Xanthine Oxidase and Xanthine Dehydrogenase from an Estivating Land Snail

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During arousal from estivation in land snails, *Otala lactea*, active metabolic functions are restored within minutes and oxygen consumption increases dramatically. During the transition from the hypoxic conditions of estivation to normoxia it is possible that xanthine oxidase (XO) in hepatopancreas contributes to the observed lipid peroxidation. Using a fluorometric assay that is based on the oxidation of pterin, the activities and some properties of XO and XO+XDH (sum of XO and xanthine dehydrogenase activities) were measured in hepatopancreas extracts. K_m values for pterin for XO and XO+XDH were 9 and 6 μM, respectively, and the K_m of XDH for methylene blue was 5 μM. Both XO+XDH and XO activities were inhibited by allopurinol (I_{50} = 2 μM), pre-incubation at 40 °C, and by 5 min H_2O_2 pre-exposure. Inclusion of azide in the reaction promoted a rise of approximately 70-fold in the inactivation power of H_2O_2 due to inhibition of high endogenous catalase activity. The I_{50} for H_2O_2 of XO+XDH and XO activities in the presence of azide was 0.04 and 0.11 mM, respectively. Unlike the situation for mammalian XO, a previous reduction of *O. lactea* XO (by pterin) was not necessary to make the enzyme susceptible to H_2O_2 effects. Interestingly, methylene blue partially prevented both heat- and H_2O_2-induced inactivation of XO+XDH activity. These data indicate that the formation of an enzyme-methylene blue complex induces protection against heat and oxidative damage at the FAD-active site. Both XO and XO+XDH activities were significantly higher in snails after 35 days of estivation compared with active snails 24 h after arousal from dormancy. The ratio of XO/(XO+XDH) activities was also slightly increased in estivating *O. lactea* (from 0.07 to 0.09; $P < 0.025$). XO activity was 0.03 nmol · min^{-1} · mg protein^{-1} in estivating snails. Compared with hepatopancreas catalase, XO activity is probably too low to contribute significantly to the net generation of oxyradicals, and hence to peroxidative damage. Rather, the low potential of XO to induce oxidative stress may constitute an adaptive advantage for *O. lactea* during arousal periods.

Introduction

Many species of land snails, including *Otala lactea* which is native to the area around the Mediterranean sea, enter a state of reduced metabolism known as estivation in order to endure the dry months of the year (Schmidt-Neilsen *et al.*, 1971; Barnhart and McMahon, 1987). During estivation water loss and heart rate are reduced, oxygen consumption falls to about 30% of the resting value while active, and activities of key enzymes of intermediary metabolism are reduced by means of reversible phosphorylation (e.g. glycogen phosphorylase, phosphofructokinase, pyruvate kinase, and pyruvate dehydrogenase) (Herreid, 1977; Whitwam and Storey, 1990, 1991; Brooks and Storey, 1990, 1992; Storey, 1993). Estivation can be rapidly broken by the reintroduction of water into the environment. Snails arouse within minutes, showing an abrupt increase in the rate of oxygen consumption and a quick recovery of active metabolic functions (Herreid, 1977; Whitwam and Storey, 1990). We have recently observed that the activities of several antioxidant enzymes in snails were significantly increased after 30 days of estivation (Hermes-Lima and Storey, 1995); included are glutathione peroxidase in hepatopancreas, catalase in foot muscle, and superoxide dismutase in both organs. In addition, lipid peroxidation increased significantly in hepatopancreas at the onset of arousal from 30 days of estivation (Hermes-Lima

Abbreviations: EDTA, ethylenediaminetetra-acetic acid; O_2^-, superoxide radical; TBARS, thiobarbituric acid reactive substances; XDH; xanthine dehydrogenase; XO, xanthine oxidase; IXPT, isoxanthopterin.

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and Storey, 1995). These results suggested that during estivation snails undergo adaptive changes that will prepare them to deal with a "physiological oxidative stress" (see Barja de Quiroga, 1992) occurring during the arousal process (Hermes-Lima and Storey, 1995).

The source of the oxygen radicals that promote lipoperoxidation in arousing snails remains unclear. A putative site for the formation of oxygen radicals is the molybdenum-dependent enzyme involved in purine catabolism, xanthine oxidase (XO). Xanthine oxidase generates superoxide radicals (O_2^-) and H_2O_2 during the oxidation of xanthine or hypoxanthine (McCord, 1985; Parks and Granger, 1986; Traystman *et al.*, 1991; Greene and Paller, 1992). It has been demonstrated that XO plays an important role in post-hypoxic injury to mammalian tissues (Patt *et al.*, 1988; Greene and Paller, 1992; Terada *et al.*, 1992; Rangan and Bulkley, 1993). Since purine metabolism is a key part of nitrogen excretion in *O. lactea* (Lee and Campbell, 1965; Speeg and Campbell, 1968), we suspected that XO and xanthine dehydrogenase (XDH) would be active in arousing animals. In fact, the presence of XDH or XO activities has been reported for most prosobranch, pulmonate and bivalve molluscs (Krenitsky *et al.*, 1974; Bishop *et al.*, 1983; Dykens and Shick, 1988; Ramesh *et al.*, 1990). Moreover, taking into consideration that the process of arousal is a transition from hypoxia (P_{O_2} is low in tissues during estivation; Barnhart, 1986a) to normoxia, the presence of XO (and/or conversion of XDH into XO) could set up a potential risk of oxidative damage.

Dykens and Shick (1988) have proposed that XO could exert oxidative stress in marine bivalves over the course of the tidal cycle. According to their proposal, molluscs presenting poor tolerance to anoxia show conversion of XDH into XO and notable adenylate degradation causing accumulation of XO substrates (xanthine and hypoxanthine) during aerial exposure. These animals would then be susceptible to oxygen reperfusion injury during reimmersion at high tides.

The present study analyzes the kinetic properties of XO and XDH from *O. lactea* hepatopancreas using pterin as a substrate, which allows for a very sensitive fluorometric quantification of the activities (Beckman *et al.*, 1989). The effects of H_2O_2 on both XO and XDH activities were analyzed since H_2O_2 is a product of the XO-catalyzed reaction and could induce XO self-inactivation (Terada *et al.*, 1991), as well as XDH damage. Maximal activities of XO and XDH were also quantified in snails after 35 days of estivation versus 24 h after arousal.

Materials and Methods

Chemicals

Allopurinol, bovine milk xanthine oxidase, EDTA, isoxanthopterin, pterin, methylene blue, phenylmethylsulfonyl fluoride, and Sephadex G-25 were purchased from Sigma Chemical Co. All other reagents were of analytical grade.

Animals

Land snails *O. lactea* were obtained from a commercial supplier in Ottawa, Canada. Animals were held in the laboratory at 22 °C in covered plastic containers lined with paper towels. Every 30–40 days animals were sprayed with dechlorinated water to induce arousal, then fed cabbage sprinkled with ground chalk; animals were then allowed to reenter dormancy over the next several days. For experimental sampling, dormant snails were sampled after 35 days of continuous dormancy as described above. At the same time another group of animals was sprayed with water, aroused and fed. After 24 h in the active state, this group of aroused snails was sampled. For sampling purposes, snails were killed by breaking the shell and organs (foot and hepatopancreas) were quickly dissected out, frozen in liquid nitrogen, and then transferred to −75 °C for storage.

Preparation of tissue extracts for enzyme assays

Frozen tissue samples were quickly weighed and immediately homogenized (1:10 w/v) in cold 50 mM potassium phosphate buffer, pH 7.0 containing 0.5 mM EDTA and with a few added crystals of phenylmethylsulfonyl fluoride. Homogenates were centrifuged at 5 °C and 25,000×*g* for 25 min. Supernatants were removed and 0.5 ml aliquots were filtered through 5 ml columns of Sephadex G-25 (with centrifugation for 1 min in a benchtop centrifuge (Helmerhorst and Stokes, 1980) to remove endogenous low molecular weight inhibitors. Extracts were then stored on ice

and used for experiments. Hepatopancreas XDH and XO activities were 40–60% and almost completely inhibited, respectively, in supernatants that were not desalted by G-25 filtration. Initial attempts were made to partially purify XO and XO+XDH using DE-52 ion exchange chromatographys (elution with a salt gradient). Both enzymes eluted in single peaks but activities were unstable, with a 50% loss of activity in only 1 day at 4 °C; hence, these were unsuitable for use.

Measurement of the activities of XO and XDH

Activities were followed by the fluorometric assay of Beckman *et al.* (1989) based on the XO/XDH-catalyzed conversion of pterin to isoxanthopterin. Assays were monitored with 345 nm excitation and 390 nm emission wavelength on a Perkin Elmer LS-50 fluorometer. In the standard assay procedure, 50 µl of enzyme extract was added to 50 mM potassium phosphate (pH 7.0) buffer containing 0.5 mM EDTA (at 25 °C). Then 10 µl of 2 mM pterin was added to record the XO activity. After 3–4 minutes, 10 µl of 1 mM methylene blue was added in order to follow the sum of XO and XDH activities (XO+XDH activity). Methylene blue was used to replace NAD^+ as the final electron acceptor for XDH. After recording the XO+XDH activity the reaction was stopped by addition of 10 µl of 4 mM allopurinol, followed by addition of 2 µl of 0.02 mM isoxanthopterin (final volume of reaction: 1.01 ml) in order to calibrate the fluorometric assay.

In order to control for accidental conversion of XDH to XO during homogenization due to sulfhydryl redox changes we tested the effect of dithiothreitol (5 mM) addition. There were no significant differences in either XO or XO+XDH activities from samples (from hepatopancreas of estivating snails) prepared with or without the addition of dithiothreitol in the homogenization buffer. These results indicated that the measured XO activity in homogenates represented an active XO in *O. lactea* hepatopancreas *in vivo*.

Analysis of data

All results were computed as mean ± S.E. of 2–4 independent experiments using different tissue extracts. One-tailed Student's t-tests were employed to analyze the data. The level of statistical significance was taken as $P < 0.05$.

Results

Hepatopancreas of *O. lactea* showed both XO and XO+XDH activities. Figure 1 shows the dependence of hepatopancreas XO activity on pterin concentration (using enzyme from 24 h aroused snails). Addition of 10 µM methylene blue after 3–4 min of monitoring XO activity allowed the further measurement of the XO+XDH activity. The apparent K_m values for pterin were 6.0 ± 0.9 µM and 8.6 ± 5.1 µM for the XO+XDH and XO activities, respectively, similar to K_m values for mammalian XO using xanthine as substrate (2–8 µM for bovine milk or mouse, human and rat liver) (Waud and Rajagopalan, 1976; Krenitsky *et al.*, 1986; Carpani *et al.*, 1990). In addition, a typical Michaelis-Menten curve for XDH was obtained with increasing concentrations of methylene blue; a K_m of 5 µM was obtained (Fig. 2).

The pH dependence of the XO+XDH activity showed a typical bell-shaped profile, and maximal activity was observed at pH 7.8 (Fig. 3). In the case of XO, a similar result was obtained, although a broader pH range (7.0–7.8) presented optimal activity. Figure 4 shows the effect of the XO inhibi-

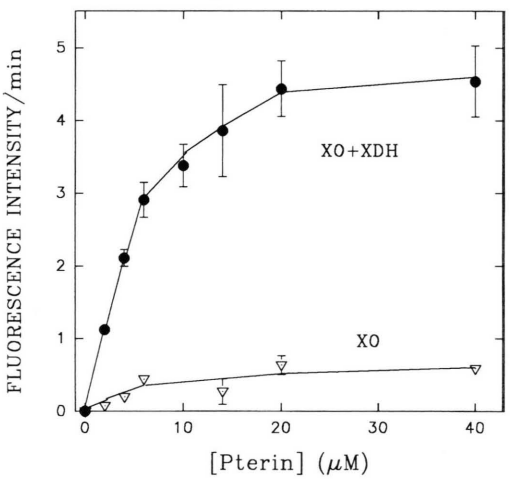

Fig. 1. Dependence of *O. lactea* hepatopancreas XO and XO+XDH activity on pterin concentration. Concentration of methylene blue in the assay for XO+XDH was 10 µM. Enzymatic activity was followed as described in Material and Methods. Values are the mean ± S.E. with $n = 3$ for XO+XDH and $n = 2–3$ for XO.

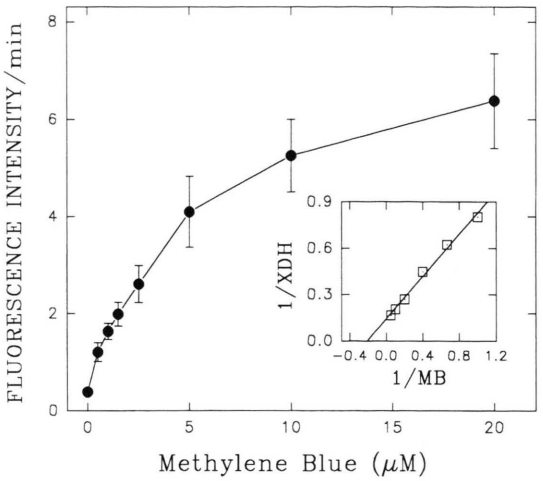

Fig. 2. Dependence of XO+XDH activity on methylene blue concentration. Concentration of pterin in the assay was 20 μM. Inset shows a Lineweaver-Burk plot of XDH activity (XDH values calculated as XO+XDH values – XO values). Values are the mean ± S.E. with $n = 3$.

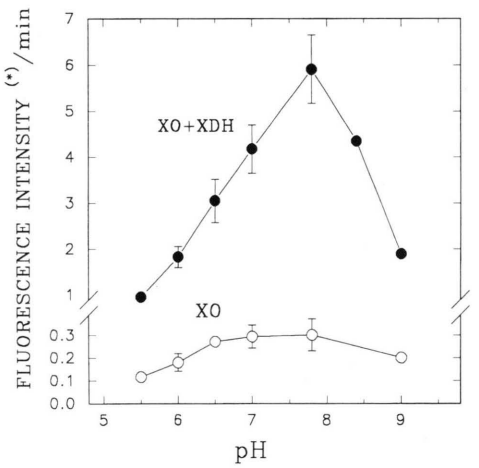

Fig. 3. Effect of pH on XO and XO+XDH activities. Buffers used were citric acid/potassium phosphate 25mM:25mM (pH 5.5–6.5); 50 mM potassium phosphate (pH 7.0 and 7.8) and 50 mM Tris-HCl (pH 8.4 and 9.0). Values are the mean ± S.E. ($n = 3$ except for pH 8.4 and 9). *: Fluorescence intensity was corrected relative to pH 7.0 values.

tor, allopurinol (Parks and Granger, 1986), on the activities of hepatopancreas XO+XDH and XO. An I_{50} value of 2 μM was found in both cases, which is similar to values for human liver XO (Krenitsky *et al.*, 1986). It is proposed that allopurinol inhibits XO activity due XO-induced con-

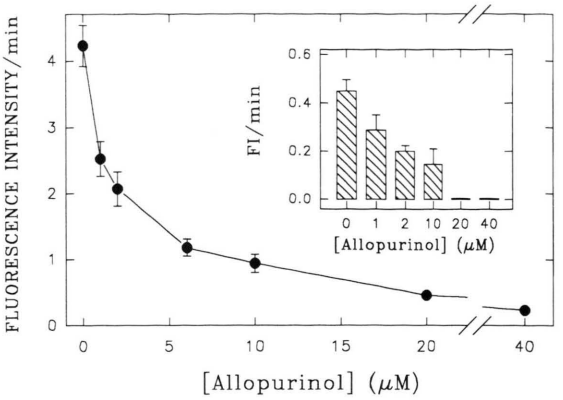

Fig. 4. Effect of allopurinol concentration on XO+XDH activity. The inset shows the inhibitory effect on XO activity alone. Allopurinol up to 0.1 mM caused no fluorescence interference. Values are the mean ± S.E. with $n = 3$.

version of allopurinol to alloxanthine (oxypurinol), which interacts with the molybdenum site of the enzyme with very high affinity (Parks and Granger, 1986).

The time course of heat (40 °C) inactivation of XO+XDH activity (Fig. 5A) exhibited a typical first order profile consisting of two-phases (Fig. 5A inset). After 15 min at 40 °C only about 35% of the original enzyme activities were still present. A similar result was observed for XO activity alone. Incubation of extracts at 26 °C induced a slower loss of XO+XDH activity (half-time of about 40 min) (Fig. 5A inset). Addition of 10 μM methylene blue to the reaction media during a 15 min heat (40 °C) exposure lead to about 54% protection of XO+XDH activity (Fig. 5B). This indicates that the formation of the enzyme-methylene blue complex stabilizes the enzyme.

Addition of hydrogen peroxide resulted in an inactivation of *O. lactea* XO+XDH activity in crude extracts (reaction media containing 50 μl extract pre-incubated for 5 min in the presence of H_2O_2 before addition of substrates). Under these conditions the I_{50} for H_2O_2 was 2.7 ± 0.6 mM (Fig. 6). However the presence of a high activity of catalase in hepatopancreas extracts (170–180 U/mg protein; Hermes-Lima and Storey, 1995) could be responsible for an underestimate of the I_{50} value for H_2O_2. Thus, the addition of 2 mM azide, which fully inhibits catalase activity, considerably enhanced the inhibitory potential of

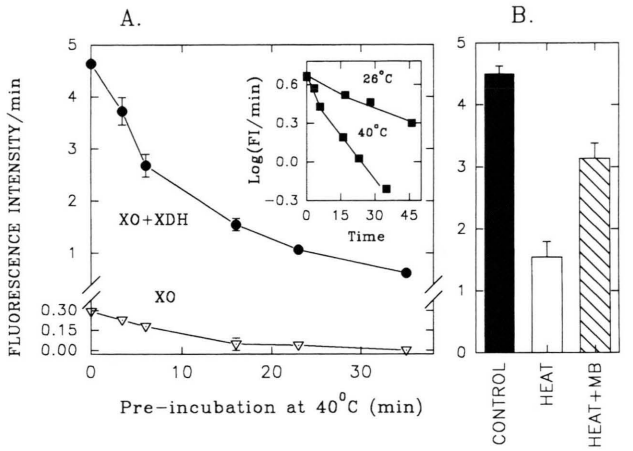

Fig. 5. (A) Time course of heat inactivation of XO+XDH and XO activities. Homogenates were pre-incubated at 40 °C for different times and 50 µl aliquots were removed for activity measurements at room temperature. The inset shows a log scale of the heat inactivation of XO+XDH at 40 °C or 26 °C. (B) Protective effect of methylene blue (MB) against 15 min heat exposure at 40 °C. Methylene blue was added at a concentration of 0.2 mM to the homogenates, and upon addition of homogenates to the reaction mixture methylene blue was diluted to 10 µM. Values are the mean ± S.E. with $n = 2-3$. The percentage protection provided by methylene blue was calculated from the formula: 100 × (heat plus MB) − (heat) / (control) − (heat).

H_2O_2 for the XO+XDH activity, and an actual I_{50} value could be measured (0.04 ± 0.01 mM). XO activity (in the presence of azide) was also highly susceptible to H_2O_2-induced damage (I_{50} about 0.1 mM; Fig. 6 inset). We have recently observed a protective effect of endogenous catalase on the ·OH-induced inactivation of glutathione S-transferase in extracts of garter snake muscle (Hermes-Lima and Storey, 1993a). Figure 7 depicts the time dependence of XO+XDH inactivation due to 0.2 mM H_2O_2 in media containing azide. Second order kinetics were observed for the inactivation (inset to Fig. 7), with a calculated $t_{1/2}$ of 1.2 ± 0.1 min.

Interestingly, 10 µM methylene blue also protected XO+XDH from inactivation promoted by 0.2 mM H_2O_2 (Table I), although pterin conferred no protection. If methylene blue was causing protection by forming an E–S complex, it would be expected that lower levels of methylene blue

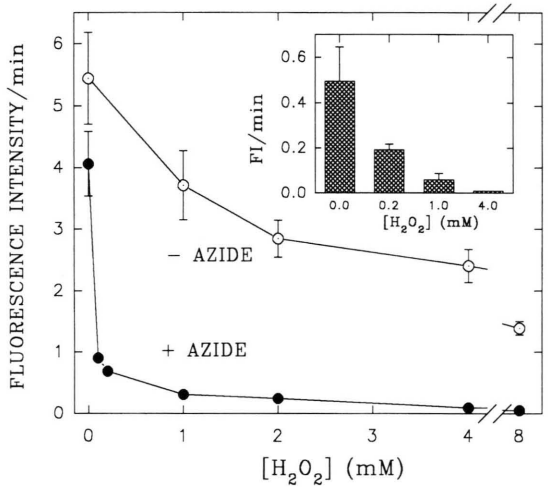

Fig. 6. Dependence of XO+XDH activity on H_2O_2 concentration in reaction media in the absence or presence of 2 mM azide. Reaction media were incubated 5 min in the presence of H_2O_2 prior to addition yof the enzyme substrates. Inset shows the effect of H_2O_2 on the activity of XO alone in media containing 2 mM azide.

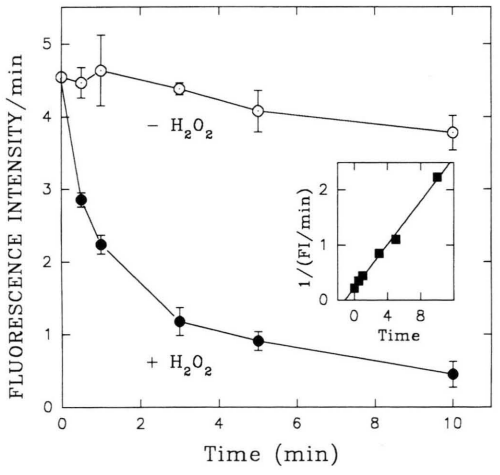

Fig. 7. Time course of H_2O_2-induced loss of XO+XDH activity. Incubations containing 50 mM potassium phosphate, 0.5 mM EDTA, 2 mM azide and 50 µl of homogenate were pre-exposed to 0.2 mM H_2O_2 prior to addition of pterin (20 µM) and methylene blue (10 µM) (filled circles). Open circles show incubations without pre-exposure to H_2O_2. The inset shows a second order kinetics profile of XO+XDH inactivation. The straight line in the graph is a computer generated linear regression.

Table I. Protective effect of methylene blue (MB) against 0.2 mM H_2O_2-induced XO+XDH inactivation.

[MB]	XO+XDH activity (fluorescence intensity/min) with MB pre-incubation[a]	without MB[b]
2.5 μM		
$-H_2O_2$	2.69 ± 0.12	3.05 ± 0.42
$+H_2O_2$	1.33 ± 0.15 (51.3 ± 6.0%)	0.57 ± 0.03 (19.1 ± 1.7%)
10 μM		
$-H_2O_2$	4.84 ± 0.55	3.18 ± 0.96
$+H_2O_2$	3.18 ± 0.96 (65.4 ± 4.3%)[c]	0.79 ± 0.11 (20.6 ± 3.8%)

[a] Buffered reaction mixtures containing 50 μl of tissue extract (with or without 0.2 mM H_2O_2) were pre-incubated in the presence of 2.5 or 10 μM methylene blue for 5 min prior to the addition of pterin.

[b] Reaction mixtures were pre-incubated for 5 min (with or without 0.2 mM H_2O_2) prior to addition of both pterin and methylene blue. Results are means ± S.E.M. (n = 3–6 different extracts) with units in relative fluorescence intensity/min. Values in brackets show the percentage of activity remaining compared with corresponding controls without H_2O_2 addition.

[c] The percentage of activity remaining is significantly different from the value for incubation with 2.5 μM methylene blue, $P < 0.05$, paired t-test.

would lead to less protection. Indeed, 2.5 μM methylene blue induced less protection than did 10 μM methylene blue ($P < 0.05$). Control experiments demonstrated that methylene blue was not destroying H_2O_2 in solution because pre-incubation of H_2O_2 with methylene blue for 1 h produced the same level of XO+XDH activity as did samples with no pre-incubation. Thus, these data suggested that H_2O_2 was inducing damage at the FAD-containing active site of XDH, where methylene blue is reduced.

Finally, Fig. 8 shows the activities of XO+XDH and XO (expressed per mg protein) in hepatopancreas of snails following 35 days of dormancy and 24 h of arousal. Both XO+XDH and XO activities were 2.3- and 3.0-fold higher, respectively, in hepatopancreas of dormant, compared with active snails. In addition, the percentage of total activity that was XO was also higher in the dormant snails, 9.6 ± 0.7 % compared with 7.0 ± 0.4 % in the snails 24 h after arousal. Very low levels of XDH, with no detectable XO activity, were found in foot muscle (30–40 times lower than activities in hepatopancreas) and the enzymatic activity was allopurinol sensitive (data not shown).

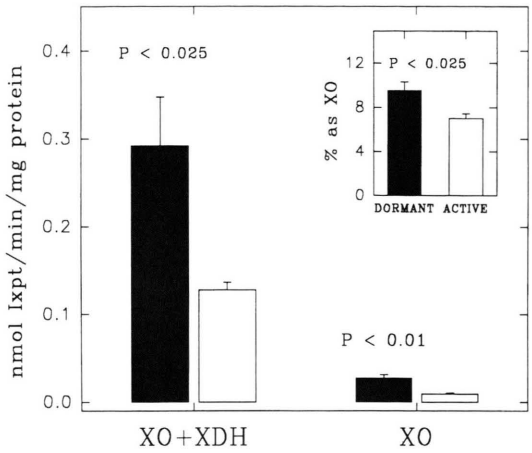

Fig. 8. Activities of XO+XDH and XO (nmol isoxanthopterin produced·min^{-1}·mg protein^{-1}) in hepatopancreas of snails after 35 days of estivation (filled bars) and 24 h after arousal (open bars). Inset shows the percentage of total XO+XDH that was XO in dormant and active *O. lactea* hepatopancreas.

Discussion

Xanthine oxidase and/or XDH participate in the catabolic pathway for the breakdown of purine nucleotides to xanthine and uric acid in most animal species (Parks and Granger, 1986). They also play an important role in post-hypoxic oxidative injury in various mammalian organs (Beckman *et al.*, 1986; Rangan and Bulkley, 1993). About 90% of the nitrogen in the excreta of pulmonate land snails is in the form of guanine, uric acid, and xanthine (Bishop *et al.*, 1983). This correlates with the fact that measurable levels of both XO and XDH were found in *O. lactea* hepatopancreas. The observed increase in XO and XO+XDH activities during estivation (Fig. 8) is a possible response to the need to produce relatively non-toxic nitrogen excretion products that can be safely accumulated in the body over long periods of dormancy. Xanthine and uric acid accumulates in the kidneys of estivating *O. lactea* at rates of 15–25 and 55–90 nmol·g whole body^{-1}·day^{-1}, respectively (Speeg and Campbell, 1968). The allopurinol-sensitive XO+XDH activity in hepatopancreas of 35 day dormant snails was about 0.3 nmol isoxanthopterin·min^{-1}·mg protein^{-1} (12 mU·g wet wt^{-1}). This pterin oxidation activity is about the same as that reported for human liver, although 6 times lower than that in rat liver (activities at 25 °C and

reported per mg protein) (Beckman *et al.*, 1989). Based on the comparative measurements of Beckman *et al.* (1989) for mammalian XO+XDH activities, we could predict that rates 2–4 fold higher (compared with pterin) would be expected for the oxidation of xanthine catalyzed by *O. lactea* XO+XDH. This expected activity of *O. lactea* XO+XDH is comparable to that reported in *Mytilus edulis*, a marine mussel that also undergoes metabolic arrest (in response to anoxia); however, this species displays only XDH (Dykens and Shick, 1988; Dykens *et al.*, 1989).

The pH optimum of XO+XDH was 7.8 (Fig. 3) but about 80 % of maximal activity was retained at pH 7. By contrast, the pH optimum of bovine milk XO (pterin as substrate) was 5.5 (Beckman *et al.*, 1989). Intracellular pH in *O. lactea* is about pH 7.5 in active animals and undoubtedly falls during estivation; hemolymph pH fell by about 0.4 units after one month of estivation (Barnhart, 1986b) and Barnhart and McMahon (1988) showed that intracellular pH paralleled extracellular pH over a wide range of P_{CO_2} values. Hence, pH effects may tend to reduce enzyme activity in estivating animals although other factors, such as elevated substrate concentrations in estivating snails may be more influential on enzyme activity *in vivo*.

The increase in the XO/(XO+XDH) activity ratio in estivation (Inset to Fig. 8) may be explained by a XDH to XO conversion via the action of proteases. It has been proposed that Ca^{2+}-dependent proteases catalyze an irreversible conversion of XDH to XO in mammals (McCord, 1985; Granger *et al.*, 1986). Interestingly, a rise in protease activities during estivation has been reported in another snail species (Ramesh *et al.*, 1990). In the case of *O. lactea*, no change in the amount of total soluble protein (measured per g wet weight) was detected after one month of estivation (Hermes-Lima and Storey, 1995). However, this does not exclude the possibility that proteases play a role in the increase in the *O. lactea* XO/(XO+XDH) ratio. Reversible oxygen-dependent conversion of XDH to XO during estivation is unlikely because of the low P_{O_2} in the tissues of estivating snails (e.g. 0.35 kPa in the lung) (Barnhart, 1986a).

Could XO and XDH be linked with an induction of oxidative stress in *O. lactea*? Many studies have demonstrated the role of XO in the generation of oxyradicals in mammalian systems under pathological situations (McCord, 1985; Granger *et al.*, 1986; Traystman *et al.*, 1991; Rangan and Bulkley, 1993). During ischemia the catabolism of ATP leads to an accumulation of xanthine and hypoxanthine (Rubin *et al.*, 1992) and Ca^{2+}-dependent conversion of XDH into XO (Traystman *et al.*, 1991). Reperfusion with oxygenated blood leads to the formation of O_2^- and H_2O_2 (catalyzed by XO or other sources of oxyradical formation) as well as highly reactive ·OH radicals (Patt *et al.*, 1988; Jaeschke and Mitchell, 1989). Hydroxyl radicals induce widespread cellular injuries including mitochondrial damage and lipid peroxidation (Traystman *et al.*, 1991; Hermes-Lima *et al.*, 1991; Henry *et al.*, 1993):

$$H_2O_2 + O_2^- \rightarrow (Fe^{3+}, Cu^{2+}) \rightarrow O_2 + OH^- + {\cdot}OH.$$

During dormancy the activity of XO in hepatopancreas increased 3-fold after 35 days of estivation (Fig. 8), reaching 0.03 nmol isoxanthopterin · min^{-1} · mg $protein^{-1}$ (1.2 mU · g wet $weight^{-1}$). In addition, the XO substrate (xanthine) accumulates slowly in the kidney of estivating snails (Speeg and Campbell, 1968), and probably also in other tissues. Upon arousal, oxygen consumption rises dramatically (Herreid, 1977) and tissue P_{O_2} quickly returns to normoxic values (Barnhart, 1986a). We have previously measured the levels of thiobarbituric acid reactive substances (TBARS) in hepatopancreas during arousal from estivation; the assay quantifies a series of aldehydic products of peroxidation including malonaldehyde and 4-hydroxynonenal (Uchiyama and Mihara, 1978; Hermes-Lima and Storey, 1995). A transient increase in TBARS content of 25 % occurred after 20 min of arousal (TBARS rose from 26.3 ± 2.7 to 32.8 ± 1.1 nmol · g wet wt^{-1}, $P < 0.05$, $n = 5$) (Hermes-Lima and Storey, 1995) indicating that damage by oxyradicals increases early in arousal. Moreover, the expected activity of *O. lactea* XO toward xanthine oxidation (see above) is also comparable with the XO activities of the marine stenoxic bivalves *Pecten maximus* and *Placopecten magellanicus* (Dykens and Shick, 1988) and it has been proposed that XO is implicated in post-anoxic injury in these animals. Furthermore, the activity of XO in estivating *O. lactea* is comparable with rat brain XO activity (accounting for 79% of the total rat

brain XO+XDH activity) (Margolin and Behrman, 1992), and is suggested to play a role in cerebral post-ischemic oxidative damage (Beckman *et al.*, 1986; Patt *et al.*, 1988; Kinuta *et al.*, 1989). Taking all these factors as a whole, it could be possible that XO contributes to oxyradicals generation in *O. lactea* during arousal.

On the other hand, based on the hepatopancreas XO activity using a natural substrate (xanthine), we can predict a rate of H_2O_2 generation by XO of $0.6–1.2\times10^{-4}$ μmol·min^{-1}·mg protein^{-1} (equivalent to 0.03 nmol pterin oxidation·min^{-1}·mg protein^{-1}) ($F_{H_2O_2}$). However, even under optimum conditions $F_{H_2O_2}$ would be overwhelmed by hepatopancreas catalase activity (170–180 μmol H_2O_2·min^{-1}·mg protein^{-1}, relative to 10 mM substrate; Hermes-Lima and Storey, 1995). This would also be true considering a catalase activity of about 2×10^{-3} μmol H_2O_2·min^{-1}. mg protein ($D_{H_2O_2}$) based on a realistic *in vivo* steady state concentration of H_2O_2 of about 10^{-7} M (Chance *et al.*, 1979). The calculated ratio $D_{H_2O_2}/F_{H_2O_2}$ (~ $0.2–0.3\times10^2$), which still underestimates the role of glutathione peroxidase and other peroxidases in the decomposition of H_2O_2, suggests that XO does not play a major role in oxyradicals generation and oxidative damage in *O. lactea*. Thus, other putative sources of oxyradicals generation during arousal need to be considered including the P450 detoxification system, which has been identified in a variety of molluscs (Livingstone, 1991), and the "electron leak" at the mitochondrial respiratory chain (Jaeschke and Mitchell, 1989; Konstantinov *et al.*, 1987). It is noteworthy that in mammalian systems, such as rat liver or pigeon heart, about 1–4% of consumed oxygen is converted to O_2^- and H_2O_2 at the mitochondrial level (Turrens *et al.*, 1982; Cadenas and Boveris, 1980; Konstantinov *et al.*, 1987), and this could also be true in the case of *O. lactea*. On the other hand, the correlation between the lack of measurable XO activity and the lack of change in TBARS levels following arousal in foot muscle of *O. lactea* (mean TBARS were only 5.7 ± 0.4 nmol·g wet weight^{-1}; Hermes-Lima and Storey, 1995) cannot be ignored.

The high susceptibility of *O. lactea* XO+XDH and XO to H_2O_2 effects (I_{50} in the presence of azide = 0.04 mM) differs from the case of purified bovine milk XO where only millimolar levels (5–30 mM) of H_2O_2 were found to be damaging (Terada *et al.*, 1991). Moreover, the requirement of xanthine (which reduces XO) for the H_2O_2-induced inactivation of bovine milk XO was not necessary in the case of *O. lactea* XO (and XDH as well). Terada *et al.* (1991) have postulated that damaging ·OH radicals would be formed at the active site of XO as electron transfer from $FADH_2$ to H_2O_2 takes place. Therefore, a reduced form of the enzyme is required for the inactivation by H_2O_2. This mechanism seems unlikely in the case of *O. lactea* XO.

The decrease in the activity of XO and XO+XDH in snails after 24 h of arousal could be only partially explained by a mechanism of H_2O_2-induced inactivation. Although endogenous antioxidant enzymes should protect hepatopancreas XO and XO+XDH activities from H_2O_2 effects, it is plausible that mitochondrial generation of oxyradicals would inflict some damage to these enzymes *in vivo* during arousal. Nevertheless, direct evidence for XO/XDH damage *in vivo* has yet to be found in *O. lactea*. The mechanism proposed by Terada *et al.* (1988) for hyperoxic injury in mammalian lung involving self-inactivation of XO (a potential feedback mechanism for cellular protection) seems unlikely in the case of *O. lactea* due to the low expected rates of XO-induced H_2O_2 formation. Finally, the reduction in hepatopancreas XO and XO+XDH activities could be interpreted as a down regulation process in order to redirect nitrogen metabolism towards pathways that could, for example, supply purines for nucleic acid biosynthesis in active snails (Speeg and Campbell, 1968; Bishop *et al.*, 1983).

In conclusion, despite the observed 3-fold increase in the activity of *O. lactea* hepatopancreas XO in estivating snails (35 days) it appears that XO only plays a secondary role in exerting oxidative stress because of (i) the low expected $F_{H_2O_2}$ values and (ii) the high $D_{H_2O_2}$ values in hepatopancreas. We have previously proposed that the increase in antioxidant enzyme activities in hepatopancreas during estivation (superoxide dismutase and glutathione peroxidase) can be regarded as a mechanism of protection against post-hypoxic stress (Hermes-Lima and Storey, 1995). Both the low activity of hepatopancreas XO and the limited conversion of XDH into XO during estivation (Fig. 8) may also represent an adaptive advantage

that minimizes oxidative stress following arousal. The lower activity of XO after 24 h arousal would make XO-mediated oxyradicals generation even less significant for active animals. Indeed, these adaptations (regarding XO/XDH and antioxidant enzymes status) may be the reason that only a very limited amount of lipid damage is observed following arousal (only 25%) (Hermes-Lima and Storey, 1995). Similarly, we have previously reported that several antioxidant enzyme activities were increased in garter snakes during anoxia or freezing tolerance as a preparation for potential oxygen reperfusion injury following reoxygenation or thawing (Hermes-Lima and Storey, 1993b; 1995). It is also important to point out that the elevated levels of uric acid in the tissues of estivating snails (Speeg and Campbell, 1968) could diminish ·OH radicals-induced injury by acting as free radical scavengers (Becker, 1993).

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