.03	0.04
$S_{0.5}$ Mg^{2+} (mM)	0.06	0.13 *
$S_{0.5}$ fructose-6-*P*, pH 7 (mM):		
control	2.18 (n_H = 2.5)	2.48 (n_H = 2.7)
+5 mM NH_4^+	1.13	1.42
+5 mM P_i	1.04	0.84
+20 μM AMP	0.60	0.78
+2.5 μM fructose-2,6-P_2	0.40	0.75 *
Activator constants (K_a):		
NH_4^+ (mM)	5.6	8.9 *
P_i (mM)	1.6	1.4
AMP (μM)	32	29
fructose-2,6-P_2 (μM)	1.1	3.9 *
I_{50} ATP (mM):		
pH 8	10.7	7.4 **
pH 7	1.2	0.77
pH 6.8	1.0	0.52 **

Apparent K_a values for activators were determined at 0.5 mM fructose-6-*P* and pH 7. I_{50} values for ATP inhibition were determined at 2 mM fructose-6-*P*.

* Values are significantly different from aerobic enzyme. $P < 0.05$.

** $P < 0.02$.

Data from Storey (1984).

mixed metabolism (both aerobic and anaerobic components) occurs, as is quite common amongst anoxia-tolerant intertidal invertebrates (Famme et al., 1981; Gnaiger, 1983).

Demonstrations of depressed metabolic rate in response to oxygen limitation are found in a wide variety of animals which naturally face periods of anoxia. The ability is not shared by closely related species with low anoxia tolerance. For example, goldfish can survive complete anoxia for 16 h at 20°C or several months at 0°C (Blazka, 1958; van den Thillart, 1982). Estimates of anoxic metabolic rate are 20–30% of normoxic rates determined by calorimetry and 1/3 of normal rates based on the sum of accumulated end products and the depletion of ATP, phosphagen and endogenous O_2 reserves (van den Thillart, 1982). Trout, on the other hand, with a very low anoxia tolerance, do not appear to depress metabolic rate in the face of anoxia (Van den Thillart, 1982). The same is true amongst intertidal marine bivalves. *Mytilus edulis* is an excellent facultative anaerobe which can survive weeks of anoxia (in air or in N_2-bubbled seawater). Both calorimetry and end-product measurements estimate anoxic metabolic rate at 5–10% of the normoxic rate (De Zwaan and Wijsman, 1976; Widdows et al., 1979; Famme et al., 1981; Shick et al., 1983; Gnaiger, 1983); a Pasteur effect is not seen (De Zwaan and Wijsman, 1976; Gade, 1983). A much less anoxia tolerant bivalve is the infaunal species, the coot clam *Mulinia lateralis*, which shows a survival time (LT_{50}) of only 2–11 days. No change in the rates of heat dissipation are found under anoxic versus normoxic conditions, implying that a Pasteur effect must support the limited anoxia tolerance of the species (Shumway et al., 1983).

Thus, metabolic depression as a survival technique for dealing with environmental anoxia is found among both vertebrates and invertebrates. The best known vertebrate example is the diving or hibernating freshwater turtle. Estimates from calorimetry indicate that diving metabolic rate is only 15% of the normal rate (Jackson, 1968); this agrees well with an incurred oxygen debt from diving which is only 1/6 of that expected if predive metabolic rates were maintained (Caligiuri et al., 1981). Such a radical metabolic depression, which predictably involves no Pasteur effect (Robin et al., 1979), allows submerged hibernation at 3°C for up to 6 months (Ultsch and Jackson, 1982). Homeothermic vertebrates in general show a very limited tolerance for anoxia or ischaemia but instances of metabolic depression as a survival technique occur. Although most free diving in seals is fully aerobic with no adjustments made to conserve oxygen, dives of unpredicted long duration and exceeding the oxygen reserve capacity (about 2% of natural dives) elicit the 'diving reflex' to conserve oxygen reserves for the brain (Kooyman et al., 1981). This includes energy savings through depression of heart rate (bradycardia) and a metabolic depression (calculated to be 1/2 to 2/3 of normal rates) coupled to anaerobic metabolism (producing lactate) in various hypoperfused peripheral tissues (Hochachka and Somero, 1984). These metabolic adjustments in seals, however, are not so much an adaptation for dealing with anoxia as adaptations for conserving, as long as possible, a limited oxygen supply. Metabolic depression as a means of energy conservation, but not as an adaptation to anoxia, also occurs in other instances among homeotherms: notably, torpor in hummingbirds and hibernation in bats and other small mammals.

Anaerobic control of glycolytic rate in systems without a Pasteur effect

Metabolic depression reduces the energy requirements of facultative anaerobes during anoxia such that an increase in the rate of fermentation is not seen. Indeed, the situation in some cases is just the opposite: glycolytic rate is depressed along with metabolism in general. Thus, for example, changes in the concentrations of fructose-6-*P* (an increase) and fructose-1,6-P_2 (a decrease) in *M. edulis* adductor (Table 1) indicate an inhibition of PFK within minutes of the initiation of anoxia (Ebberink and De Zwaan, 1980) with a concomitant reduction in glycolytic flux. This occurs despite a decrease in the concentrations of inhibitors of PFK (ATP, arginine phosphate, citrate) and an increase in the concentrations of activators (ADP, AMP, P_i) over the initial minutes of anoxia. In fact, the effects of anoxia on the behaviour of these effectors of PFK in *M. edulis* muscle is exactly the same as is seen in mammalian tissues (Table 1). How, then, can these changes be reconciled with an inactivation of glycolysis in the one case and an activation (Pasteur effect) in the other?

Kinetic properties of PFK in anoxia tolerant molluscs

PFK has been purified from several marine mollusc sources: oyster (*Crassostrea virginica*) adductor muscle (Storey, 1976), *M. edulis* adductor muscle (Ebberink, 1982), and whelk (*Busycotypus canaliculatum*) foot muscle (Storey, 1984). Properties of the whelk enzyme are shown in Table 2. The enzyme displays all the common properties of PFK (see reviews of the mammalian enzyme: Ramaiah, 1974; Uyeda, 1979) including sigmoidal fructose-6-*P* kinetics at pH 7, substrate inhibition by ATP, potent and synergistic activations by AMP and fructose-2,6-P_2 as well as effects by other common activators (NH_4^+, P_i, ADP) and inhibitors (*P-enol*pyruvate, glycerol-3-*P*). It shares with most invertebrate PFKs (see Storey, 1984; 1985b), as opposed to the vertebrate enzyme, a relative insensitivity to citrate and fructose-1,6-P_2 as effectors and a low affinity for fructose-6-*P* such that activity in vivo (under physiological fructose-6-*P* and ATP concentrations) is virtually obligately linked to the actions of positive modulators (Storey, 1984). No unusual modulators of the enzyme occur. As is common for PFK from other sources (Uyeda, 1979), a decrease in pH decreases enzyme affinity for fructose-6-*P* and increases inhibition by ATP. Ebberink (1982) suggested that the decrease in cellular pH which accompanies anoxia could, therefore, aid inactivation of PFK; however, pH change is a slow and long-term process (decreasing only about 0.1 and 0.3 units over the first 1 and 12 h of anoxia, respectively, (Ellington, 1983)) which could not effect an inhibition of PFK in the initial minutes of anoxia.

The role of fructose-2,6-P_2

The behaviour of fructose-2,6-P_2 content in cells has been characterized over the aerobic–anaerobic transition in yeast, mammalian tissues and tissues of marine molluscs. In yeast, anoxia elicits a rapid rise in fructose-2,6-P_2 content (Furuya et al., 1982b); here the compound is apparently acting as a signal of the abundance of

glucose and potentiating a Pasteur effect based on the catabolism of extracellular glucose. Anoxia or ischaemia in mammalian tissues, however, results in a rapid decrease in fructose-2,6-P_2 content (to less than 10% of control levels within 10 min of anoxic incubation of hepatocytes) (Hue, 1982); this indicates that fructose-2,6-P_2 cannot be a control factor in the Pasteur effect in mammalian tissues. Rather, fructose-2,6-P_2 regulation of PFK controls the use of carbohydrate for biosynthetic purposes at times of plentiful energy (Hue, 1982) and its effects are withdrawn under anoxia. Tissues of marine molluscs show the same response during anoxia. Fructose-2,6-P_2 content in soft tissues falls rapidly in the early hours of anoxia (Storey, 1985a); in the oyster, content decreases to 10–19% and 2.5–4% of control values after 3 and 24 h of anaerobiosis, respectively (Table 3). As in mammals, then, fructose-2,6-P_2 appears to signal the availability of carbohydrate for anabolic purposes. During anoxia, anabolism is probably largely curtailed as part of the general metabolic depression, and the fall in fructose-2,6-P_2 content in soft, synthetic tissues would strongly depress glycolytic rate, leaving glycolysis responsive only to the maintenance energy requirements of these tissues. In muscle tissues of the oyster, low levels of fructose-2,6-P_2 occur in both aerobic and anaerobic tissues with no effect of anoxia on content (Table 3) (Storey, 1985a). However (as will be discussed in the next section), PFK isolated from anoxic muscle has a higher K_a for fructose-2,6-P_2 and shows a lesser effect of fructose-2,6-P_2 on $S_{0.5}$ for fructose-6-*P* than does the enzyme from aerobic muscle. The result is that the bisphosphate is a much less effective activator of PFK in anoxic muscle (Storey, 1984).

Overall, then, fructose-2,6-P_2 appears to have a significant role in decreasing glycolytic rate (at least in soft tissues) during anoxia, in depressing carbohydrate availability for anabolic purposes and therefore in indirectly limiting the activities of anabolic pathways in soft tissues. The strong synergistic interactions between fructose-2,6-P_2 and AMP (or other activators) (Storey, 1985b) would also mean that AMP, NH_4^+ and P_i are less effective in activating PFK in the anoxic tissue which

TABLE 3

Levels of fructose-2,6-P_2 (pmol/g wet wt) in tissues of the oyster, *Ostrea edule*, over the course of anoxia and aerobic recovery

	Control	Anoxia				Recovery (24 h)
		3 h	6 h	24 h	96 h	
Mantle	433 ± 62	83 ± 8	46 ± 6	12 ± 4	61 ± 2	423 ± 53
Gill	696 ± 75	73 ± 6	76 ± 11	27 ± 12	75 ± 3	610 ± 17
Hepatopancreas	806 ± 97	110 ± 6	50 ± 2	20 ± 2	73 ± 7	667 ± 99
Phasic adductor	75 ± 3	60 ± 8	56 ± 7	70 ± 8	98 ± 10	75 ± 7
Catch adductor	47 ± 3	47 ± 3	50 ± 6	53 ± 9	20 ± 5	93 ± 7

Data are means ± S.E., $n = 5$. Similar results were found for soft tissues and muscle of *M. edulis* over a course of anoxia while *Littorina littorea* showed a decline in fructose-2,6-P_2 in soft tissues during anoxia but a rise in foot muscle. Data from Storey (1985a).

again would promote a decreased glycolytic rate during anoxia. However, it is still obvious that changes in adenylates, arginine phosphate and P_i should activate PFK in anoxia in marine molluscs and promote a Pasteur effect. The absence of a Pasteur effect, I feel, results from higher levels of control on PFK and other glycolytic enzymes superceding allosteric control. These are (1) covalent modification of enzymes by protein phosphorylation and (2) enzyme association/dissociation with subcellular particles. These are not only key to glycolytic rate control during anoxia, but also have important implications for general metabolic rate depression in facultative anaerobes.

Covalent modification

Enzyme and protein modification by the covalent incorporation of phosphate is a widespread phenomenon with important consequences for metabolic control in vivo (Cohen, 1980). Covalent modification provides a means for large scale change in enzyme activity or enzyme kinetic properties over a short time scale. Phosphorylation is stimulated by cAMP and/or Ca^{2+}/calmodulin, making covalent modification responsive to extracellular signals (e.g., hormones). In mammals, regulatory control by covalent modification occurs most frequently in multifunctional tissues and serves to regulate the relative activities of competing pathways. In liver, for example, the flow of carbohydrate through glycolytic versus gluconeogenic reactions is partially regulated via protein kinase-mediated phosphorylation of pyruvate kinase (PK) to produce a less active enzyme form (Engstrom, 1978). A variety of other regulatory enzymes in liver are also subject to covalent modification including glycogen phosphorylase, glycogen synthase, phosphofructokinase, acetyl-CoA carboxylase and triglyceride lipase (Cohen, 1980; Sakakibara and Uyeda, 1983). Analogous glycolytic enzymes in skeletal muscle (e.g., M-type PK and PFK) are often not good substrates for phosphorylation, apparently because muscle metabolism most often tends to be unidirectional and unifunctional, dedicated to substrate catabolism for energy production.

Covalent modification by phosphorylation/dephosphorylation of enzymes appears to play a major role in the control of glycolysis with respect to aerobic versus anaerobic flux in anoxia-tolerant marine molluscs. Phosphorylation of enzymes is apparently important in reducing carbon flux through glycolysis during anoxia, in determining the pathways of carbohydrate catabolism and, very probably, in regulating the depression of a variety of metabolic processes during anoxia.

Glycogen phosphorylase

Covalent modification converts glycogen phosphorylase from the inactive (AMP dependent) *b* form to the active *a* form (Cohen, 1980). Activation of glycogenolysis in tissues is most often accompanied by the rapid cAMP- or Ca^{2+}/calmodulin-stimulated conversion of phosphorylase *b* to *a*. Ebberink and Salimans (1982) examined glycogen phosphorylase activity in adductor muscle of *M. edulis* over a time course of up to 18 h of anoxia and found no change in the percentage of phosphorylase in the *a* form (6–8%) as a result of anaerobiosis. Although elevated

levels of AMP in anoxic tissues (Ebberink and De Zwaan, 1980) might result in increased activity of phosphorylase *b* during anoxia, it appears most probable that glycogenolysis is not stimulated by the aerobic/anaerobic transition in marine molluscs. This suggests that the rate of carbohydrate catabolism does not increase during anaerobiosis and is consistent with the absence of a Pasteur effect.

Phosphofructokinase

PFK isolated from anoxic foot muscle of the whelk *B. canaliculatum* shows some significant differences in kinetic properties compared to the enzyme from aerobic muscle. These include: (a) decreased activating effects by NH_4^+ and fructose-2,6-P_2 (for fructose-2,6-P_2 an increased K_a and a reduced effect in lowering $S_{0.5}$ for fructose-6-P), (b) increased substrate inhibition by ATP, and (c) reduced affinity for Mg^{2+} (Table 2) (Storey, 1984). These same kinetic differences characterize the dephosphorylated versus phosphorylated forms of mammalian PFK, the phosphorylated enzyme being more sensitive to inhibition by ATP and citrate and less sensitive to the actions of activators (Foe and Kemp, 1982; Sakakibara and Uyeda, 1983). It appears, then, that the aerobic and anoxic forms of PFK in whelk foot muscle are, respectively, the dephosphorylated and phosphorylated enzyme forms. This is backed up by the effect of alkaline phosphatase on the anoxic enzyme form; phosphatase treatment reduced the K_a for fructose-2,6-P_2 by 5 fold to a value typical of the aerobic enzyme form (Storey, 1984).

Thus, the data strongly support the hypothesis of covalent modification of PFK during anoxia with phosphorylation producing a less active enzyme form. While energy status during anoxia (including ATP, ADP, AMP and P_i levels) would appear to favour activation of the enzyme (see Table 1), the effects of phosphorylation could override this by mechanisms including (a) increased ATP inhibition and decreased fructose-2,6-P_2 activation of the phosphorylated enzyme and (b) loss of the powerful synergistic interactions between fructose-2,6-P_2 and other activators when content of the bisphosphate is decreased. Phosphorylation of the key rate-limiting enzyme of glycolysis may be one of the major reasons for the reduction in glycolytic flux during anaerobiosis in marine molluscs and may, at least partially, account for the absence of a Pasteur effect.

Pyruvate kinase

Covalent modification as a regulatory mechanism for glycolysis during anaerobiosis also extends to another control enzyme, pyruvate kinase (PK). Evidence for the occurrence of two interconvertible forms of PK in anoxia-tolerant marine molluscs has been accumulating over recent years (Siebenaller, 1979; Holwerda et al., 1981, 1983, 1984). The proportions of the two PK variants in *M. edulis* adductor muscle have been shown to respond to anoxia or high temperature stress (Holwerda et al., 1983). Recent work in my laboratory has shown that at least seven tissues of the whelk *B. canaliculatum* contain distinct aerobic and anoxic variants of PK which differ in both kinetic and electrophoretic properties (Plaxton and Storey, 1985a), in all instances consistent with the known properties of the dephosphorylated versus phosphorylated forms of mammalian L (liver) -type PK (Engstrom, 1978). These

TABLE 4

Some properties of aerobic (dephosphorylated) and anoxic (phosphorylated) forms of pyruvate kinase isolated from whelk, *B. canaliculatum*, radular retractor muscle and hepatopancreas

	Muscle PK		Hepatopancreas PK	
	Aerobic	Anoxic	Aerobic	Anoxic
Purified activity (U/mg)	368	186	14	2.3
Affinity for *P-enol*pyruvate:				
$S_{0.5}$	0.07 mM	0.85 mM	0.38 mM (18)	1.1 mM (2.0)
n_H	0.99	2.57	1.15	1.19
$S_{0.5}$ + 50 μM fructose-1,6-P_2	0.04 mM	0.04 mM	0.12 mM (167)	0.11 mM (2.4)
$S_{0.5}$ + 3 mM aspartate	—	—	0.17 mM (233)	0.28 mM (2.5)
Activator constants (K_a):				
fructose-1,6-P_2	0.054 μM	1.3 μM	0.16 μM	0.48 μM
aspartate	—	—	1.5 mM	1.5 mM
Inhibitor constants:				
alanine	24.5 mM	0.05 mM	3.9 mM	0.48 mM
Mg ATP	2.7 mM	0.8 mM	2.4 mM	3.0 mM

Aspartate is a potent activator of hepatopancreas PK but does not affect muscle PK. Activators greatly increase maximal velocity (relative V_{max} given in brackets) of the aerobic form of hepatopancreas PK but have little effect on the anoxic form and do not alter V_{max} of the muscle enzyme. Inhibitor and activator constants were determined with respect to *P-enol*pyruvate. Inhibitor constants are K_i values for muscle PK and I_{50} values, determined at 0.4 mM *P-enol*pyruvate, for hepatopancreas PK. Data from Plaxton and Storey (1984a, 1985b).

variants are found for each of the three tissue-specific isozymes. Aerobic and anoxic variants of PK from radular retractor muscle (red muscle isozyme) (Plaxton and Storey, 1984a) and hepatopancreas (soft tissue isozyme) (Plaxton and Storey, 1985b) have been purified and characterized (Table 4). That the aerobic and anoxic forms differ due to the covalent incorporation of phosphate has been shown conclusively for the radular retractor enzyme. [^{32}P]Orthophosphate was readily incorporated into the enzyme under anoxic conditions producing an *O*-phospho-L-threonine residue (Plaxton and Storey, 1984b). Treatment of the anoxic form with alkaline phosphatase to remove covalently bound phosphate restores an enzyme which is kinetically and physically indistinguishable from the aerobic PK variant.

The result of phosphorylation of whelk radular retractor PK is a much less active enzyme form. Compared to the aerobic form the anoxic variant showed: (a) a lower maximal activity (39 versus 116 units/g wet weight), (b) a greatly reduced affinity for *P-enol*pyruvate ($S_{0.5}$ 12-fold higher), (c) a greatly reduced activation by fructose-1,6-P_2 (K_a 26-fold higher) and (d) an extremely potent inhibition by alanine (K_i 490-fold lower) (Table 4) (Plaxton and Storey, 1984a). These kinetic differences between dephosphorylated and phosphorylated forms of whelk muscle PK are also those typical of the two forms of mammalian L-type PK (Engstrom, 1978). Of note, however, is the fact that the differences in alanine effects between aerobic and anoxic forms of whelk PK are much more pronounced than those seen for the liver

enzyme; this is consistent with the key role of alanine in the anaerobic metabolism of marine molluscs.

Phosphorylation of PK during anoxia is not only important in reducing glycolytic flux but is also apparently the key mechanism regulating the so-called '*P-enol*pyruvate branchpoint'. Early stages of anoxia require continued PK activity to account for the synthesis of alanine as a glycolytic end product: in later stages, however, alanine synthesis stops and glycolytic carbon is instead diverted through *P-enol*pyruvate carboxykinase (PEPCK) into succinate (Fig. 1). Early studies on molluscan PK suggested that inhibitory control of PK during anoxia resulted from alanine inhibition (increased as alanine accumulated as an end product) and pH effects (the low pH of anoxic tissues favouring PEPCK over PK) (Mustafa and Hochachka, 1971). Anoxia-induced phosphorylation of PK, however, has considerably more powerful inhibitory effects on the enzyme (see above) and these are greatly enhanced by the accumulation of alanine as an anaerobic end product. Thus, only covalent modification during anoxia could effectively allow a successful competition by PEPCK over PK (2.9 vs. 116 U/g, respectively, in aerobic radular retractor) for glycolytic carbon. There is no evidence for covalent modification of PEPCK in response to anoxia (Plaxton and Storey, 1985a).

Marine molluscs have co-opted the use of phosphorylation of PK to direct the aerobic versus anoxic routing of *P-enol*pyruvate catabolism. Mammalian tissues, however, use covalent modification of PK to regulate glycolytic versus gluconeogenic flux (Engstrom, 1978). How then is PK regulated to allow gluconeogenic carbon flow in the soft tissues of marine molluscs? To answer this we examined the properties of hepatopancreas PK from the whelk (Table 4) (Plaxton and Storey, 1985b). The enzyme from anoxic animals is again the phosphorylated form, although kinetic differences between aerobic and anoxic forms are not as pronounced as for the radular retractor enzyme; thus, anoxic PK, compared to aerobic PK, showed (a) a lower maximal activity (0.92 vs. 3.4 U/g), (b) a 3-fold higher K_m for *P-enol*pyruvate, (c) a 3-fold higher K_a for fructose-1,6-P_2, and (d) an 8-fold lower I_{50} for alanine. The weaker effects of phosphorylation on this isozyme (gill and kidney share the same isozyme form) probably reflect the PK/PEPCK activity ratio in aerobic hepatopancreas of only 4:1 (compared to 40:1 in radular retractor): PK inactivation need not be as strong in this tissue to allow effective competition by PEPCK. Properties of the aerobic (dephosphorylated) enzyme form are quite unusual, however, and these probably determine enzyme regulation under conditions of glycolytic versus gluconeogenic flux in the aerobic state. Thus, activity of the aerobic enzyme is modulated by a wide variety of allosteric effectors and in particular by two powerful activators, fructose-1,6-P_2 and aspartate, which greatly increase V_{max} of the enzyme (by 9–13-fold), decrease $S_{0.5}$ for *P-enol*pyruvate (by 2–3-fold) and override effects of inhibitors (Table 4). In the aerobic state, then, glycolytic flux could be favoured in the presence of high levels of activators; gluconeogenic flux could be potentiated in the absence of effectors.

Our studies of hepatopancreas PK are the first reported instance of an animal PK which is strongly affected by the amino acid aspartate (Plaxton and Storey, 1985b), although we have subsequently found aspartate activation of PK in several other

marine invertebrates (Storey, 1986). PK from radular retractor muscle, however, is not aspartate activated. As a key substrate of anaerobic metabolism, aspartate control of PK could allow a very sensitive regulation of carbon flow through this locus. Under anaerobic conditions aspartate depletion as a substrate would slowly withdraw activation of PK at the same time as an opposite accumulation of alanine as an end product would increase enzyme inhibition. These effects would become more pronounced as phosphorylation of PK proceeded. Rerouting of glycolytic carbon from alanine to succinate as the end product could therefore be very precisely co-ordinated with the exhaustion of aspartate reserves. During aerobic recovery from anoxia, gluconeogenic reactions utilizing succinate or alanine as substrates could be strongly favoured by low tissue levels of aspartate (and also perhaps fructose-1,6-P_2) which would suppress PK activity.

Control of metabolic depression by covalent modification

Covalent modification has been very effectively put to use in all tissues of marine molluscs as a mechanism for decreasing glycolytic rate in response to anoxia.

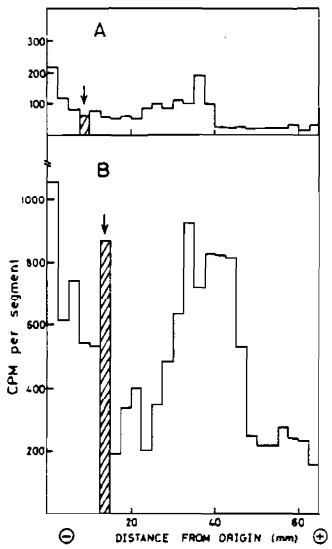

Fig. 2. Profile of [^{32}P]orthophosphate incorporation into proteins in dialyzed crude extracts of whelk, *B. canaliculatum*, radular retractor muscle from animals held under (A) aerobic versus (B) anoxic (21 h in N_2-bubbled seawater) conditions. Separation is by cellulose acetate electrophoresis. The segment which stained for pyruvate kinase activity is shown by an arrow. Taken from Plaxton and Storey (1984b), reprinted with permission of the European Journal of Biochemistry.

Normal allosteric controls by adenylates, etc. which would tend in mammalian systems to activate glycolysis in anoxia can apparently be overridden, at least in part, by phosphorylation-dependent inactivation of enzymes. It also appears likely that covalent modification of enzymes/proteins is put to a wider use during anaerobiosis in inactivating a number of metabolic processes and contributing to the overall metabolic depression which characterizes anaerobiosis. Thus we have found a high level of incorporation of covalently bound ^{32}P in extracts of anoxic muscle of the whelk (Fig. 2) (Plaxton and Storey, 1984b) which strongly suggests that a wide variety of proteins and enzymes may be subject to anoxia-induced phosphorylation.

Enzyme association/dissociation with the particulate fraction

The second concept in metabolic control which appears to be of major consequence in regulating aerobic versus anaerobic glycolytic rate (and also likely in regulating overall metabolic rate) is enzyme and pathway regulation via the association of enzymes with subcellular structures. A variety of new evidence is leading to the rejection of the old concept of the cytoplasm as containing a random distribution of all soluble enzymes and metabolites. Various studies have now shown associations, apparently physiologically relevant, between many of the so-called 'soluble' enzymes of glycolysis and membrane fractions, glycogen particles or structural proteins in the cell (Knull, 1978; Moses, 1978; Wilson et al., 1982). The microcompartmentation which can result from such associations can provide an effective mechanism for enzyme and pathway control by (1) channeling substrates/products between consecutive enzymes in a pathway, and (2) altering enzyme kinetic properties due to conformational changes occurring during binding (Arnold and Pette, 1970; Masters, 1985). For example, the key enzyme regulating the Pasteur effect, PFK, shows appreciable binding to the particulate structure and as a result of binding, its kinetics change from allosteric to Michaelis-Menten (Masters, 1985). Studies on mammalian muscle have found that metabolic situations which increase glycolytic flux (tetanic contraction, anoxia, ischaemia) result in an increase in the proportions of several glycolytic enzymes associated with the particulate fraction (Knull et al., 1973; Clarke et al., 1980; 1984). We have found the same result on the initiation of flight in cockroach flight muscle (K.B. Storey, unpublished results). Therefore, it appears that an alteration of the location of enzymes from the soluble to the particulate (bound) phase is an important feature in the activation of glycolysis, interacting with and complementing other control features such as allosteric regulation and covalent modification.

We have applied two of the techniques used to assess enzyme binding to an examination of the behaviour of glycolytic enzymes during environmental anoxia in whelk (*B. canaliculatum*) muscle (Plaxton and Storey, 1986). The technique of Clarke et al. (1984) involves rapid homogenization of tissues in a sucrose medium to preserve enzyme-particle associations, followed by centrifugation to separate soluble and particulate fractions and finally, release of the enzyme content of each fraction by resuspension in dilute buffer. The technique of Ross and Hultin (1980) forces soluble enzymes out of tissue strips by high-speed centrifugation, leaving bound

enzymes behind in the tissue to be later released by homogenization in dilute buffer. Fig. 3 shows the percentages of eight glycolytic enzymes associated with the particulate fraction in whelk ventricle as assessed by the technique of Clarke et al. (1984) under three conditions: (1) aerobic (sampled directly from oxygenated seawater), (2) 21 h anoxia in N_2-bubbled seawater, and (3) 2 h recovery in aerated seawater after 21 h anoxia. Anoxia resulted in a significant decrease in the percentage of each enzyme associated with the particulate fraction. The effect was most pronounced for hexokinase, a decrease from 45 (aerobic) to 10% (anoxic) bound, and for aldolase, a decrease from 41 to 9% bound. With the exception of lactate dehydrogenase, the effect had been reversed, with a significant increase in enzyme binding, as a result of 2 h of aerobic recovery. Very similar results were obtained when foot muscle was assessed by this same technique (Plaxton and Storey, 1986). The method of Ross and Hultin (1980) was used to assess enzyme binding by six glycolytic enzymes under aerobic versus anoxic (21 h) conditions. This technique showed a significant decrease in the percentages of hexokinase, aldolase and pyruvate kinase associated with the particulate fraction during anoxia in ventricle but no effect on the binding of the three terminal dehydrogenases (lactate, octopine and alanopine dehydrogenases) (Plaxton and Storey, 1986).

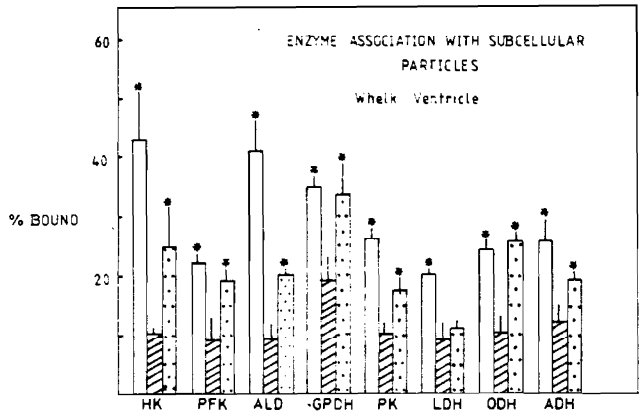

Fig. 3. Percentage of recovered enzyme activity associated with the particulate fraction of the cell in whelk, *B. canaliculatum*, ventricle under three conditions: aerobic (□), anoxic (21 h in N_2-bubbled seawater) (▨), and 2 h aerobic recovery from anoxia (▩). Separation of enzyme activities into soluble and bound fractions was based on the procedure of Clarke et al. (1984) in which tissue homogenization in a sucrose medium preserves enzyme associations with subcellular particles. Enzymes assayed are hexokinase (HK), phosphofructokinase (PFK), aldolase (ALD), glycerol-3-*P* dehydrogenase (GPDH), pyruvate kinase (PK), lactate dehydrogenase (LDH), octopine dehydrogenase (ODH) and alanopine dehydrogenase (ADH). Asterisks denote values which are significantly different from the anoxic state. $P < 0.10$.

It is apparent, then, that the aerobic/anaerobic transition in whelk tissues leads to a decrease in the association of glycolytic enzymes with the particulate fraction, the probable result being a decrease in enzyme and pathway organization promoting a decrease in glycolytic flux. The opposite result, an increase in the association of glycolytic enzymes with the particulate fraction, characterizes anoxia or ischaemia in mammalian heart (in ischaemia the percentages of PFK and aldolase bound rise from 13 and 29.5% to 24.5 and 78%, respectively) promoting the known increase in glycolytic flux (Pasteur effect) (Clarke et al., 1984). Both mammalian and molluscan studies, then, show that binding of glycolytic enzymes to the particulate fraction of the cell is decreased when glycolytic flux is reduced and increased when glycolysis is activated. This mechanism of enzyme/pathway control should receive much more attention and may prove to have a major role in the regulation of the overall metabolic depression which occurs during anaerobiosis in many anoxia-tolerant animals.

The major subcellular site of glycolytic enzyme binding in mammalian systems appears to be the myofibril proteins, F-actin as well as troponin and tropomyosin (Walsh et al., 1980; Stewart et al., 1980; Westrin and Backman, 1983) although membrane fractions and glycogen particles are other possible sites. For example, hexokinase has long been known to reversibly bind to mitochondria with binding increased during ischaemia (Purich et al., 1973; Knull et al., 1973) and several studies have documented aldolase binding to F-actin (Arnold and Pette, 1970; Westrin and Backman, 1983). The subcellular site(s) of glycolytic enzyme binding in whelk muscle have not yet been determined. However, we have ruled out glycogen particles as the binding site; glycogen particles isolated from whelk ventricle by the method of Lyon (1984) contained less than 7% of the measurable activities of eight glycolytic enzymes and no change in enzyme content of the fraction occurred as a result of 21 h anoxia (Plaxton and Storey, 1986).

Signals mediating the biochemical response to anoxia

Various processes occurring during anoxia have been discussed: general metabolic depression, decreased glycolytic flux, phosphorylation of enzymes and decreased association of enzymes with subcellular particles. In part, the reduced metabolic rate of anoxia is due to a natural cessation of physical activities (e.g. locomotion, feeding) which are not possible in the anaerobic state. This obviously results in energy savings. But additionally, specific metabolic rate reduction is brought into play. What signals link the loss of oxygen with subsequent events, for example the phosphorylation of enzymes? In mammalian tissues events such as the phosphorylation of enzymes are often hormone mediated with cAMP and Ca^{2+} acting as the intracellular messengers. Hormonal involvement in the initiation of anaerobic metabolism in marine molluscs does not seem likely as studies have shown equivalent responses of tissues in the intact animal or in in vitro preparations. However, intracellular changes in Ca^{2+} and cAMP as a result of anoxia should be quantitated. Intracellular pH may also be a factor. Over the long term intracellular pH falls during anaerobiosis by perhaps 0.1 to 0.4 pH units (Ellington, 1983). This might

facilitate the phosphorylation of enzymes or the dissociation of enzymes from the particulate fraction. In mammalian systems aldolase binding to myofibrils is increased as pH decreases (Clarke et al., 1984); perhaps an opposite effect in anoxia-tolerant molluscs would facilitate decreased enzyme binding during anoxia.

Conclusion

The Pasteur effect is not the universal response of cells to anoxia. The activation of fermentative metabolism when oxygen supply fails is one response which benefits organisms under certain circumstances. Thus, the Pasteur effect characterizes organisms which rely on high rates of aerobic energy generation to support a very active lifestyle; these organisms rarely encounter low oxygen conditions and have only one option for surviving brief periods of anoxia – an attempt to compensate for normal energy demands with an activation of glycolytic energy production. The other circumstance where the Pasteur effect is useful is under conditions of unlimited exogenous substrate supply, as may often typify the growth conditions of yeasts and microorganisms. Control of the Pasteur effect can largely be accounted for through allosteric regulation of PFK and hexokinase although, as pointed out by Racker (1974), this still may not be entirely adequate.

Systems with a high natural tolerance for environmental anoxia generally do not show a Pasteur effect with the transition from aerobic to anaerobic conditions, despite metabolite level changes which should produce a Pasteur effect. This present article proposes that this is because 'override' mechanisms, superceding allosteric effects, are brought into play to depress glycolytic rate as part of the energy conservation strategy used for long-term anaerobic survival. At least two mechanisms are crucial in this regard: covalent modification of key glycolytic enzymes and enzyme association/dissociation interactions with subcellular particles. My review has concentrated on the roles of these mechanisms in controlling glycolytic rate in anoxia-tolerant marine molluscs (Fig. 4). These mechanisms are both very powerful: both have much stronger and farther reaching effects on glycolysis than do previous types of control mechanisms proposed for the regulation of anaerobic metabolism in

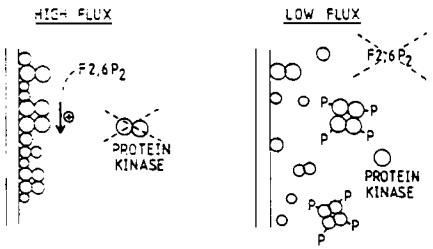

Fig. 4. Diagrammatic representation of proposed roles of covalent modification and enzyme association/dissociation with subcellular structures in controlling high versus low flux states of glycolysis.

marine molluscs. Thus, the effects of covalent modification on the kinetic properties of PFK and PK are very much more powerful in depressing enzyme activity (at physiological levels of substrates and effectors) than are the minor changes in cellular pH and metabolite (e.g., alanine) concentrations which have for many years been suggested as the controlling factors. The large scale changes that occur in PFK effector binding and in PK substrate and effector binding as a result of enzyme phosphorylation (Storey, 1984; Plaxton and Storey, 1984a) make the minor effects on these parameters of a slowly decreasing intracellular pH redundant as a useful regulation of enzyme activity. With respect to enzyme binding, not only is this a major factor in glycolytic control but this mechanism, although not yet tested, could hold the key to the organization of mitochondrial tricarboxylic acid cycle enzymes during anaerobiosis. The association of appropriate enzymes into membrane-bound complexes may be the means by which forward versus reverse functioning of the tricarboxylic acid cycle can be made responsive to the presence versus absence of oxygen.

Covalent modification of enzymes and enzyme binding to subcellular particles are obviously powerful mechanisms for regulating metabolism. Unlike allosteric controls which must be tailored to individual enzymes, these types of controls could be applied to all enzymes and pathways in the cell and used to regulate cellular responses to a variety of stimuli. Thus covalent modification in mammalian systems is largely known as a mechanism for controlling competing pathways in multifunctional tissues. It is likely however that covalent modification and enzyme/particle binding are not only involved in the control of glycolytic rate in anoxia tolerant molluscs but are also generalized mechanisms responsible for depressing the activities of many metabolic pathways to bring about the generalized metabolic rate depression which characterizes long-term anaerobiosis (Fig. 4). These types of overriding metabolic controls will undoubtedly apply to long-term anaerobiosis in many other animal systems and are also likely to be key to the gross depression of metabolism which occurs in many other situations (e.g., anhydrobiosis, freeze tolerance, torpor, hibernation and estivation). Comparative biochemistry must begin to look above the level of allosteric/kinetic control of enzymes and pathways and examine these higher tiers of metabolic control and the potentially major roles that these could play in keying metabolic responses to environmental stresses.

Acknowledgements

Thanks to J.M. Storey and W.C. Plaxton for excellent ideas and research which led to this article. Research performed in my laboratory was supported by an operating grant from the N.S.E.R.C. Canada.

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