The Journal of Experimental Biology 206, 1241-1249

doi:10.1242/jeb.00226

Metabolic enzyme activities across an altitudinal gradient: an examination of pikas (genus *Ochotona*)

Brandon A. Sheafor
Department of Biology, Mount Union College, Alliance, OH 44601, USA
e-mail: sheafobr@muc.edu

Accepted 13 January 2003

Summary

Changes in metabolic enzyme activities were examined in three species of pikas that occur over a range of altitudes. Because these closely related mammals live in comparable ecosystems and face similar environmental factors regardless of altitude, modifications of metabolic machinery are probably due to differences in oxygen availability. Citrate synthase (CS), β-hydroxyacyl CoA dehydrogenase (HOAD) and lactate dehydrogenase (LDH) activities were measured in heart, diaphragm, vastus lateralis, gastrocnemius and soleus muscles. Additionally, the activity levels of both M-LDH (skeletal muscle type) and H-LDH (heart type) isozymes were quantified in tissue samples. Pikas from high altitude had greater CS and HOAD activities in heart and diaphragm when compared with pikas from low altitude, while activity levels did not differ in skeletal muscles. The increase in oxidative enzyme activities in tissues with high metabolic demand is thought to enhance oxygen utilization when oxygen availability is low and may reflect greater metabolic demand on heart and diaphragm tissue. Pikas from high altitude were also found to have greater total LDH activities in all tissues examined. High altitude animals had dramatically higher H-LDH activity (2.3–3.8 times greater) while M-LDH activity was more comparable (1.8 times lower to 1.7 times greater) when compared with low altitude animals. High total LDH activity enables pikas to perform short bouts of anaerobic activity, while high levels of H-LDH isozymes may serve to enhance lactate removal and decrease recovery time in animals living at high altitude.

Key words: hypobaric hypoxia, citrate synthase, β-hydroxyacyl CoA dehydrogenase, lactate dehydrogenase isozymes, lactate removal, *Ochotona*, pika.

Introduction

The ability of organisms to maintain energy production in the absence of an adequate oxygen supply has been a heavily researched topic for the past few decades. Most studies have focused on how hypoxia affects metabolic processes and how ATP synthesis is able to continue despite reduced oxygen availability. One possible site of metabolic modification is at the level of metabolic enzyme activities. Alteration of activity levels can produce an adjustment of not only the rate and/or efficiency of a specific metabolic pathway but also regulation of the extent to which that pathway is utilized. Previous research has shown that metabolic enzyme activities can be modified by exposure to hypoxia, but which pathways are adjusted and the direction and magnitude of the changes differ depending on the experimental protocol used. Adjustment of metabolic enzyme activities may be of particular importance to mammals that inhabit high altitudes. Not only are these animals continuously subjected to low partial pressures of oxygen but they also have the added metabolic cost of maintaining body temperature while being exposed to extremely low ambient temperatures.

In general, one of two approaches has been taken in the examination of enzyme modification in response to low oxygen levels. The first approach has been to examine the effects of acute hypoxia on organisms that ordinarily encounter normoxic environments. Laboratory rats and humans are most commonly used in this type of experiment. Short-term exposure to low oxygen levels through either exercise (in rats: Constable et al., 1987; in humans: Holloszy, 1967; Baldwin et al., 1973; Terrados et al., 1990), normobaric hypoxia (in rats: Daneshrad et al., 2000; in humans: Desplanches et al., 1993) or hypobaric hypoxia (in humans: MacDougall et al., 1991; Hoppeler and Desplanches, 1992; Howald et al., 1990) has produced mixed results. Previous studies have demonstrated that activities of oxidative marker enzymes can be elevated (in humans: Holloszy, 1967; Desplanches et al., 1993; Terrados et al., 1990), decreased (in humans: Hoppeler and Desplanches, 1992; Howald et al., 1990) or show no change (in rats: Daneshrad et al., 2000; in humans: Holloszy, 1975) during the process of hypoxic acclimation. Activities of enzymes that catalyze glycolytic and anaerobic reactions have been shown to either decrease (in rats: Constable et al., 1987; in humans: Baldwin et al., 1973; Holloszy, 1975; Terrados et al., 1990) or

remain constant (in humans: Holloszy and Oscai, 1969; Howald et al., 1990) when animals are acclimated to hypoxia. Despite the inconclusive results of previous experiments, researchers have inferred that a modification of enzyme activity is inherently adaptive and, therefore, advantageous to organisms subjected to chronic hypoxia, such as animals living at high altitude (Holloszy, 1975; Pette and Dölken, 1975; MacDougall et al., 1991). However, it has not been demonstrated which, if any, of these alterations impart functional, long-term benefits to animals that are continually exposed to low oxygen levels. Furthermore, since the experimental subjects examined (e.g. laboratory rats or humans) are not known to have been exposed to chronic hypoxia at any time in their genetic history, there is no reason to expect that they have evolved the appropriate responses needed to cope with this phenomenon.

The second method for determining the effects of hypoxia on metabolic enzyme activity has been to investigate the biochemical makeup of animals that inhabit hypoxic environments, such as guinea pigs (Harris et al., 1970; Mensen de Silva and Cazorla, 1973; Barrie et al., 1975) or high altitude ungulates (Hochachka et al., 1982). It is assumed that animals exposed to a hypoxic environment over an evolutionary time frame have, through natural selection, developed and maintained characters that are beneficial to maintaining ATP synthesis despite continually limited oxygen availability. These studies have reported an elevated level of oxidative enzyme activity in all cases and a depressed (Mensen de Silva and Cazorla, 1973; Hochachka et al., 1982) or unchanged (Reynafarje, 1962) anaerobic enzyme activity level when compared with animals living at low altitude. However, the low altitude species used for comparison in these studies (e.g. laboratory rats or rabbits) do not share a close phylogenetic relationship with the high altitude animals to which they are compared.

The shortcomings associated with comparing two distantly related species and drawing adaptive conclusions have been well described by Garland and Adolph (1994). In short, the conclusions made in these studies assume that, were it not for the effects of the environmental parameter being analyzed, the two species would be physiologically identical. However, the genetic differentiation that occurs during speciation can affect many physiological traits. Which changes are due to a particular environmental factor and which are due to the countless other selective pressures that an organism encounters are unclear. Therefore, to demonstrate adaptation to hypoxia convincingly, a comparison among closely related species that have similar selective pressures but differ in oxygen availability is needed.

Pikas, small lagomorphs of the Family Ochotonidae, are ideal animals for the study of physiological adaptation to high altitude. In North America, these mammals are restricted to the rocky, talus fields of tundra/alpine ecosystems found above treeline. In the lower latitudes of their distribution, treeline occurs only at high altitude. However, as one moves towards the poles, this ecosystem exists at elevations as low as sea level. As pikas can be found throughout this latitudinal gradient, it is possible to examine their physiology over a range of altitudes. Environmental temperatures over this range are similar, which eliminates the confounding effects of differing ambient temperatures on metabolism (Greenland, 1989; Barry et al., 1981). As a result, it is possible to compare closely related species in environments that differ markedly in the amount of oxygen available but are comparable in other environmental parameters (Table 1).

Table 1. *Environmental, ecological and behavioral data for* Ochotona princeps *and* Ochotona collaris

	Ochotona princeps	*Ochotona collaris*
Altitude at which collected (m)	3350	1070
Approximate barometric pressure (kPa)	66.7	89.3
Mean annual temperature (°C)	–3.71±0.5[a]	–3.47±0.8[b]
Mean annual snowfall (mm)	930.0±54.2[a]	1764.8±77.5[b]
Home territory size (m^2)	720±15.7[c]	700±12.1[d]
Above ground activity ($h\,day^{-1}$)	4.1±0.8[c]	4.5±0.6[d]

References: [a]Greenland (1989); [b]Alaska Climate Research Center/ NOAA-CIRES Diagnostic Center (1990; http://www.cdc.noaa.gov/); [c]Smith and Ivins (1986); [d]Broadbooks (1965).

Pikas are extremely active foragers year-round and have high metabolic rates (MacArthur and Wang, 1973) with no form of metabolic reduction such as daily torpor, aestivation or hibernation (Krear, 1965; MacArthur and Wang, 1973). Thus, their demand for a high rate of ATP turnover is not decreased by metabolic reduction strategies. While most mammals at high altitude have adequate oxygen for sustaining resting metabolism, oxygen quantities are frequently not sufficient to support activity and exercise at a level comparable with that achieved at low altitude (West, 1982). High altitude pikas have a scope of activity comparable with their low altitude counterparts and other low altitude mammals (Table 1) and therefore must have some way to compensate for their hypoxic surroundings. Alterations in the activities of metabolic enzymes or in the proportions of metabolic isozymes are likely sites for this compensation and are explored in this paper. It is hoped that this information will present an insight into the crucial physiological modifications that allow an organism to maintain ATP synthesis in a continuously hypoxic environment.

Materials and methods

Animals

Three closely related species of pikas were examined in this study: *Ochotona princeps* (the North American pika), *Ochotona collaris* (the collared pika) and *Ochotona hyperborea* (a Russian species of pika). Early taxonomic analyses placed all three forms within one species, *Ochotona alpina* (Gureev, 1964; Corbet, 1978), while others gave *O. hyperborea* its own designation and considered *O. collaris* and

O. princeps to be the same species (Broadbooks, 1965; Youngman, 1975). Although current morphometric analyses have led to the splitting of *O. collaris* and *O. princeps* into two separate species (Hall, 1981; Weston, 1981), it is clear that all three species are closely related.

Ochotona princeps is the high altitude species investigated in this study. Eight adult *O. princeps* (mean body mass, 148.41 g; range, 145.9–170.7 g) were live-trapped using Tomahawk live traps baited with apples. All animals were trapped at the University of Colorado Mountain Research Station on Niwot Ridge, Colorado (40°02′ N, 105°35′ W; 3350 m). Nine adult *O. collaris* (mean body mass, 125.37 g; range, 113.6–130.8 g) were similarly live-trapped from Eagle Summit, Alaska (65°29′ N, 145°25′ W; 1070 m) and are considered to be low altitude animals in this study. Heart tissue from four adult *O. hyperborea* was obtained from the frozen tissue collection at the University of Alaska at Fairbanks. *O. hyperborea* were snap-trapped 18 km north of Magadan, Russia (59°45′ N, 150°53′ E; 0 m). Tissues were immediately removed from animals upon trapping and frozen in liquid nitrogen. In this study, *O. hyperborea* is designated a sea level species. All pikas were trapped between the months of June and August.

Tissue extraction

Five muscle tissues were examined in this study. Three skeletal muscles were chosen (vastus lateralis, gastrocnemius and soleus) in order to compare muscles with varying compositions of oxidative and glycolytic muscle fibers. Diaphragm and ventricular cardiac muscles were also investigated because they are intimately involved with oxygen delivery and are excellent indicators of an animal's overall metabolic status.

After capture, *O. princeps* and *O. collaris* were anesthetized on site with methoxyflurane and carried to a four-wheel drive vehicle outfitted to operate as a mobile laboratory. Pikas were then injected with pentobarbital intraperitoneally (50 mg kg^{-1}). As soon as the animal was fully under anesthesia, a 50–200 mg sample was taken of each tissue and frozen on dry ice. Identical procedures were performed in the laboratory on eight adult laboratory rabbits (*Oryctolagus cuniculus*). Rabbit data are used as an intrafamiliar outgroup control.

O. hyperborea cardiac muscle was removed upon trapping, placed in 2 ml cryovials and frozen in liquid nitrogen until it could be stored in a –70°C freezer at the University of Alaska at Fairbanks.

All tissues remained frozen until they were homogenised in 19 volumes (w/v) of homogenisation buffer (175 mmol l^{-1} KCl, 2 mmol l^{-1} EDTA, 10 mmol l^{-1} Tris, pH 7.0) using refrigerated ground glass tissue homogenisers. Between three and six separate aliquots of the 1/20 homogenate were placed into 0.7 ml Eppendorf tubes for each tissue. Homogenates were stored at –70°C until immediately before analysis of enzyme activities, when they were thawed and diluted with homogenisation buffer to the desired concentrations. Assays performed on fresh homogenates showed no difference in enzyme activity when compared with assays using frozen homogenates.

Protein concentrations were determined spectrophotometrically using a modification of the Lowry protein assay (Peterson, 1977). Bovine serum albumin was used as a standard and the 1/20 homogenate was used as the tissue source. Protein assays showed no significant difference in protein content between homologous tissues.

Enzyme activity

Enzyme activities were measured on a Gilford 260 spectrophotometer (Oberlin, OH, USA) that was kept at a constant temperature by a circulating water bath (Isotemp Model 900; Fisher Scientific, Pittsburgh, PA, USA). All reactions took place in pre-warmed, quartz cuvettes and had a final volume of 1 ml. Enzyme activities were measured at both 38°C (rabbit operating body temperature) and at 40°C (pika operating body temperature) for all species. Analysis of enzyme Q_{10} showed no differences among species. Therefore, comparisons were made at 40°C.

Citrate synthase (CS; EC 4.1.3.7) and β-hydroxyacyl CoA dehydrogenase (HOAD; EC 1.1.1.35) activities were measured using a final dilution of 1/1000 for heart and diaphragm tissues and 1/200 for skeletal muscles. For CS, the 1 ml reaction volume contained 0.3 mmol l^{-1} acetyl CoA, 0.1 mmol l^{-1} DTNB 5,5′-dithio-bis(2-nitrobenzoic acid), 100 mmol l^{-1} Tris-HCl buffer at pH 8.0 and 100 μl diluted homogenate. The reaction was initiated by adding 100 μl of 0.5 mmol l^{-1} oxaloacetate. Absorbance was measured at 412 nm (Srere, 1969). For HOAD, the final 1 ml reaction volume contained 0.5 mmol l^{-1} EDTA, 0.23 mmol l^{-1} NADH, 100 mmol l^{-1} triethanolamine-HCl buffer at pH 7.0 and 100 μl diluted homogenate. The reaction was started by the addition of 100 μl of 0.1 mmol l^{-1} acetoacyl-CoA. Absorbance was measured at 340 nm (Bass et al., 1969). Total lactate dehydrogenase activity (LDH; EC 1.1.1.27) was measured using a final dilution of 1/1000 for all tissues. The 1 ml reaction volume consisted of 0.25 mmol l^{-1} NADH, 100 mmol l^{-1} potassium phosphate buffer at pH 7.0 and 50 μl diluted homogenate. The addition of 100 μl of 10 mmol l^{-1} sodium pyruvate initiated the reaction. The disappearance of NADH was measured at 340 nm (Gleeson and Harrison, 1986).

This study assumes that, for homologous tissues, CS activity g^{-1} fresh tissue produces a measure of relative overall oxidative capacity, HOAD activity g^{-1} fresh tissue yields an estimation of relative fatty acid oxidative capacity, and LDH activity g^{-1} fresh tissue provides a measure of relative anaerobic metabolic capacity.

Isozyme analysis

LDH isozymes were separated by native polyacrylamide gel electrophoresis on a vertical gel electrophoresis system (Model V16, Bethesda Research Laboratories, Gaithersburg, MA, USA). Gels consisted of a separating gel of 7.5% acrylamide in 1.5 mol l^{-1} Tris-HCl (pH 8.5) and a stacking gel of 5% acrylamide in 0.5 mol l^{-1} Tris-HCl (pH 6.8). Homogenates

were diluted with a sample buffer of 62.5 mmol l^{-1} Tris-HCl, 10% glycerol and 0.002% bromophenol blue. A running buffer of 250 mmol l^{-1} Tris-HCl and 190 mmol l^{-1} glycine was used in all trials. Electrophoresis was carried out for 12 h at 4°C using a constant current power supply (Model 3-1500; Haake-Buchler, Saddleback, New Jersey) set at 15 mA. Gels were stained with a solution containing 0.33 mmol l^{-1} NAD^+, 0.10 mmol l^{-1} phenazine methosulfate, 0.27 mmol l^{-1} nitro blue tetrazolium, 25.0 mmol l^{-1} lactic acid and 20 mmol l^{-1} Tris-HCl (pH 8.2).

Quantification of relative isozyme activity followed the protocol of Klebe (1975). For each of the five muscle tissues examined, samples from three animals of each species were tested. Serial twofold dilutions of samples were electrophoretically separated in consecutive lanes and then stained until a visual endpoint was reached for each tetramer. The relative percentage of activity produced by a given tetramer was calculated by dividing the dilution factor of the last visible band by the sum of the dilution factors for all five tetramers. The contribution of each isozyme to the relative percentage of activity produced by a tetramer was calculated by multiplying the relative percentage by the proportion of each isozyme within the tetramer (e.g. M1:H3=0.25 M-type and 0.75 H-type). Values for isozyme activity were calculated by multiplying the total isozyme contribution by the total LDH activity.

Measurements of enzyme and isozyme activities were compared among species by simple one-way analysis of variance (ANOVA) and a *post-hoc* Scheffe's multiple range test. Statistical analyses were computed using SPSS statistical programs (SPSS Inc., Chicago, IL, USA).

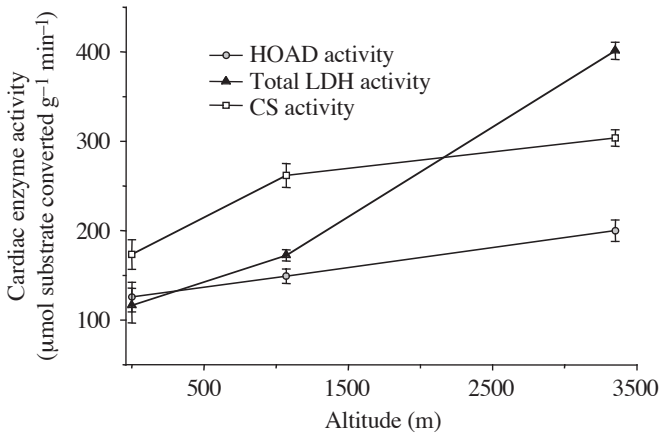

Fig. 1. Enzyme activities from ventricular cardiac muscle of pikas collected across an altitudinal gradient: Russian pikas (*Ochotona hyperborea*) residing at sea level (*N*=4), collared pikas (*Ochotona collaris*) residing at 1070 m (*N*=9) and North American pikas (*Ochotona princeps*) residing at 3350 m (*N*=8). Values are means ± S.E.M. CS, citrate synthase; HOAD, β-hydroxyacyl CoA dehydrogenase; LDH, lactate dehydrogenase.

Results

Enzyme activity

Enzyme activities for the three pika species, as well as for rabbit, are reported in Table 2. Citrate synthase activity is positively correlated with altitude in both heart and diaphragm tissues (Table 2). The high-altitude-adapted *O. princeps* had a cardiac CS activity level that was significantly higher than the low altitude *O. collaris* and the sea-level-adapted *O.*

Table 2. *Muscle enzyme activities from* Ochotona princeps *residing at 3350 m,* Ochotona collaris *residing at 1070 m,* Ochotona hyperborea *residing at sea level and laboratory rabbits (*Oryctolagus cuniculus*)*

	Heart	Diaphragm	Vastus	Gastrocnemius	Soleus
Citrate synthase					
O. princeps	303.71±9.17^{a}	160.16±7.23^{a}	50.00±2.69^{a}	52.16±4.60^{a}	60.68±6.19^{a}
O. collaris	261.69±13.30^{b}	123.42±1.98^{b}	46.84±3.40^{a}	46.71±2.28^{a}	55.91±2.10^{a}
O. hyperborea	173.36±16.62^{c}	–	–	–	–
Rabbit	131.27±3.86^{c}	53.48±2.88^{c}	22.33±1.86^{b}	23.85±3.49^{b}	42.07±2.56^{b}
β-hydroxyacyl CoA dehydrogenase					
O. princeps	199.98±11.99^{a}	97.49±3.99^{a}	22.58±1.04^{a}	27.97±1.23^{a}	42.42±2.80^{a}
O. collaris	149.09±8.02^{b}	70.78±3.22^{b}	31.11±1.12^{b}	31.35±2.46^{a}	36.46±2.02^{a}
O. hyperborea	125.71±16.61^{a,b}	–	–	–	–
Rabbit	216.94±9.91^{a}	62.95±3.46^{b}	24.06±1.93^{a,b}	29.86±3.58^{a}	53.10±3.36^{b}
Lactate dehydrogenase					
O. princeps	401.26±9.66^{a}	592.42±15.57^{a}	1011.43±6.11^{a}	1216.04±64.50^{a}	1145.15±85.77^{a}
O. collaris	172.40±6.35^{b}	289.15±13.63^{b}	590.01±13.29^{b}	586.22±20.63^{b}	505.68±21.76^{b}
O. hyperborea	116.29±19.35^{b}	–	–	–	–
Rabbit	151.05±8.05^{b}	523.63±6.81^{c}	1123.94±31.16^{c}	963.08±14.17^{c}	177.51±9.21^{c}

Values are means ± S.E.M., measured in μmol substrate converted g^{-1} wet tissue min^{-1}. Statistical analyses were performed to compare activities of each enzyme between species within each tissue. Values within each column which do not have the same superscript letter differ significantly (P<0.05) as determined by analysis of variance and Scheffe's multiple range test. N is 8 for *O. princeps* and rabbit, 9 for *O. collaris* and 4 for *O. hyperborea*.

hyperborea (Table 2; Fig. 1). Cardiac CS activity in *O. hyperborea* was not significantly different from rabbit. CS activity in the diaphragm of *O. princeps* was significantly higher than *O. collaris*, while both pika species showed significantly greater activity than did rabbit. There was no significant difference in CS activity of skeletal muscles between high and low altitude pikas, yet the pika values were consistently greater than those for rabbit.

β-hydroxyacyl CoA dehydrogenase activity also showed a positive relationship with altitude in cardiac tissue for the three species of pikas (Table 2; Fig. 1). HOAD activity in the cardiac and diaphragm tissue of *O. princeps* was significantly greater than that of *O. collaris*. As with citrate synthase, HOAD activity in skeletal muscle showed little correlation with altitude.

Within pika species, total lactate dehydrogenase activity showed a positive correlation to altitude in all tissues examined (Table 2; Fig. 1). All tissues showed a significant difference between high and low altitude pikas, with the high altitude animals having higher LDH activity. Within each pika species, LDH activity levels were similar across all skeletal muscles. Rabbits followed the expected mammalian pattern of high LDH activity in vastus lateralis, moderate activity in gastrocnemius and low activity in soleus muscle (Pagliassotti and Donovan, 1990).

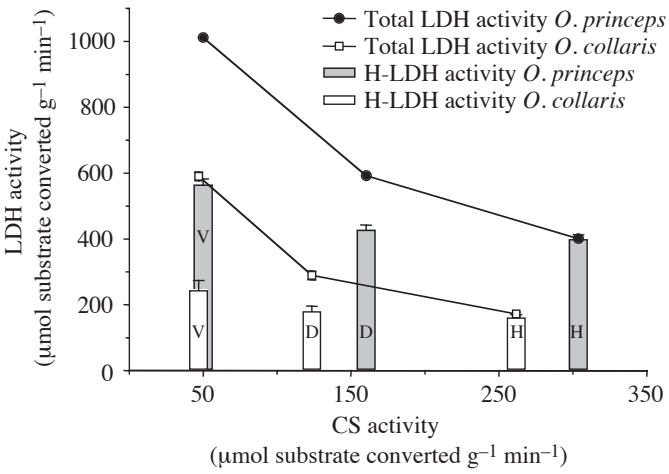

Fig. 2. Differences in metabolic enzyme and isozyme activities between pika species residing at high altitude (*Ochotona princeps* from 3350 m) and low altitude (*Ochotona collaris* from 1070 m). Activities are shown for three tissues: heart (H), diaphragm (D) and vastus lateralis (V). Values are means ± S.E.M. Error bars for total LDH activities are smaller than the symbols used. *N*=8 for *O. princeps* total LDH values, *N*=9 for *O. collaris* total LDH values. *N*=3 for all isozyme analyses. CS, citrate synthase; LDH, lactate dehydrogenase; H-LDH, heart-type lactate dehydrogenase.

Isozyme analysis

Lactate dehydrogenase isozyme activities for *O. princeps*, *O. collaris*, *O. hyperborea* and *O. cuniculus* are reported in Table 3. In all tissues, H-LDH activity was significantly greater in *O. princeps* when compared with the other species (Table 3; Fig. 2).

In heart muscle, *O. princeps* had H-LDH activity that was nearly 2.5 times greater than cardiac tissue values in any other species examined. Cardiac M-LDH activity for *O. princeps* was significantly lower than in other pika species. High-altitude-adapted pikas had diaphragm tissue values that were greater in both H-LDH and M-LDH activity when compared with both pikas and rabbits adapted to low altitude (Table 3; Fig. 2).

H-LDH activity in skeletal muscle was up to 3.8 times greater in *O. princeps* than in *O. collaris* and was significantly greater in all skeletal muscles examined. *O. princeps* had significantly higher H-LDH activity in all skeletal muscles when compared with rabbits. M-LDH activity in skeletal muscle was greater in *O. princeps* when compared with *O. collaris* in vastus lateralis and gastrocnemius, but there was no activity difference between pika species in soleus muscle

Table 3. *Lactate dehydrogenase isozyme activities from* Ochotona princeps *residing at 3350 m,* Ochotona collaris *residing at 1070 m,* Ochotona hyperborea *residing at sea level and laboratory rabbits (*Oryctolagus cuniculus*)*

	Heart	Diaphragm	Vastus	Gastrocnemius	Soleus
H-LDH activity					
O. princeps	399.85±13.27[a]	428.43±14.24[a]	565.57±17.15[a]	689.75±36.46[a]	886.24±46.33[a]
O. collaris	162.24±8.17[b]	181.65±14.33[b]	245.61±28.39[b]	245.55±17.35[b]	229.59±22.36[b]
O. hyperborea	109.31±14.08[c]	–	–	–	–
Rabbit	147.48±12.11[b]	407.96±9.85[c]	161.38±84.64[c]	422.54±11.94[c]	128.25±23.56[c]
M-LDH activity					
O. princeps	5.97±0.20[a]	157.62±15.86[a]	453.60±21.61[a]	537.29±32.73[a]	256.16±49.90[a]
O. collaris	10.54±0.53[b]	101.00±3.83[b]	373.94±46.67[b]	310.10±41.31[b]	273.06±44.59[a]
O. hyperborea	16.39±2.59[c]	–	–	–	–
Rabbit	4.02±0.54[d]	128.10±5.05[c]	959.69±130.09[c]	529.99±13.66[a]	40.57±4.56[b]

Values are means ± S.E.M., measured in μmol substrate converted g^{-1} wet tissue min^{-1}. Statistical analyses were performed to compare activities of each enzyme between species within each tissue. Values within each column which do not have the same superscript letter differ significantly ($P<0.05$) as determined by analysis of variance and Scheffe's multiple range test. *N*=3 for all species.

(Table 3). In all skeletal muscles examined, *O. princeps* had greater H-LDH activity than M-LDH activity within a muscle, while *O. collaris* had more M-LDH than H-LDH activity (Table 3; Fig. 2).

Discussion

Oxidative enzymes

For both heart and diaphragm tissues, oxidative metabolic enzyme activities are scaled upwards in response to high altitude (Table 2; Figs 1, 2). These findings are in agreement with enzymatic changes observed in humans acclimated to hypoxia (Barnard and Peter, 1971; Desplanches et al., 1993; Terrados et al., 1990), as well as to mammals that have evolved in hypoxic environments (Harris et al., 1970; Barrie et al., 1975; Hochachka et al., 1982). No apparent correlation was found between altitude and oxidative enzyme activities in skeletal muscle.

Efficient synthesis of ATP is critical in heart and diaphragm muscles that are continuously active. Therefore, oxidative rather than glycolytic pathways are favored, creating a high oxidative demand in these tissues. In order for an animal at high altitude to maintain adequate oxidative metabolism in these tissues, particularly during exercise, modifications in the ability of the tissues to utilize available oxygen are necessary to sustain function at a level comparable with that achieved under normoxic conditions. Skeletal muscle, on the other hand, can be rested and may rely to a greater extent on the less economical glycolytic pathway for a portion of its energy production. Due to the lower oxidative demands on skeletal muscle tissues, modification of oxygen utilization may not be imperative to maintain a reasonable scope of activity.

Citrate synthase activities provide a measure of relative overall oxidative capacity. The site of oxidative metabolism is within the mitochondria and, therefore, an increase in CS activity is considered to be an indication of an increase in mitochondrial density and/or mitochondrial size. A positive relationship between an increase in CS activity and mitochondrial density has been demonstrated during acclimation to hypoxia *via* exercise (Holloszy, 1967; Gollnick and King, 1969), normobaric hypoxia (Desplanches et al., 1993) and hypobaric hypoxia (Ou and Tenney, 1970). Under hypoxic conditions, a greater mitochondrial density is thought to be advantageous due to the increased probability that an oxygen molecule will come in contact with an oxidative enzyme site (i.e. cytochrome *c* oxidase) in a short time interval and, subsequently, increase the ability of the cell to utilize available oxygen (Ou and Tenney, 1970). Therefore, it is not surprising to find that tissues with a high oxidative demand, such as heart and diaphragm, show a correlation between altitudinal hypoxia and CS activity (Table 2; Figs 1, 2). By increasing CS activity and/or mitochondrial density in tissues with large oxidative demands, high altitude pikas allow the tissues to maintain aerobic metabolism at a sufficient level without a reliance on glycolytic means of ATP synthesis in tissues critical for oxygen transport. Skeletal muscle in pikas is not used for sustained activities but is used in short bursts during foraging forays and predator avoidance. These tissues have a low oxidative demand and can rely on glycolytic energy production in the face of low oxygen availability. Therefore, changes in skeletal muscle CS activity and mitochondrial density may not be obligatory in pikas found at altitude (Table 2; Fig. 2). An alternative explanation would be that high-altitude-adapted pikas increase ventilation and heart rate in order to maintain oxygen transport despite reduced oxygen availability. The increase of ventilation and cardiac output might lead to a training effect in heart and diaphragm, which would increase oxidative enzyme activity in these tissues.

HOAD activity indicates relative fatty acid oxidation capacity. While this is a gauge of oxidative metabolism, it does not give an indication of total oxidative capacity as does CS but measures only the portion derived from fat oxidation. Pikas feed on leaves and flowers from alpine/tundra plants and have low levels of fats in their natural diet (Dearing, 1997). Subsequently, they do not rely heavily on lipid metabolism, which is reflected in their relatively low HOAD activities when compared with laboratory animals (Table 2). However, among pika species, HOAD activity corresponds to altitude in the highly oxidative heart and diaphragm tissues but has no relationship to altitude in skeletal muscles (Table 2). This pattern is similar to that found in CS activity and further emphasizes an adaptive upward scaling of oxidative enzyme activity in animals at altitude, particularly in tissues that rely heavily on oxidative pathways.

Anaerobic function

One possible response that an animal exposed to hypoxia could develop in order to maintain cellular function is reliance upon anaerobic means of ATP synthesis. This strategy is employed by some bacteria (Gottschalk, 1979), yeast (Hochachka and Somero, 1984) and invertebrates (De Zwaan and Wijsman, 1976; Collicutt and Hochachka, 1977; Saz, 1981) that encounter hypoxic environments. However, anaerobic pathways are not extensively used in terrestrial vertebrates because fermentation by-products such as lactate can induce a decrease in cell function through pH imbalance and inhibition of glycolysis (Hochachka and Mommsen, 1983). Therefore, it is not surprising that increases in anaerobic capacity have not been demonstrated in mammals acclimated to hypoxic conditions (Constable et al., 1987; Howald et al., 1990; Terrados et al., 1990). It has been proposed that skeletal muscles exposed to hypoxia are modified to function more like heart tissue, confining ATP synthesis to oxidative pathways and limiting glycolytic energy production in order to minimize lactate accumulation (Baldwin et al., 1973; Holloszy, 1975).

Studies comparing mammals adapted to high altitude with lowland species have reported similar reductions in anaerobic capacities (Mensen de Silva and Cazorla, 1973; Hochachka et al., 1982, 1991). A downregulation of anaerobic metabolic capacity and the ensuing reduction in lactate accumulation in response to hypoxic conditions, dubbed the lactate paradox (West, 1986; Hochachka, 1988), have been observed in all high

altitude species examined thus far. The effect is thought to be caused by a more efficient coupling between ATP demand and ATP supply, allowing for a more effective integration between glycolysis and oxidative metabolism (Hochachka, 1988, 1994). However, pikas do not follow the lactate paradox pattern. Lactate dehydrogenase activity, an indicator of relative anaerobic metabolic capacity, shows a positive correlation with altitude in pikas rather than the negative relationship associated with the lactate paradox (Table 2; Fig. 2). This indicates that the lactate paradox is not necessarily a required modification for success at altitude.

The maintenance of a high anaerobic potential in pika tissues may be dictated by their ecology. Pikas are heavily preyed upon by a variety of animals and avoid predation by sprinting from foraging grounds to the safety of nearby rock piles (Broadbooks, 1965; Krear, 1965; Ivins and Smith, 1983). This makes survival dependent upon the ability to generate burst activity for short periods of time. The rapid ATP synthesis derived through glycolytic rather than oxidative means largely powers burst activity. Therefore, pikas may not be able to reduce their anaerobic capacity and still maintain the sprinting ability necessary for survival in their specific environment. In other words, it may be beneficial for a pika to contend with lactate build-up in order to avoid becoming a meal.

LDH isozyme activity

While predatory avoidance may explain why it is beneficial for all pikas to maintain a high anaerobic capacity, it does not explain why high altitude animals have a higher total LDH activity than do low altitude animals. Since burst activity is equally necessary at any altitude and is not dependent on oxygen availability, anaerobic capacities should be comparable among pika species. Yet, in all tissues examined, *O. princeps* have LDH activities that are over twofold higher than those of *O. collaris* (Table 2). The phenomenon appears to be explained by differences in LDH isozyme ratios between the two species.

H-LDH is the isozyme found predominantly in mammalian heart and liver tissue. It preferentially converts lactate to pyruvate, and high levels of H-LDH in cardiac tissue would allow lactate to be used as a metabolic substrate, which removes the possibility of cardiac muscle fatigue due to pH imbalances (Hochachka and Somero, 1984). M-LDH is found predominantly in mammalian skeletal muscle and preferentially converts pyruvate to lactate. High levels of M-LDH are necessary for the maintenance of redox balance within tissues anaerobically synthesizing ATP.

LDH isozyme patterns in *O. princeps* suggest that muscle tissues in high altitude pikas are behaving in a fashion similar to cardiac muscle. In all tissues examined, *O. princeps* had higher H-LDH activity than M-LDH activity. In *O. collaris*, H-LDH activity was only greater than M-LDH in highly oxidative heart and diaphragm. All skeletal muscles in *O. collaris* had higher M-LDH than H-LDH activity (Table 3).

Rabbit tissues follow more characteristically mammalian LDH isozyme patterns when compared with pikas (Pagliassotti and Donovan, 1990; Tables 2, 3). Highly oxidative tissue such as heart, diaphragm, and soleus have a high H-LDH to M-LDH ratio, while glycolytic tissue such as vastus has a great deal more M-LDH than H-LDH activity. The gastrocnemius muscle of the rabbit is composed of both oxidative and glycolytic fibers and, consequently, has similar H-LDH and M-LDH activities.

It has been proposed that the high H-LDH isozyme activities found in the skeletal muscles of animals such as hummingbirds function to reduce lactate accumulation during prolonged exercise (Hochachka et al., 1992). Because pika muscle is rarely involved in sustained activity, high levels of H-LDH are more likely to be a mechanism for accelerating the reconversion of acquired lactate after anaerobic bouts of activity rather than as a means of reducing accumulation of lactate in tissues. Hypoxia impedes the rate at which lactate can be oxidized and increases the time it takes animals at altitude to clear lactate from tissues where it has been produced (Hochachka and Mommsen, 1983).

An increase in skeletal muscle H-LDH subunits would facilitate lactate reconversion and, consequently, reduce the time that an animal would be required to remain inactive while lactate is eliminated. In normoxic mammals, the majority of accumulated lactate is thought to be removed through the lactate shuttle (Brooks, 1986). In this process, lactate is transported *via* the circulatory system from its production site to tissues with high respiratory rates where it is utilized as a metabolic substrate. This removal strategy may not be sufficient for pikas at altitude because, even at rest, tissues such as heart that typically utilize lactate as a substrate are hypoxically restricted and cannot contribute substantially to lactate removal. Skeletal muscle does not typically play a major role in lactate removal in mammals. For example, <7% of lactate in rat skeletal muscle has been shown to be oxidized *in situ* (McLane and Holloszy, 1979). Therefore, the high levels of H-LDH in pika skeletal muscle may facilitate lactate reconversion to pyruvate that can then be oxidized at the site of production. Excess pyruvate could also be converted to glucose through glyconeogenic pathways *in situ* (Guppy et al., 1979; McLane and Holloszy, 1979) and glyconeogenic capabilities should be further analyzed. Hepatic and renal conversion of lactate to glucose through the Cori cycle is a prevalent site of lactate removal in mammals and should be investigated in future experiments.

The findings presented in this paper allow for both general and specific conclusions to be drawn about the modification of metabolic enzyme activity in response to hypoxia. The upward scaling of oxidative metabolic enzyme activity appears to be a physiological response general to mammals in low oxygen environments. This shift is prominent in tissues that are highly aerobic and requires constant, steady ATP synthesis but may not be evident in all tissues. Increased oxidative capacities in tissues with high metabolic demands serve to facilitate oxygen utilization when oxygen availability is low. While a downregulation of anaerobic enzyme activity has been viewed as a common physiological response of

hypoxic mammals, this study demonstrates that, at least for pikas, the opposite is true. Pikas show a distinct positive correlation between total LDH activity and hypoxia. However, the majority of the increase in total activity is due to an increase in H-LDH isozyme, which may function to expedite the removal of lactate in muscle tissue and allow pikas to return to resting levels within a short time interval. Although this strategy for maintaining energy production in a hypoxic environment may be specific to pikas, it demonstrates that there are many ways in which metabolic processes can be modified in response to environmental stress.

The author wishes to thank Dr Gordon Jarrell, Dr Joseph Cook and Dr Chris Conroy at the University of Alaska at Fairbanks for assistance and for supplying *Ochotona hyperborea* tissue samples. Special thanks to Dr Gregory Snyder, Dr Jason Podrabsky, Kurt Raese, Dr Cheryl Plumb and Dr Todd Gleeson for technical assistance.

References

Baldwin, K. M., Winder, W. W., Terjung, R. L. and Holloszy, J. O. (1973). Glycolytic enzymes in different types of skeletal muscle: adaptation to exercise. *Am. J. Physiol.* **225**, 962-966.

Barnard, J. R. and Peter, J. B. (1971). Effects of exercise on skeletal muscle III. Cytochrome changes. *J. Appl. Physiol.* **31**, 904-908.

Barrie, E., Heath, D., Arias Stella, J. and Harris, P. (1975). Enzyme activities in red and white muscles of guinea-pigs and rabbits indigenous to high altitudes. *Environ. Physiol. Biochem.* **5**, 18-26.

Barry, R. G., Courtin, G. M. and Labine, C. (1981). Tundra climates. In *Tundra Ecosystems: A Comparative Analysis* (ed. L. C. Bliss, J. B. Cragg, D. W. Heal and J. J. Moore), pp. 81-114. Cambridge: Cambridge University Press.

Bass, A., Brdiczka, D., Eyer, P., Hofer, S. and Pette, D. (1969). Metabolic differentiation of distinct muscle types at the level of enzymatic organization. *Eur. J. Biochem.* **10**, 198-206.

Broadbooks, H. E. (1965). Ecology and distribution of the pikas of Washington and Alaska. *Am. Mid. Nat.* **73**, 299-335.

Brooks, G. A. (1986). Lactate production under fully aerobic conditions: the lactate shuttle during rest and exercise. *Fed. Proc.* **45**, 2924-2929.

Collicutt, J. M. and Hochachka, P. W. (1977). The anaerobic oyster heart: Coupling of glucose and aspartate fermentation. *J. Comp. Physiol.* **115**, 147-157.

Constable, S. H., Favier, R. J., McLane, J. A., Fell, R. D., Chen, M. and Holloszy, J. O. (1987). Energy metabolism in contracting rat skeletal muscle: adaptation to exercise training. *Am. J. Physiol.* **253**, C316-C322.

Corbet, G. B. (1978). *The Mammals of the Palaearctic Region: a Taxonomic Review*. London, Ithaca: British Museum (Natural History) and Cornell University Press.

Daneshrad, Z., Garcia-Riera, M. P., Verdys, M. and Rossi, A. (2000). Differential responses to chronic hypoxia and dietary restriction of aerobic capacity and enzyme levels in the rat myocardium. *Mol. Cell. Biochem.* **210**, 159-166.

Dearing, M. D. (1997). Effects of *Acomastylis rossii* tannins on a mammalian herbivore, the North American pika, *Ochotona princeps*. *Oecologia* **109**, 122-131.

Desplanches, D., Hoppeler, H., Linossier, M. T., Denis, C., Claassen, H., Dormois, D., Lacour, J. R. and Geyssant, A. (1993). Effects of training in normoxia and normobaric hypoxia on human muscle ultrastructure. *Pflügers Arch.* **425**, 263-267.

De Zwaan, A. and Wijsman, T. C. M. (1976). Anaerobic metabolism in Bivalvia (Mollusca). *Comp. Biochem. Physiol. B* **54**, 313-324.

Garland, T., Jr and Adolph, S. C. (1994). Why not to do two-species comparative studies: limitations on inferring adaptation. *Physiol. Zool.* **67**, 797-828.

Gleeson, T. T. and Harrison, J. M. (1986). Reptilian skeletal muscle: fiber-type composition and enzymatic profile in the lizard, *Iguana iguana*. *Copeia* **1986**, 324-332.

Gollnick, P. D. and King, D. W. (1969). Effect of exercise and training on mitochondria of rat skeletal muscle. *Am. J. Physiol.* **216**, 1502-1509.

Gottschalk, G. (1979). *Bacterial Metabolism*. New York: Springer-Verlag.

Greenland, D. (1989). The climate of Niwot Ridge, Front Range, Colorado, USA. *Arctic Alpine Res.* **21**, 380-391.

Guppy, M., Hulbert, W. C. and Hochachka, P. W. (1979). Metabolic sources of heat and power in tuna muscles. II. Enzyme and metabolite profiles. *J. Exp. Biol.* **82**, 303-320.

Gureev, A. A. (1964). *Lagomorpha. Fauna USSR, Mammals*. Moscow: Science Publishing House.

Hall, E. R. (1981). *The Mammals of North America*. Second edition. New York: John Wiley & Sons.

Harris, P., Castillo, Y., Gibson, K., Heath, D. and Arias-Stella, J. (1970). Succinic and lactic dehydrogenase activity in myocardial homogenates from animals at high and low altitude. *J. Mol. Cell. Cardiol.* **1**, 189-193.

Hochachka, P. W. (1988). The lactate paradox: analysis of underlying mechanisms. *Ann. Sports Med.* **4**, 184-189.

Hochachka, P. W. (1994). *Muscles as Molecular and Metabolic Machines*. Boca Raton: CRC Press.

Hochachka, P. W., Stanley, C., Merkt, J. and Sumar-Kalinowski, J. (1982). Metabolic meaning of elevated levels of oxidative enzymes in high altitude adapted animals: an interpretive hypothesis. *Respir. Physiol.* **52**, 303-313.

Hochachka, P. W. and Mommsen, T. P. (1983). Protons and anaerobiosis. *Science*. **219**, 1391-1397.

Hochachka, P. W. and Somero, G. N. (1984). *Biochemical Adaptation*. Princeton: Princeton University Press.

Hochachka, P. W., Stanley, C., Matheson, G. O., McKenzie, D. C., Allen, P. S. and Parkhouse, W. S. (1991). Metabolic and work efficiencies during exercise in Andean natives. *J. Appl. Physiol.* **70**, 1720-1730.

Hochachka, P. W., Stanley, C., McKenzie, D. C., Villena, A. and Monge, C. (1992). Enzyme mechanisms for pyruvate-to-lactate flux attenuation: study of Sherpas, Quechuas, and hummingbirds. *Int. J. Sports Med.* **13**, S119-S123.

Holloszy, J. O. (1967). Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. *J. Biol. Chem.* **242**, 2278-2282.

Holloszy, J. O. (1975). Adaptation of skeletal muscle to endurance exercise. *Med. Sci. Sports* **7**, 155-164.

Holloszy, J. O. and Oscai, L. B. (1969). Effect of exercise on α-glycerophosphate dehydrogenase activity in skeletal muscle. *Arch. Biochem. Biophys.* **130**, 653-656.

Hoppeler, H. and Desplanches, D. (1992). Muscle structural modifications in hypoxia. *Int J. Sports Med.* **13**, S166-S168.

Howald, H., Pette, D., Simoneau, J. A., Hoppeler, H. and Cerretelli, P. (1990). Effects of chronic hypoxia on muscle enzyme activities. *Int. J. Sports Med.* **11**, S10-S14.

Ivins, B. L. and Smith, A. T. (1983). Responses of pika (*Ochotona princeps*: Lagomorpha) to naturally occurring terrestrial predators. *Behav. Ecol. Sociobiol.* **13**, 277-287.

Klebe, R. J. (1975). A simple method for the quantification of isozyme patterns. *Biochem. Gen.* **13**, 805-812.

Krear, H. R. (1965). An ecological and ethological study of the pika (*Ochotona princeps saxatilis* Bangs) in the Front Range of Colorado. *Ph.D. Thesis*. Boulder: University of Colorado, Boulder.

MacArthur, R. A. and Wang, L. C. H. (1973). Physiology of thermoregulation in the pika, *Ochotona princeps*. *Can. J. Zool.* **51**, 11-16.

MacDougall, J. D., Green, H. J., Sutton, J. R., Coates, G., Cymerman, A., Young, P. and Houston, C. S. (1991). Operation Everest II: structural adaptations in skeletal muscle in response to extreme simulated altitude. *Acta Physiol. Scand.* **142**, 421-427.

McLane, J. A. and Holloszy, J. O. (1979). Glycogen synthesis from lactate in the three types of skeletal muscle. *J. Biol. Chem.* **254**, 6548-6553.

Mensen de Silva, E. and Cazorla, A. (1973). Lactate, α-GP, and Krebs cycle in sea-level and high-altitude native guinea pigs. *Am. J. Physiol.* **224**, 669-672.

Ou, L. C. and Tenney, S. M. (1970). Properties of mitochondria from hearts of cattle acclimatized to high altitude. *Respir. Physiol.* **8**, 151-159.

Pagliassotti, M. J. and Donovan, C. M. (1990). Role of cell type in net lactate removal by skeletal muscle. *Am. J. Physiol.* **258**, E635-E642.

Peterson, G. L. (1977). Amplification of the protein assay method of Lowry et al., which is more generally applicable. *Anal. Biochem.* **83**, 346-356.

Pette, D. and Dölken, G. (1975). Some aspects of regulation of enzyme levels in muscle energy-supplying metabolism. *Adv. Enz. Reg.* **13**, 355-375.

Reynafarje, B. (1962). Myoglobin content and enzymatic activity of muscle and altitude adaptation. *J. Appl. Physiol.* **17**, 301-305.

Saz, H. J. (1981). Energy metabolism of parasitic helminths. *Ann. Rev. Physiol.* **43**, 323-341.

Smith, A. T. and Ivins, B. L. (1986). Territorial intrusions by pikas (*Ochotona princeps*) as a function of occupant activity. *Anim. Behav.* **34**, 392-397.

Srere, P. A. (1969). Citrate synthase. *Methods Enzymol.* **13**, 3-5.

Terrados, N., Jansson, E., Sylvén, C. and Kaijser, L. (1990). Is hypoxia a stimulus for synthesis of oxidative enzymes and myoglobin? *J. Appl. Physiol.* **68**, 2369-2372.

West, J. B. (1982). Diffusion at high altitude. *Fed. Proc.* **41**, 2128-2130.

West, J. B. (1986). Lactate during exercise at extreme altitude. *Fed. Proc.* **45**, 2953-2957.

Weston, M. L. (1981). The *Ochotona alpina* complex: a statistical re-evaluation. In *Proceedings of the World Lagomorph Conference* (ed. K. Myers and C. D. MacInnes), pp. 73-89. Guelph, Ontario: Guelph University Press.

Youngman, P. M. (1975). *Mammals of the Yukon Territory*. Ottawa: National Museum of Natural Sciences, National Museums of Canada, Publications in Zoology.