Four kingdoms on glacier ice: convergent energetic processes boost energy levels as temperatures fall

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A diverse group of glacially obligate organisms coexist on temperate glaciers between Washington State and Alaska. A fundamental challenge for these and other cold-adapted species is the necessity to maintain an energy flux capable of sustaining life at low physiological temperatures. We show here that ice-adapted psychrophiles from four kingdoms (Animalia, Eubacteria, Fungi, Protista) respond to temperature fluctuations in a similar manner; namely, ATP levels and the total adenylate pool increase as temperatures fall (within their viable temperature limits, respectively), yet growth rate increases with temperature. By contrast, mesophilic representatives of each kingdom respond in an opposite manner (i.e. adenylates increase with temperature). These observations suggest that elevated adenylate levels in psychrophiles may offset inherent reductions in molecular diffusion at low physiological temperatures.

Keywords: psychrophile; adaptation; ATP; ice worm; convergent evolution

1. INTRODUCTION

The ice worm, *Mesenchytraeus solifugus* (kingdom Animalia), its bacterial microbiota (kingdom Eubacteria) and several algal (kingdom Protista) and fungal (kingdom Fungi) species coexist on temperate Alaskan glaciers. Particularly striking is the ability of these psychrophilic organisms to thrive in their extreme habitat: algal blooms cover the glacial surface during the summer and ice worms are observed at densities of up to 1000 m⁻² at some locations (Goodman 1971; Shain *et al.* 2000). A shared metabolic challenge for these and other psychrophiles is the necessity to generate sufficient levels of energy (e.g. ATP) to sustain life at low physiological temperatures, where biological processes cease to function in most organisms (Belehradek 1935; Willis 1987). This is particularly true for ice worms, the largest glacially obligate metazoan, which displays activity levels (e.g. motor and neural) at 0 °C comparable with their temperate counterparts living between 10–20 °C, for example, the earthworm (Goodman 1971; Shain *et al.* 2001).

We have demonstrated previously that ice worms respond to temperature change in an unusual manner; specifically, ATP levels spike and the total adenylate pool (i.e. AMP, ADP, ATP) increases as temperatures drop, a

response opposite to that observed in mesophilic annelids (Napolitano *et al.* 2004). In this study, our aim was to examine the energetic response of other psychrophiles and their mesophilic counterparts in an effort to uncover a possible underlying bioenergetic strategy of cold adaptation. Our results indicate that disparate psychrophiles (e.g. algae, bacteria, fungi) raise adenylate levels as temperatures fall, in what appears to be a compensatory mechanism that offsets inherent reductions in molecular diffusion associated with cold temperature.

2. MATERIAL AND METHODS

(a) Specimen culture and identification

Psychrophilic bacterial strains *Psp1*, 2 (*Pseudomonas* sp.) were obtained by dissociating ice worms between two glass slides and plating the contents on luria broth agar at 0 °C for approximately one week. Psychrophilic algae and fungi were isolated by plating snow or ice from Byron Glacier, AK on carbon-free (culture collection of marine phytoplankton DY-V) and yeast extract peptone dextrose medium, respectively; plates were maintained in an illuminated growth chamber at 0 °C for approximately one month. Ice worms were collected and maintained as described (Shain *et al.* 2000). The antarctic algal strain *Cdsp* (*Chlamydomonas* sp.) was purchased from CCMP (no. 1619 from Lake Bonney, Antarctica). Byron Glacier microbiota were identified to the genus level by rRNA sequencing (Accugenix, Newark, DE, USA). Mesophilic species *Chlamydomonas moerussi* (Carolina Biological), *Escherichia coli* (strain HB101), *Lumbriculus variegatus* (BugFarm) and *Saccharomyces cerevisiae* (Fisher) were cultured by standard procedures.

(b) Adenylate measurements

Specimens were weighed to the nearest 0.1 mg. Adenylates were extracted rapidly by homogenization in 200 µl of cold 7% perchloric acid and neutralized to *ca.* pH 7 with a solution containing 3 M KOH, 0.4 M Tris and 3 M KCl (*ca.* 40 µl), as described in Lowry & Passonneau (1972). AMP and ADP were converted to ATP with myokinase/pyruvate kinase (Sigma), as described in Adam (1963). For ATP levels, specimens were deproteinized in 50 mM of HEPES, pH 7.4 (170 µl) containing 50 mAU ml⁻¹ of proteinase K (Novagen) at 50 °C for 30 min (this procedure generated comparable results in side-by-side comparisons with perchloric acid extractions, but proved more reproducible when using milligram quantities of the starting material). Total adenylates were obtained by enzymatic conversion of AMP and ADP, as described above. All samples were coupled with luciferin–luciferase (Cal-Biochem) assays as described in Lowry & Passonneau (1972) and monitored with a Beta-Scout luminometer (Perkin-Elmer).

(c) Growth rate

Specimens grown at appropriate temperatures were monitored for changes in optical density at specified time intervals using an ultraviolet/visible spectrophotometer (Abs₅₅₀ and Abs₆₀₀; Beckman).

(d) Statistical analysis

Data points are reported as the mean ± s.e.m. At least three independent measurements were obtained for each sample, comprising between 1 and 3 mg of tissue or cells (e.g. bacterial, algal, fungal) per data point. ANOVA analysis and Student's *t*-tests were conducted where appropriate.

3. RESULTS

ATP levels were monitored as a function of temperature change among psychrophiles (ice-adapted algae, bacteria, fungi, worms) and also in mesophilic representatives of five kingdoms (Animalia, Eubacteria, Fungi, Plantae, Protista). Among the mesophiles, ATP levels increased invariably with temperature gain (figure 1a). By contrast, all psychrophilic species elevated ATP levels as physiological temperatures were lowered. It is noteworthy that psychrophiles maintained ATP at levels comparable with their mesophilic counterparts at normal physiological temperatures, respectively, but a clear disparity was observed at lower temperatures where psychrophilic ATP levels

Figure 1. Energetic distinctions between psychrophilic and mesophilic organisms. (a) Psychrophiles (blue, ice adapted (Alaska); green, ice adapted (Antarctica)) elevated ATP levels as temperatures declined, while mesophiles (red, temperate) elevated ATP levels as temperatures increased ($p < 0.05$). Each species was acclimatized under growth conditions for *ca.* 24 h or longer at respective temperatures before measuring [ATP]. Representative slopes are shown based on at least three data points within the viable temperature range of each species, including the temperature most frequently encountered by each species (e.g. *Escherichia coli*: 37 °C; lines with only two data points were truncated to avoid overlap). (b) Cold shock rapidly changed ATP levels in psychrophiles and mesophiles, but in opposite directions ($p < 0.05$). *Cdsp* equilibrated at 12 °C elevated ATP levels *ca.* 66% after 2 h at 0 °C (12 °C $\rightarrow$ 0 °C; +66%). Similar gains were observed in other psychrophiles: *Rn* (12 °C $\rightarrow$ 0 °C; +46%); *Psp1* (12 °C $\rightarrow$ 0 °C; +25%); *Ms* (0 °C $\rightarrow$ -6 °C; +32%). ATP levels dropped in mesophiles upon cold shock: *Lv* (22 °C $\rightarrow$ 0 °C; -42%); *Ec* (37 °C $\rightarrow$ 0 °C; -49%); *Cm* (22 °C $\rightarrow$ 0 °C; -25%). (c) Growth rates increased upon temperature gain (despite decreasing ATP levels in psychrophiles) within the viable range of all species examined ($p < 0.01$). *Cm*, *Chlamydomonas moewussi* (Protista); *Cdsp*, *Chlamydomonas* sp. (Protista; CCMP 1619 from Lake Bonney, Antarctica); *Crsp*, *Cryptococcus* sp. (Fungi); *Ec*, *E. coli* (strain HB101; Eubacteria); *Gm*, *Geranium maculatum* (Plantae); *Lv*, *Lumbriculus variegatus* (Animalia); *Ms*, *Mesenchytraeus solifugus* (Animalia); *Rn*, *Raphidonema nivale* (Protista); *Sc*, *Saccharomyces cerevisiae* (Fungi); *Psp1*, *Psp2*, *Pseudomonas* sp. 1, 2 (Eubacteria).

were consistently higher. This was particularly evident among ice worms, which displayed ATP levels between 2–12-fold higher than all of the mesophiles examined, and considerably higher than other psychrophiles (figure 1a). It is important to note, however, that several mammalian cell types (e.g. hepatocytes, muscle) maintain ATP levels well above that observed in ice worms (i.e. 2–10 mM; Storey & Kelly 1995; Fedorow *et al.* 1998); comparable levels were corroborated here with mouse liver and muscle (3.0 ± 0.443 and 2.9 ± 0.587 nmol ATP mg⁻¹,

respectively). Nevertheless, ATP levels decline with temperature in mammalian cells (Fedorow *et al.* 1998; English & Storey 2000), as observed in mesophilic taxa (figure 1a).

Strikingly, ATP levels spiked in all psychrophiles (*ca.* 25–65%) upon cold shock but fell (*ca.* 25–50%) in mesophilic species (figure 1b), identifying rapid physiological responses to cold temperature in both classes of organisms, but in opposite directions. Growth rate, however, increased with temperature in all species examined (within

Figure 2. Adenylate profiles of Alaskan psychrophilic versus mesophilic species. (a) Algae, (b) Fungi, (c) Eubacteria and (d) Animalia (some ice worm data are included for comparison; Napolitano *et al.* 2004). Acclimatized temperature points are shown in the centre and left side of each panel; shaded regions represent a 120 min cold shock at indicated temperatures, respectively. Psychrophiles (bottom of each panel) elevated TAN levels as temperatures fell (left side, white) and elevated AEC upon cold shock (right side, shaded) ($p < 0.05$); the opposite trend was observed in counterpart, mesophilic species, respectively (top of each panel) ($p < 0.05$).

their viable temperature ranges, respectively), including psychrophilic algae, bacteria and fungi (figure 1c).

An examination of adenylate ratios as a function of temperature change revealed that the adenylate energy charge (AEC) (a measure of energy state defined by the equation $AEC = [ATP] + 1/2[ADP]/[ATP] + [ADP] + [AMP]$; Atkinson (1968)) rose sharply upon a temperature drop among psychrophiles but fell in mesophiles (figure 2). After

an acclimatization period of *ca.* 24 h, corresponding gains were also observed in [ADP] and [AMP] generating a net increase in total adenylate nucleotides (TAN) among psychrophiles, and a net decrease among mesophiles (figure 2).

4. DISCUSSION

Adaptations to cold temperature occur at multiple levels including changes in membrane composition (Nichols *et*

al. 1995; Russell 1997), metabolism (Pörtner & Playle 1998; Peck 2002) and modifications in protein structure or function (Marshall 1997; Smalas *et al.* 2000; D'Amico *et al.* 2002). The rapid, temperature-dependent changes in adenylate levels observed in this study (e.g. greater than 40% gains in ATP levels upon cold shock in some psychrophiles) suggest fundamental differences in energy metabolism between psychrophilic and mesophilic species. Importantly, respiratory rates generally decrease with temperature in organisms that have been examined, including psychrophiles (e.g. Goodman 1971; Pörtner *et al.* 1999; Salomon & Buchholz 2000; Peck 2002), yet adenylate levels rose as temperatures fell in all psychrophiles examined here. This is interpreted as evidence that components of ATP synthetic machinery (e.g. F_1F_0 ATP synthase, glycolytic enzymes) in psychrophiles may be well adapted to function at a low temperature, either as a result of structural modifications or changes in the cellular environment, for example pH (Pörtner *et al.* 1999). Thus, gains in ATP appear to result when relative rates of ATP synthesis exceed that of ATP consumption, even though actual rates of ATP production are likely to decline (i.e. in parallel with declining respiratory rates). These observations are perplexing in light of temperature-dependent growth rates observed in psychrophilic algae, bacteria and fungi (which grow faster as temperatures increase), as observed in other psychrophiles (e.g. Bosch *et al.* 1987; Stanwell-Smith & Peck 1998). Clearly, cell growth is correlated with an overall increase in energy demand, yet the psychrophiles examined here paradoxically increased adenylate levels at lower temperatures (i.e. slower growth rates) within their viable temperature ranges, respectively.

One consequence of increasing the adenylate pool size is the potential to maintain biochemical processes at viable levels under conditions of reduced molecular diffusion (e.g. low physiological temperature). By raising TAN levels, psychrophiles may compensate for reductions in diffusion by increasing the number of molecules (i.e. adenylate nucleotides) available to drive biochemical reactions. In principle, this strategy could offset, at least in part, the inherent lethargy usually associated with cold temperature. It is noteworthy that ice worms maintain relatively high ATP levels in comparison with other psychrophilic species (see figure 1a) and are particularly active on glacier ice (Goodman 1971; Shain *et al.* 2001).

That psychrophiles from four kingdoms respond similarly to temperature loss (i.e. all raise adenylate levels) indicates that this response has arisen multiple times over the course of evolution. Because a similar response was observed in an Antarctic algal species, this putative adaptation is clearly not restricted to psychrophiles residing on Alaskan glaciers; rather, it may represent an energetic signature of other psychrophilic species. The evolutionary significance of these convergent energetic processes is two-fold. First, the molecular mechanism(s) underlying this response is probably the result of relatively few (and perhaps only one) evolutionary step(s), consistent with theorems of punctuated equilibrium (Gould & Eldredge 1993). Second, the ability to generate high [ATP] at low physiological temperature appears to represent an

important component of cold-temperature adaptation that crosses biological kingdoms.

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- Adam, H. 1963 Adenosine-5'-diphosphate and adenosine-5'-monophosphate. In *Methods of enzymatic analysis* (ed. H. U. Bergmeyer), pp. 573–577. New York: Academic.
- Atkinson, D. E. 1968 The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. *Biochemistry* **11**, 4030–4034.
- Belehradek, J. 1935 *Temperature and living matter*. Berlin: Gebrüder Borntraeger.
- Bosch, I., Beauchamp, K. A., Steele, M. E. & Pearse, J. S. 1987 Development, metamorphosis and seasonal abundance of embryos and larvae of the Antarctic sea urchin *Sterechinus neumayeri*. *Biol. Bull.* **173**, 126–135.
- D'Amico, S., Claverie, P., Collins, T., Georlette, D., Gratia, E., Hoyoux, A., Meuwis, M.-A., Feller, G. & Gerday, C. 2002 Molecular basis of cold adaptation. *Phil. Trans. R. Soc. Lond. B* **357**, 917–925. (DOI 10.1098/rstb.2002.1105.)
- English, T. E. & Storey, K. B. 2000 Enzymes of adenylate metabolism and their role in hibernation of the white-tailed prairie dog, *Cynomys leucurus*. *Arch. Biochem. Biophys.* **376**, 91–100.
- Fedorow, C. A., Churchill, T. A. & Kneteman, N. M. 1998 Effects of hypothermic hypoxia on anaerobic energy metabolism in isolated anuran livers. *J. Comp. Physiol. B* **168**, 555–561.
- Goodman, D. 1971 Ecological investigations of ice worms on Caseiment Glacier, Southeast Alaska. Ohio State University Research Foundation, Institute of Polar Studies, report 39, pp. 1–59.
- Gould, S. J. & Eldredge, N. 1993 Punctuated equilibrium comes of age. *Nature* **366**, 223–227.
- Lowry, O. H. & Passonneau, J. V. 1972 *A flexible system of enzymatic analysis*. New York: Academic.
- Marshall, C. J. 1997 Cold-adapted enzymes. *Trends Biotechnol.* **15**, 359–364.
- Napolitano, M. J., Nagele, R. O. & Shain, D. H. 2004 Ice worms elevate adenylate levels at low physiological temperature. *Comp. Biochem. Physiol. A* **137**, 227–235.
- Nichols, D. S., Nichols, P. D., Russell, N. J., Davies, N. W. & McMeekin, T. A. 1995 Polyunsaturated fatty acids in the psychrophilic bacterium *Shewanella gelidimarina* ACAM 456T: molecular species analysis of major phospholipids and biosynthesis of eicosapentaenoic acid. *Biochim. Biophys. Acta* **1347**, 164–176.
- Peck, L. S. 2002 Ecophysiology of Antarctic marine ectotherms: limits to life. *Polar Biol.* **25**, 31–40.
- Pörtner, H. O. & Playle, R. C. 1998 *Cold ocean physiology*. Cambridge University Press.
- Pörtner, H. O., Peck, L. S., Zielinski, S. & Conway, L. Z. 1999 Intracellular pH and energy metabolism in the highly stenothermal Antarctic bivalve *Limopsis marionensis* as a function of ambient temperature. *Polar Biol.* **22**, 17–30.
- Russell, N. J. 1997 Psychrophilic bacteria: molecular adaptations of membrane lipids. *Comp. Biochem. Physiol. A* **118**, 489–493.
- Salomon, M. & Buchholz, F. 2000 Effects of temperature on the respiration rates and the kinetics of citrate synthase in two species of Idotea (Isopoda, Crustacea). *Comp. Biochem. Physiol. B* **125**, 71–81.
- Shain, D. H., Carter, M. R., Murray, K. P., Maleski, K. A., Smith, N. R., McBride, T. R., Michalewicz, L. A. & Saidel, W. M. 2000 Morphologic characterization of the ice worm *Mesenchytraeus solifugus*. *J. Morphol.* **246**, 192–197.
- Shain, D. H., Farrell, A., Mason, T. & Michalewicz, L. A. 2001 Distribution and behavior of ice worms (*Mesenchytraeus solifugus*) in south-central Alaska. *Can. J. Zool.* **79**, 1813–1821.
- Smalas, A. O., Leiros, H. S., Os, V. & Willassen, N. P. 2000 Cold adapted enzymes. *Biotech. A. Rev.* **6**, 1–57.
- Stanwell-Smith, D. P. & Peck, L. S. 1998 Temperature and embryonic development in relation to spawning and field occurrence of larvae of three Antarctic echinoderms. *Biol. Bull.* **194**, 44–52.
- Storey, K. B. & Kelly, D. A. 1995 Glycolysis and energetics in organs of hibernating mice (*Zapus hudsonius*). *Can. J. Zool.* **73**, 202–207.
- Willis, J. S. 1987 Cold tolerance in mammalian cells. In *Temperature and animal cells* (ed. K. Bowler & B. J. Fuller), pp. 285–309. Southampton, Hants, UK: The Company of Biologists Ltd, The Society for Experimental Biology.