Patterns of protein synthesis and phosphorylation during anoxia in the land snail *Otala lactea*

S.P.J. BROOKS[1] AND K.B. STOREY[2]

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

Received April 30, 1993
Accepted April 12, 1994

BROOKS, S.P.J., and STOREY, K.B. 1994. Patterns of protein synthesis and phosphorylation during anoxia in the land snail *Otala lactea*. Can. J. Zool. **72**: 856–862.

Protein labeling patterns with [^{32}P]phosphorous and [^{35}S]methionine plus [^{35}S]cysteine were examined in normoxic and anoxic tissues of *Otala lactea*. Analysis of protein phosphorylation showed differences between normoxic and anoxic individuals in both the extent of labeling and the pattern of protein phosphorylation. These changes were confined to the hepatopancreas where anoxia caused overall protein phosphorylation to decrease by a factor of 3. Subcellular fractionation of the hepatopancreas showed that this overall decrease in protein phosphorylation was due to a lower level of protein phosphorylation in individual fractions. Thus, the level of protein phosphorylation during anoxia was 45% (plasma membrane fraction), 21% (mitochondrial fraction), 24% (microsomal fraction), and 22% (cytosolic fraction) of that measured in normoxic tissues. Isoelectric focusing and sodium dodecyl sulfate polyacrylamide gel electrophoresis showed several changes in protein phosphorylation patterns. For example, the relative phosphorylation of cytosolic proteins of molecular mass 65, 49, 30, 25, 21, and 18 kDa was increased in normoxic individuals. Cytosol from anoxic snails showed a relative increase in the phosphorylation of proteins of molecular mass 68, 52, 44, 37, 18, and 16 kDa. No differences in the protein labeling patterns of the mitochondrial and microsomal fractions were apparent. In contrast, analysis of proteins labeled with [^{35}S]methionine plus [^{35}S]cysteine showed no differences when anoxic and normoxic snail tissues were compared.

BROOKS, S.P.J., et STOREY, K.B. 1994. Patterns of protein synthesis and phosphorylation during anoxia in the land snail *Otala lactea*. Can. J. Zool. **72** : 856–862.

Les marqueurs de protéines [^{32}P]phosphore et [^{35}S]méthionine plus [^{35}S]cystéine ont été examinés dans des tissus normoxiques et anoxiques chez *Otala lactea*. L'analyse de la phosphorylation des protéines a mis en lumière des différences entre les individus normoxiques et anoxiques, aussi bien dans l'importance du marquage que dans le pattern de phosphorylation des protéines. Ces changements étaient restreints à l'hépatopancréas où les conditions d'anoxie ont provoqué une diminution par un facteur de 3 de la phosphorylation globale des protéines. Le fractionnement subcellulaire de l'hépatopancréas a démontré que cette diminution généralisée de la phosphorylation des protéines était attribuable à une diminution de la phosphorylation des protéines dans les différentes fractions. La phosphorylation des protéines durant l'anoxie était de 45% (fraction de la membrane plasmatique), 21% (fraction mitochondriale), 24% (fraction microsomique) et 22% (fraction cytosolique) de la valeur mesurée dans les tissus normoxiques. La focalisation isoélectrique et l'électrophorèse sur gel de polyacrylamide–dodécyl sodium de sulfate ont mis en lumière plusieurs modifications des patterns de phosphorylation des protènes. Par exemple, la phosphorylation relative des protéines cytosoliques de masses moléculaires de 64, 49, 30, 25, 21 et 18 kDa était plus importante chez les individus normoxiques. Chez les individus anoxiques, la phosphorylation relative des protéines cytosoliques a augmenté dans le cas des protéines de masses moléculaires de 68, 52, 44, 37, 18 et 16 kDa. Aucune différence n'a été enregistrée dans les patterns de marquage des protéines des fractions mitochondriale et microsomique. En revanche, l'analyse des protéines marquées à la [^{35}S]méthionine plus [^{35}S]cystéine n'a révélé aucune différence entre les tissus anoxiques et normoxiques des gastropodes.

[Traduit par la Rédaction]

Introduction

When water and food supplies become low, snails retreat into their shells and enter a dormant state, termed estivation, that is characterized by a metabolic rate 5–30% of the corresponding rate in active snails (Herreid 1977; Barnhart and McMahon 1987; Rees and Hand 1990). The biochemical mechanisms involved in coordinating this metabolic rate depression have received considerable attention recently (see Storey and Storey 1990 for a review). In studies with *Otala lactea*, several mechanisms involved in regulating glycolytic rate during estivation have been identified, including (*i*) reversible phosphorylation of enzymes to reduce their activity (Brooks and Storey 1990; Whitwam and Storey 1990, 1991), (*ii*) changes in the level of enzyme binding to subcellular structures (Brooks and Storey 1990), (*iii*) control of carbohydrate utilization via fructose 2,6-bisphosphate regulation of phosphofructokinase (Brooks and Storey 1990), and (*iv*) changes in the total activities of some glycolytic enzymes (Brooks and Storey 1990). The universality of these mechanisms in mediating metabolic rate depression has been demonstrated by their discovery in several animal models including hibernating mammals, anoxic turtles, and frozen frogs as well as estivating snails (see Storey and Storey 1990).

The mechanisms responsible for coordinating changes in enzyme phosphorylation and changes in protein synthesis patterns during metabolic arrest are difficult to study directly. We have, therefore, chosen to study these mechanisms indirectly by studying the end products of these pathways. The study of protein phosphorylation is justified by the fact that several enzyme activities are regulated by reversible changes in the degree of protein phosphorylation (Edelman et al. 1987; Blackshear et al. 1988; Shenolikar 1988). For example, protein kinases, structural components such as myosin light chain, enzymes of protein synthesis, membrane ion channels, membrane receptors, and a variety of enzymes of intermediary metabolism such as glycogen phosphorylase, phosphofructokinase, pyruvate kinase, pyruvate dehydrogenase, α-ketoacid dehydrogenase, hydroxymethylgutaryl-CoA reductase,

[1]Present address: Nutrition Research Division, Food Directorate, Health Protection Branch, Health Canada, Banting Research Centre, Tunney's Pasture, Ottawa, ON K1A 0L2, Canada.

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

glycogen synthase, and isocitrate dehydrogenase are regulated by reversible phosphorylation.

Changes in protein synthesis patterns are also well characterized in animals exposed to extreme heat or cold shock, or to toxic concentrations of metals. The resulting protein synthesis patterns often show the presence of several stress-related proteins (SRPs). These SRPs are identified and characterized mainly by autoradiographic analysis of polyacrylamide gels; labeled proteins are classified by molecular mass determined in the presence of sodium dodecyl sulfate (SDS) in the first dimension and by isoelectric point in the second dimension (see Pipkin et al. 1988). More recently, however, some SRPs have been positively demonstrated to take part in protein folding and transport into organelles (see Gething and Sambrook 1992 for a review).

Estivation is characterized by hypoxia, hypercapnia, and extracellular acidosis with primary reliance on carbohydrates to fuel metabolism (Herreid 1977; Barnhart 1983, 1986*a*, 1986*b*; Umezurike and Iheanacho 1983; Livingstone and de Zwaan 1983). Perhaps, as a partial consequence to this, *O. lactea* also show a substantial tolerance for anoxia, readily surviving 2–3 days under an N_2 atmosphere (Brooks and Storey 1990). In this respect they are similar to many marine molluscs that readily survive 1–2 days of total anoxia (Storey and Storey 1990). Studies of enzyme phosphorylation (Whitwam and Storey 1990, 1991), enzyme binding, fructose 2,6-bisphosphate regulation of phosphofructokinase, changes in total enzyme concentration (Brooks and Storey 1990), and changes in intermediary metabolite levels (Churchill and Storey 1989) have shown that similar biochemical mechanisms operate to control metabolic rates during anoxia and estivation. The present study was undertaken to investigate changes in protein phosphorylation and protein synthesis in anoxic snails. The advantages of studying the effects of anoxia are threefold: (*i*) anoxia is an easily imposed and easily controlled stress, (*ii*) snails readily survive prolonged periods of anoxia, and (*iii*) the biochemical mechanisms involved in anoxia survival are likely to be the same as those used to survive hypoxia (Thillart et al. 1992; Zwaan et al. 1991). Work in our laboratory has previously compared the biochemical changes associated with anoxia and estivation to determine the universality of these mechanisms. As part of this comparison we are also investigating the phosphorylation and protein labeling patterns in anoxic and estivating snails. This paper presents the data from anoxic snails.

Materials and methods

Animals and chemicals

Snails (*O. lactea*) were graciously provided by Dr. M.C. Barnhart from an introduced population in San Diego, California. Animals were held in the laboratory at 21°C in covered plastic containers lined with moist paper towels. Snails were fed cabbage and ground chalk every 15–20 days. Snails were aroused by the introduction of food and water and maintained in an aroused state for 24 h by repeated spraying with water to maintain a high humidity in the chamber. Radioactive ^{35}S EXPRESS protein labeling mix (1240 Ci/mmol L-[^{35}S]methionine plus 1210 Ci/mmol L-[^{35}S]cysteine in a 77%:18% mixture; 1 Ci = 37 GBq) and [^{32}P]phosphorous (neutralized acid) were obtained from New England Nuclear. NCS tissue solubilizer and ACS-II scintillation cocktail were purchased from Amersham. Other chemicals were obtained from either Sigma Chemical Co. (St. Louis, Mo.) or Boehringer-Mannheim (Montréal, Que.).

Experimental protocol

Between 6 and 10 μL (9 μCi) of carrier-free radioactivity (either [^{35}S]methionine plus [^{35}S]cysteine or [^{32}P]phosphorous) was injected into the snails. To minimize trauma to animals we chose to deliver the radioactivity between the mantle edge and the shell at a site close to the pneumostome. This was done by slipping the tip of a 25-μL Hamilton syringe between the mantle and shell while keeping the needle tip pointed towards the shell. The needle was inserted approximately 4 mm past the mantle edge. Throughout the experiment the snails were maintained in the active state by placing them in containers lined with moist paper toweling to keep the humidity high. Injected snails were separated into two groups. Normoxic snails were held in large containers with the covers placed slightly ajar to maintain air flow. Anoxic snails were placed in a tightly sealed chamber filled with N_2 gas. At the end of a 45-h exposure period, animals were quickly removed from the chambers and sacrificed. For whole tissue studies, haemolymph, hepatopancreas, mantle, and foot muscle were quickly sampled and immediately frozen in liquid N_2. In some experiments, the hepatopancreas was not frozen but fresh tissue was fractionated as described below.

Subcellular fractionation

To measure the ^{32}P distribution among subcellular fractions of hepatopancreas in active and anoxic snails, tissue samples (0.3–0.5 g) were immediately homogenized 1:4 in ice cold TE buffer (0.2 M Tris-HCl, 5 mM $MgCl_2$, 10 mM ethylenediaminetetraacetic acid (EDTA), 50 mM NaF, pH 7.4) containing 0.25 M sucrose. All fractionation steps were carried out on ice. The homogenate (crude fraction) was layered over an equal volume of TE buffer containing 0.34 M sucrose and centrifuged at 1000 × *g* for 10 min at 4°C. The resulting pellet was washed once by resuspending it in TE buffer containing 0.25 M sucrose and 1% Triton X-100 and recentrifuging. The final pellet was saved as the plasma membrane fraction (PM). The supernatant from the 1000 × *g* spin was further fractionated into mitochondrial (MT), microsomal (MC), and cytosolic (CY) fractions as follows. Firstly, the supernatant was centrifuged at 17 000 × *g* to pellet mitochondria. The MT fraction was washed once in TE buffer containing 0.25 M sucrose before being stored on ice. The supernatant from the 17 000 × *g* spin was centrifuged at 100 000 × *g* for 1 h to pellet microsomal activity. The supernatant from the 100 000 × *g* spin was defined as the CY fraction.

Enzyme and protein assays

To measure the relative purity of the different subcellular fractions, the specific activities of the following four marker enzymes were monitored: 5′-nucleotidase (plasma membrane), succinate – cytochrome *c* reductase (mitochondria), rotenone-insensitive NADPH – cytochrome *c* reductase (microsomes), and lactate dehydrogenase (cytosol). Enzymes were assayed as follows: 5′-nucleotidase according to Morre (1971), succinate – cytochrome *c* reductase according to Sottocasa et al. (1967), and NADPH – cytochrome *c* reductase according to Strobel and Dignam (1978), except that the assay contained 1.5 μM rotenone, and lactate dehydrogenase according to Brooks and Storey (1990). All assays were performed at room temperature.

Protein concentrations were measured using the micro Bio-Rad protein assay method (modified Bradford assay) with bovine gamma globulin as the standard.

Radioactivity determinations

Samples were homogenized 1:3 (w/v) in 5% (w/v) trichloroacetic acid (TCA) – 10 mM H_3PO_4 and centrifuged at 15 000 × *g* for 20 min to separate incorporated from unincorporated radioactivity. For ^{35}S incorporation experiments, the supernatants were removed and mixed with 9 volumes of scintillation cocktail. Pellets were solubilized by adding 0.7 mL of NCS tissue solubilizer and incubated overnight at 40°C. The solubilized pellet was mixed with 10 mL of scintillation cocktail plus 20 μL of glacial acetic acid. Samples were then counted in a Canberra-Packard 1900-CA scintillation counter using ET-DPM methodology. For ^{32}P incorporation, supernatants

TABLE 1. Activities of subcellular marker enzymes in *O. lactea* hepatopancreatic fractions

Enzyme	IU/g protein in fraction				
	Crude	PM	MT	MC	CY
5′NT (PM)	8.5±1.1	19.7±4.5	7.7±1.6	4.4±0.9	7.7±0.4
ScR (MT)	13.5±3.0	nd	66.1±20.3	6.0±2.4	7.0±1.6
NcR (MC)	2.2±0.4	5.5±3.9	6.1±3.5	31.8±13.9	6.4±3.1
LDH (CY)	155±15	67±12	130±65	140±65	327±21

NOTE: Values are given as means ± SEM for 5 determinations. 5′NT, 5′-nucleotidase; ScR, succinate – cytochrome *c* reductase; NcR, NADPH – cytochrome *c* reductase, LDH; lactate dehydrogenase; nd, not detected.

TABLE 2. ^{32}P Incorporation (dpm/μg wet mass) into normoxic and anoxic *O. lactea* tissues 45 h post-injection

	Total		Acid precipitable	
	Normoxic	Anoxic	Normoxic	Anoxic
Foot	4.19±1.20	3.29±0.61	1.83±0.56 (44)	1.18±0.21 (36)
Mantle	18.7±4.4	24.4±4.1	7.47±1.90 (40)	9.83±1.40 (40)
Haemolymph	0.81±0.16	0.81±0.12	0.09±0.01 (11)	0.09±0.01 (11)
Hepatopancreas				
Crude	8.13±1.56	4.12±0.47[a]	2.74±0.69 (34)	0.93±0.16 (23)[a]
PM	0.71±0.09	0.44±0.05[a]	0.25±0.04 (35)	0.12±0.04 (26)[a]
MT	0.93±0.19	0.26±0.08[a]	0.62±0.15 (65)	0.120±0.04 (49)[a]
MC	1.09±0.21	0.34±0.02[a]	0.83±0.19 (74)	0.20±0.02 (59)[a]
CY	4.82±0.50	2.58±0.24[a]	0.50±0.07 (11)	0.12±0.01 (5)[a]

NOTE: Values are given as means ± SEM for 4 separate determinations. Values in parentheses represent the percentage of the total radioactivity associated with the pellet.

[a]Significantly different from normoxic value at the $P < 0.05$ level as determined by the two-tailed Student's *t* test.

were removed and both supernatants and pellets were counted using Cerenkov methodology. The total number of disintegrations per minute (dpm) per microgram wet mass of tissue was determined by adding the total counts in the supernatant and in the pellet (1 dpm = 0.0167 Bq).

SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970), using 10% (w/v) polyacrylamide gels. Samples with approximately 25 000 dpm were loaded onto each lane. For autoradiography, gels were first stained with Coomassie Brilliant Blue R-250 and treated with Enlightening (New England Nuclear) prior to being dried in a gel dryer. Gels were then exposed to Kodak X-OMAT X-ray film for 35 d at −80°C prior to being developed. Molecular mass standards were as follows: phosphorylase *b* (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde 3-phosphate dehydrogenase (36 kDa), bovine red blood cell carbonic anhydrase (29 kDa), and bovine α-lactalbumin (14.2 kDa). For direct determination of radioactivity in gels, gels were first stained with Coomassie Brilliant Blue R-250 to visualize standards. Individual gel lanes were then cut into slices approximately 2 mm long and 2 cm wide, and incubated with 1 mL of a 9:1 (v/v) NCS:water solution at 50°C for 2 h in capped vials. Samples were counted after addition of 10 mL of scintillation cocktail.

Isoelectric focusing

Preparative isoelectrofocusing (IEF) was performed by the method of Vesterberg (1971), using an LKB 8101 column (110 mL). Gradients of pH 3.5–10 (for PM, MT, and MC fractions) or 4–6.5 (for the CY fraction) were used to separate proteins. Samples representing fractions from two or three hepatopancreas preparations were desalted prior to being loaded onto columns (see Helmerhorst and Stokes 1980). Columns were developed at 300 V (pH 3.5–10) or 500 V (pH 4–6.5) for 17 h at 6°C. All buffers for PM, MT, and MC runs contained 1% Triton X-100 to solubilize membrane-associated proteins. After the column had developed, it was drained and fractions of approximately 1.5 mL were collected. The radioactivity of ^{32}P-labeled proteins was measured directly by Cerenkov counting methodology. For IEF analysis of ^{35}S-labeled proteins a gel-based procedure was used. Samples with 10 000 – 15 000 dpm were loaded onto a 5.5% polyacrylamide – 0.14% bisacrylamide gel in 10% glycerol, with 5.5% ampholines (of the appropriate pH range). Gel polymerization was initiated with *N,N,N′,N′*-tetramethylethylenediamine (TEMED) and ammonium persulfate and allowed to continue for 1 h. The polymerized gel was prerun for 15 min at 200 V, 15 min at 300 V, and 30 min at 400 V. The gel was developed for 16 h at 400 V (constant voltage). Gels were stained for protein with Coomassie Brilliant Blue G-250, destained, cut, and counted as described for SDS-PAGE. The pH gradient of the acrylamide IEF gels was monitored using IEF standards of pH 3.5–10 obtained from Sigma.

Results

Subcellular fractionation

Table 1 presents the distribution of the different subcellular marker enzymes among the PM, MT, MC, and CY fractions of *O. lactea* hepatopancreas. The data show enrichment of all fractions when marker specific activities in individual fractions were compared with the corresponding crude value or with activities in other subcellular fractions. Specific enrichments were as follows: 2.3-fold (PM), 4.9-fold (MT), 14.5-fold (MC), and 2.1-fold (CY).

^{32}P incorporation experiments

Table 2 presents measurements of total and protein-associated ^{32}P radioactivity in tissues of animals assessed 45 h

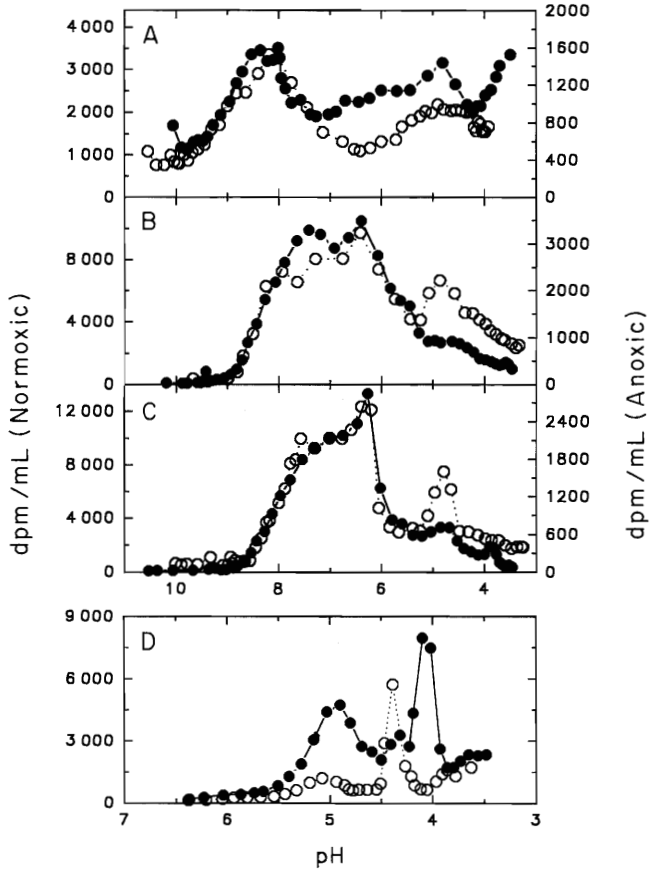

FIG. 1. IEF chromatography of ^{32}P-labeled proteins from 45-h normoxic and anoxic hepatopancreas subcellular fractions. ^{32}P was incorporated into the plasma membrane (A), mitochondrial (B), microsomal (C), and cytosolic (D) fractions from normoxic (●) and anoxic (○) snails. Incorporation (dpm/mL of column fraction) (see Materials and methods) is plotted as a function of the pH value of the fraction. Note that the pH scale in D is different from that in A–C. Three separate runs were performed. The standard deviation of the three runs was estimated to be approximately ±8% of the absolute value reported. This estimate was obtained by interpolating the values of the IEF profiles to identical pH values. The panels each represent a single IEF run.

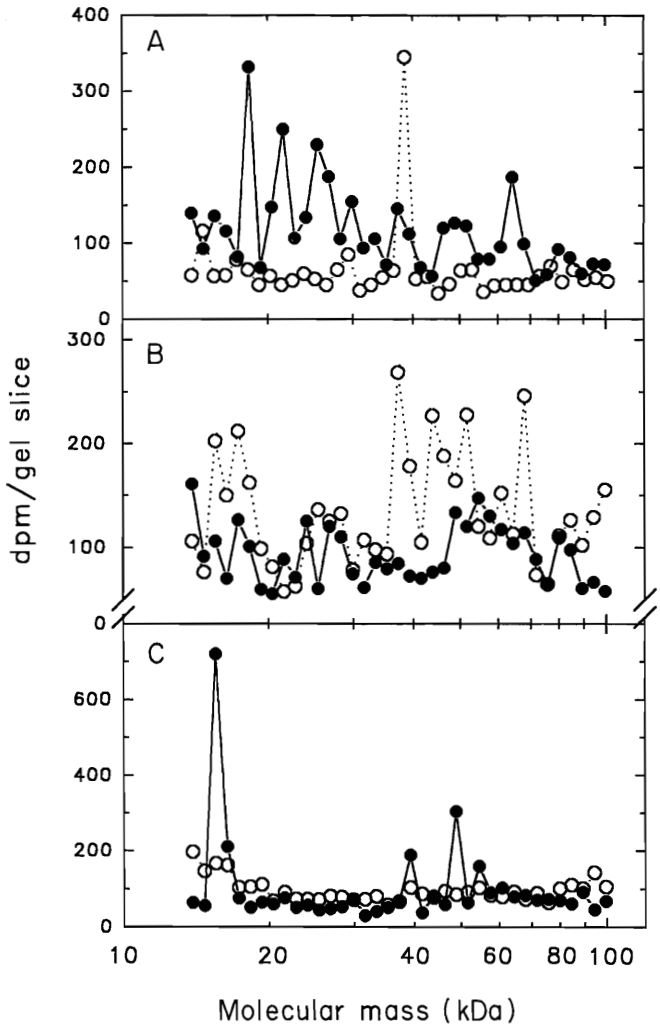

FIG. 2. SDS polyacrylamide gel electrophoresis of ^{32}P-labeled proteins from cytosolic IEF peak fractions. Individual fractions at a single pH value from normoxic (●) and anoxic (○) IEF chromatography columns (Fig. 1) were analyzed by SDS-PAGE. The peaks from the CY fraction at pH 4.9 (A), pH 4.4 (B), and pH 4.1 (C) were electrophoresed on 10% polyacrylamide gels. Gels were sliced and individual slices counted by Cerenkov methodology. A curve of molecular mass versus retardation factor (R_f) was obtained from standards and this was used to calibrate the sample lanes. Three separate runs were performed. The standard deviation of the three runs was estimated to be approximately ±10% of the absolute value reported. This estimate was obtained by interpolating the values of the IEF profiles to identical pH values. The panels each represent a single SDS-PAGE lane.

post-injection. The results show tissue-specific differences in the radioactivity of total homogenates and TCA pellets from hepatopancreas. In particular, ^{32}P incorporation into anoxic hepatopancreas was 51% of that into normoxic hepatopancreas. The percentage of radioactivity in the acid pellet of anoxic hepatopancreas was also reduced to 68% of normoxic values. These results translate into a decrease in protein phosphorylation to only 34% of normoxic levels. Subcellular fractionation of hepatopancreas into PM, MT, MC, and CY fractions showed that total and protein-associated radioactivity was lower in all subcellular fractions from anoxic individuals. Specifically, the hepatopancreas from anoxic animals had 45% (PM), 21% (MT), 24% (MC), and 22% (CY) of the normoxic value (Table 2).

The subcellular fractions from 45-h control and anoxic hepatopancreas samples were further analyzed by IEF to reveal potential differences in individual protein phosphorylation. The results of these analyses are shown in Fig. 1. Changes in the protein phosphorylation patterns were evident in all subcellular fractions. For example, a relative increase in the phosphorylation of proteins with pI values between pH 4.2 and 7.4 was observed in PM from normoxic individuals. A relative increase in the phosphorylation of proteins with a pI of 4.9 was observed in anoxic mitochondria and of proteins with a pI of 4.8 in anoxic microsomes. IEF analysis of ^{32}P-labeled normoxic and anoxic CY proteins showed several differences. Relative increases in the phosphorylation of proteins with a pI range between 4.6 and 5.1 and between 3.9 and 4.1 were observed in normoxic individuals. Phosphorylation of CY proteins with a pI of 4.4 was relatively higher in anoxic individuals.

Selected IEF peaks from the CY fraction were further analyzed by SDS-PAGE and the results are presented in Fig. 2. Analysis of the IEF peak for pH 4.9 showed increased phosphorylation of proteins with a molecular mass of 64, 49,

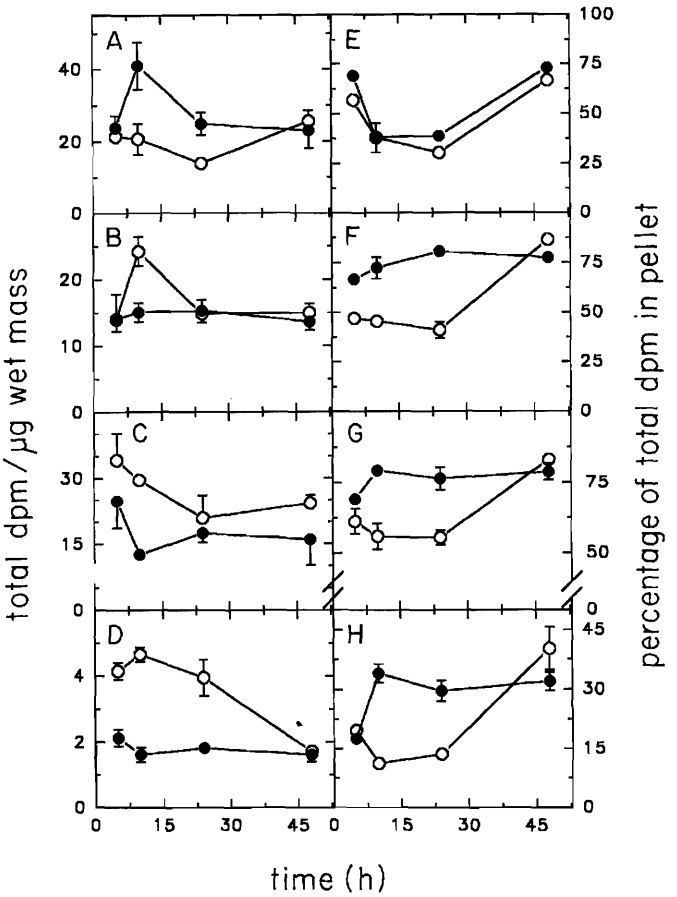

FIG. 3. The total uptake of [^{35}S]methionine plus [^{35}S]cysteine into tissues (A–D) and the percentage of the total radioactivity associated with the acid precipitable pellet (E–H) are plotted as a function of time post-injection. Results are shown for normoxic (●) and anoxic (○) conditions for hepatopancreas (A, E), foot (B, F), mantle (C, G), and haemolymph (D, H). The values represent means ± SEM for 4 separate determinations. The absence of error bars indicates that the SEM was smaller than the symbol size.

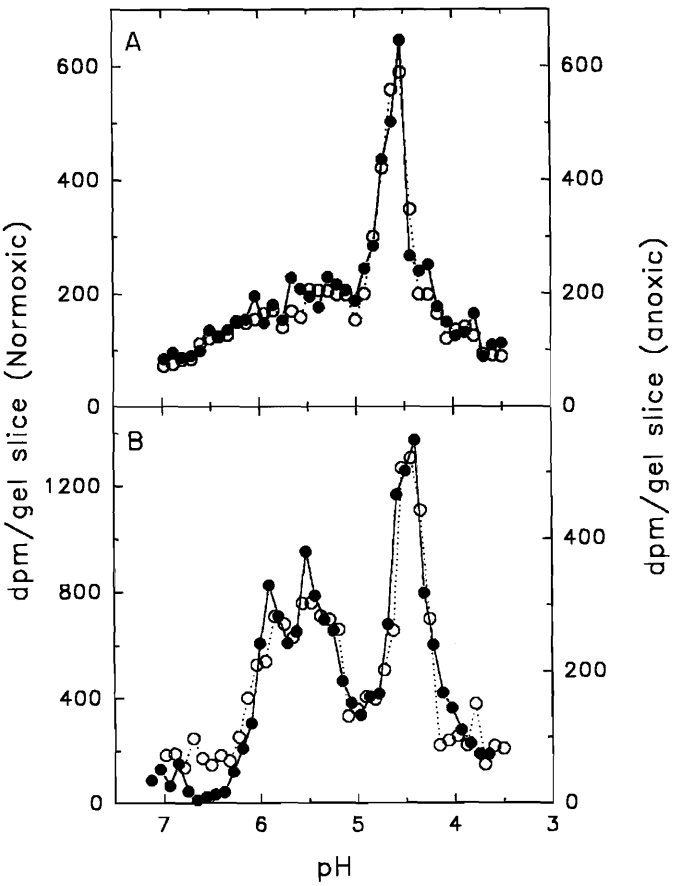

FIG. 4. Isoelectric focusing gel of proteins labeled with [^{35}S]methionine plus [^{35}S]cysteine from 10-h animals: comparison of normoxic (●) and anoxic (○) protein labeling patterns in hepatopancreas (A) and foot muscle (B). IEF was performed in polyacrylamide gels, the gels were sliced, and individual slices were counted for radioactivity as detailed in the Materials and methods section.

30, 25, 21, and 18 kDa in normoxic individuals. Anoxic hepatopancreas cytosol showed increased phosphorylation of a 37-kDa protein. Analysis of the IEF peak of the pH 4.4 CY fraction showed increased phosphorylation of several proteins in anoxic individuals. Peaks were apparent at 68, 52, 44, 37, 18, and 16 kDa. Analysis of the IEF peak of the pH 4.1 CY fraction showed only minor differences between anoxic and normoxic cytosol. Increased phosphorylation of two proteins (49 and 16 kDa) was apparent in cytosol from normoxic individuals. Analysis of the IEF peak of the pH 4.9 MT fraction and the peak of the pH 4.8 MC fraction showed no reproducible differences in the protein phosphorylation pattern.

^{35}S *incorporation experiments*

The total [^{35}S]methionine plus [^{35}S]cysteine incorporation into *O. lactea* hepatopancreas, foot, mantle, and haemolymph is shown Fig. 3. These results demonstrate that the [^{35}S]methionine plus [^{35}S]cysteine was rapidly taken up by normoxic *O. lactea* tissues so that incorporation was maximal by 5–10 h post-injection (depending on the tissue) and remained fairly constant for the remainder of the experiment. Total radioactivity in haemolymph and mantle from anoxic snails, on the other hand, was high in the early hours and then declined, although it stabilized in mantle by 24 h post-injection. These results may reflect the site of injection (cavity near the mantle) and the time required for distribution of radioactivity. By 45 h post-injection, approximately 22% of the injected radioactivity was associated with the hepatopancreas, 28% with the foot, 15% with the mantle, and 1% with the haemolymph. The data of Fig. 3 also show the percentage of the total radioactivity that was acid precipitable as a function of time post-injection. These data show that protein-associated radioactivity in normoxic foot and mantle tissue was statistically higher than that in the corresponding anoxic tissues at times prior to 48 h. Analysis of haemolymph revealed no difference in the percentage of protein-associated radioactivity at 5 and 48 h but normoxic values at 10 and 24 h were statistically higher than the corresponding anoxic values. Protein-associated radioactivity in hepatopancreas was similar for both normoxic and anoxic animals.

Figure 4 presents IEF profiles of the proteins labeled with [^{35}S]methionine plus [^{35}S]cysteine 10 h post-injection because this time point showed the greatest difference in total ^{35}S incorporation between normoxic and anoxic animals. No apparent differences were observed between protein labeling patterns in foot and hepatopancreas when tissues from

normoxic and anoxic snails were compared. SDS-PAGE and autoradiographic analysis of hepatopancreas and foot tissues at 5, 10, 14, and 48 h also showed no differences between normoxic and anoxic snails (data not shown).

Discussion

The results of the present paper show that *O. lactea* responds to anoxia by altering its protein phosphorylation patterns. Marked changes in protein phosphorylation were localized to the hepatopancreas where differences in normoxic and anoxic snail extracts could be demonstrated. Overall, these changes were consistent with a reduced level of enzyme phosphorylation during anoxia: the degree of protein phosphorylation during anoxia was only 20–30% of that found in normoxic animals (Table 2). A relatively higher degree of protein phosphorylation, however, was observed in the MT, MC, and CY fractions from anoxic hepatopancreas, showing that both a general decrease in protein phosphorylation and a specific targeting of individual proteins are important aspects of a successful anoxia tolerance strategy. No changes in total or protein-associated phosphorylation were observed in haemolymph, foot, or mantle tissues. The lack of change in foot and mantle tissues does not indicate that protein phosphorylation events do not play a major role in regulating the proteins of these tissues. In fact, several foot-associated proteins are phosphorylated when snails are injected with [^{32}P]phosphorous (data not shown). However, because of the difficulty in fractionating foot and mantle tissues into subcellular components, it was not possible to obtain detailed information on changes in protein phosphorylation associated with these subcellular fractions.

It is not surprising that specific changes in enzyme phosphorylation were observed during anoxia. Several previous studies have characterized stable kinetic changes in enzyme activity as a response to anoxia exposure; stable changes are consistent with covalent modification of the enzyme. For example, decreases in the activity of phosphofructokinase (Whitwam and Storey 1991), pyruvate kinase (Whitwam and Storey 1990), glycogen phosphorylase (Brooks and Storey 1990), and pyruvate dehydrogenase (Brooks and Storey 1992) were observed during anoxia. For three of these enzymes (pyruvate kinase, pyruvate dehydrogenase, and glycogen phosphorylase) this covalent modification is known to be an enzyme phosphorylation event; these enzymes are phosphorylated during anoxia. Figure 2 shows that the phosphorylation state of several proteins from the CY fraction is altered during anoxia. This suggests a complex, concerted response to lack of oxygen in this tissue. However, without specific antibodies it is difficult to absolutely identify these protein species. As indicated above, changes in enzyme phosphorylation patterns are not confined to the hepatopancreas. For example, decreases in the activity of pyruvate kinase and phosphofructokinase from foot and mantle (Whitwam and Storey 1990, 1991) and glycogen phosphorylase from foot (Brooks and Storey 1990) have been observed during anoxia. However, owing to the difficulties in fractionating foot and hepatopancreas into subcellular fractions, it was not possible to analyze these tissues beyond the level of gross changes in protein phosphorylation. SDS-PAGE analysis of whole tissue foot and mantle homogenates, however, did not reveal any differences in the protein phosphorylation pattern (data not shown).

The snail shows different metabolic responses to anoxia and to estivation. In *O. lactea*, estivation is characterized by intermittent episodes of oxygen uptake and carbon dioxide release. However, oxidative metabolism clearly continues throughout dormancy, albeit at a reduced rate when compared with that in the active state (Herreid 1977; Barnhart and McMahon 1987). Stored carbohydrate is the major fuel during dormancy (Livingstone and De Zwaan 1983; Umezurike and Iheanacho 1983) and anaerobic end products do not accumulate (Churchill and Storey 1989). On the other hand, the initial response of snails to declining oxygen concentrations is a compensatory one; snails show a Pasteur effect where glycolytic flux increases rapidly in response to allosteric activation of phosphofructokinase. This response is subsequently followed by a profound metabolic depression as oxygen falls below some critical lower limit. Anaerobic end products such as D-lactate, alanine, and succinate accumulate rapidly during the first 2 h of anoxia and continue to accumulate throughout the anoxic episode (Churchill and Storey 1989). nevertheless, the biochemical mechanisms that down-regulate metabolism during estivation and anoxia are similar in nature and include changes in enzyme phosphorylation. One can also observe similarities when protein phosphorylation patterns from anoxic and estivating (S.P.J. Brooks and K.B. Storey, unpublished results) snails are compared. It is possible that these results indicate a similarity in the overall response of snails to anoxia and estivation. However, note that a detailed comparison between phosphorylated protein peaks must be performed before any definite conclusion can be drawn.

The present paper showed that the IEF pattern of anoxic animals was similar to that of normoxic animals after 10 h of anoxia. On the other hand, during estivation one can observe the appearance of a 50-kDa protein of higher radioactivity in hepatopancreas and of 70- and 30-kDa proteins in foot muscle after 1 day (S.P.J. Brooks and K.B. Storey, unpublished observation). The identification of stress-related proteins during estivation in an estivation-tolerant animal suggests that they are important components of an overall estivation survival strategy in *O. lactea*. The absence of these proteins during exposure to environmental anoxia may indicate that they appear earlier during estivation (none were observed in 45-h tissues, data not shown) or may indicate important differences in the biochemical response of snails to anoxia and estivation.

Acknowledgments

Thanks to Dr. M.C. Barnhart for supplying the snails as well as information on their care. Thanks to J.M. Storey for critical reading of the manuscript. This work was supported by a Natural Sciences and Engineering Council of Canada operating grant and a National Institutes of Health (GM 43796, U.S.A.) grant to K.B.S.

Barnhart, M.C. 1983. Gas permeability of the epiphragm of a terrestrial snail, *Otala lactea*. Physiol. Zool. **56**: 436–444.

Barnhart, M.C. 1986*a*. Control of acid–base status in active and dormant land snails, *Otala lactea* (Pulmonata, Helicidae). J. Comp. Physiol. B, **156**: 347–354.

Barnhart, M.C. 1986*b*. Respiratory gas tensions and gas exchange in active and dormant land snails, *Otala lactea*. Physiol. Zool. **59**: 733–745.

Barnhart, M.C., and McMahon, B.R. 1987. Discontinuous CO_2 release and metabolic depression in dormant land snails. J. Exp. Biol. **128**: 123–138.

Blackshear, P.J., Nairn, A.C., and Kuo, J.F. 1988. Protein kinases 1988: a current perspective. FASEB J. **2**: 2957–2969.

Brooks, S.P.J., and Storey, K.B. 1990. Glycolytic enzyme binding and metabolic control in estivation and anoxia in the land snail *Otala lactea*. J. Exp. Biol. **151**: 193–204.

Brooks, S.P.J., and Storey, K.B. 1992. Properties of pyruvate dehydrogenase from the land snail, *Otala lactea*: control of enzyme activity during estivation. Physiol. Zool. **65**: 620–633.

Churchill, T.A., and Storey, K.B. 1989. Intermediary energy metabolism during dormancy and anoxia in the land snail *Otala lactea*. Physiol. Zool. **62**: 1015–1030.

Edelman, A.M., Blumenthal, D.K., and Krebs, E.G. 1987. Protein serine/threonine kinases. Annu. Rev. Biochem. **56**: 567–613.

Gething, M.-J., and Sambrook, J. 1992. Protein folding in the cell. Nature (London), **355**, 33–45.

Helmerhorst, E., Stokes, G.B. 1980. Microcentrifuge desalting: a rapid, quantitative method for desalting small amounts of protein. Anal. Biochem. **104**: 130–135.

Herreid, C.F. 1977. Metabolism of land snails (*Otala latea*) during dormancy, arousal and activity. Comp. Biochem. Physiol. A, **56**: 211–215.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), **227**: 680–685.

Livingstone, D.R., and De Zwaan, A. 1983. Carbohydrate metabolism in gastropods. *In* The Mollusca. Vol. 1. *Edited by* K.M. Wilbur. Academic Press, New York. pp. 177–242.

Morre, D.J. 1971. Isolation of golgi apparatus. Methods Enzymol. **22**: 131–144.

Pipkin, J.L., Anson, J.F., Hinson, W.G., Burns, E.R., and Casciano, D.A. 1988. Comparative immunochemical and structural similarities of five stress proteins from various tissue types. Comp. Biochem. Physiol. B, **89**: 43–50.

Rees, B.B., and Hand, S.C. 1990. Heat dissipation, gas exchange and acid–base status in the land snail *Oreohelix* during short-term estivation. J. Exp. Biol. **152**: 77–92.

Shenolikar, S. 1988. Protein phosphorylation: hormones, drugs and bioregulation. FASEB J. **2**: 2753–2764.

Sottocasa, G.L., Kuylenstierna, B., Ernster, L., and Bergstrand, A. 1967. An electron transport system associated with the outer membrane of liver mitochondria. A biochemical and morphological study. J. Cell Biol. **32**: 415–438.

Storey, K.B., and Storey, J.M. 1990. Metabolic rate depression and biochemical adaptation in anaerobiosis, hibernation and estivation. Q. Rev. Biol. **65**: 145–174.

Strobel, H.W., and Dignam, J.D. 1978. Purification and properties of NADPH-cytochrome *c* P-450 reductase. Methods Enzymol. **52**: 89–96.

Thillart, G., van den, Lieshout, G., van, Storey, K.B., Cortesi, P., and Zwaan, A., de. 1992. Influences of long term hypoxia on the energy metabolism of the HB-containing bivalve *Scapharca inaequivalvis*: critical O_2 levels for metabolic depression. J. Comp. Physiol. B, **162**: 297–304.

Umezurike, G.M., and Iheanacho, E.N. 1983. Metabolic adaptations in the estivating giant African snail (*Achatina achatina*). Comp. Biochem. Physiol. B, **74**: 493–498.

Vesterberg, O. 1971. Isoelectrofocusing of protein. Methods Enzymol. **22**: 389–412.

Whitwam, R.E., and Storey, K.B. 1990. Pyruvate kinase from the land snail *Otala lactea*: regulation by reversible phosphorylation during estivation and anoxia. J. Exp. Biol. **154**: 321–337.

Whitwam, R.E., and Storey, K.B. 1991. Regulation of phosphofructokinase during estivation and anoxia in the land snail *Otala lactea*. Physiol. Zool. **64**: 595–610.

Zwaan, A., de, Cortesi, P., Thillart, G., van den, Roos. J., and Storey, K.B. 1991. Differential sensitivities to hypoxia by two anoxia tolerant marine molluscs: a biochemical analysis. Mar. Biol. (Berlin), **111**: 343–351.