CATECHOL OXIDASE LATENCY IN NIDAMENTAL GLAND EXTRACTS FROM THE LITTLE SKATE (RAJA ERINACEA)

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Egg capsules of the little skate (Raja erinacea) are formed from protein precursors secreted by the nidamental gland. Sclerotization of the capsule involves the production of catechols and their subsequent oxidation to quinones by catechol oxidase. This enzyme is latent when incorporated into the capsular matrix, and is activated coordinately with increasing capsule catechol content and color development (Koob & Cox, J. mar. biol. Assoc. U.K. 70, 395-411, 1990). In nidamental gland lysates, catechol oxidase activity is typically preceded by a latent period of one to several minutes following substrate addition. Treatment of catechol oxidase with α-chymotrypsin significantly reduced the latent period and concomitantly increased initial rates of substrate oxidation. Conversely, treatment of nidamental gland extracts with a serine proteinase inhibitor caused substantial increases in oxidase latency and reduction in activity. Thus, a proteinase or proteinase sensitive factor might influence catechol oxidase activity in vivo (Koob & Cox, Biol. Bull. 175, 202-211, 1988: Cox & Koob, Comp. Biochem. Physiol. 95B, 767-771, 1990). Subsequent investigations of enzyme latency revealed that nidamental gland lysates contain an endogenous factor which promotes latency without affecting the initial rate of substrate oxidation (Straus & Cox, Bull. Mt. Desert Is. Biol. Lab. 30, 23-24, 1991). The latency factor was shown to extend the latent period in a concentration dependent manner. Further characterization revealed that the factor was insensitive to heat denaturation, destroyed by acid hydrolysis, and apparently was of low molecular weight. Our continued studies have focused on the effects of substrate, ions and chelation on catechol oxidase latency, and on the molecular properties of the latency factor.

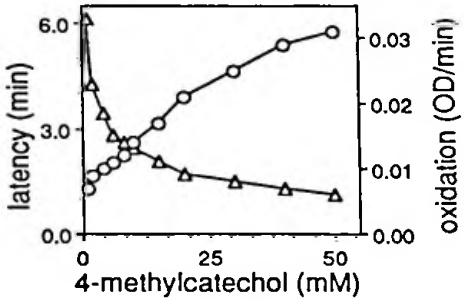
Nidamental gland lysates were prepared by homogenization as previously described (Koob & Cox, 1988, op cit.) in 0.5 M NaCl buffered at pH 7 with 50 mM Tris-HCl or 10 mM HEPES. Glands were divided into equivalent sections for homogenization in Tris extraction buffer in the presence or absence of 25 mM ethylenediaminetetraacetic acid (EDTA). Five ml aliquots of the control and EDTA extracts were dialyzed against extraction buffer (to a dilution factor of 4 X 10^{11}) at 4° C with Spectrapor 2 membrane to remove small molecules and chelating agent. Enzyme activity was determined by following the oxidation of 4-methylcatechol at 400 nm as previously described (Koob & Cox, 1988, op cit.).

Extracts prepared with EDTA exhibited a six-fold increase in the latent period following substrate addition, and they oxidized substrate (1 mM 4-methylcatechol) at rates 6.5% (N=12) of control extracts. It should be noted that dialysis of EDTA extracts restored catechol oxidase activity to the same levels as dialyzed control extracts. Dialysis eliminated the initial latent period in both control and EDTA extracts, and increased oxidation rates 2.2- and 2.7-fold respectively over those of undialyzed control extracts.

Pre-incubation of control extracts with physiological concentrations of $CaCl_2$ (6 mM) or $MgCl_2$ (2.5 mM) for 15 min at ambient temperature lengthened the initial latent phase by about three-fold and diminished oxidase activity by 50 - 60%. EDTA extracts were similarly affected

by treatment with $CaCl_2$ and unaffected by treatment with $MgCl_2$. Pre-incubation of dialyzed and control extracts with $MgCl_2$ had no apparent effect on either latency or activity. Conversely, pre-incubation of dialyzed extracts with $CaCl_2$ for 15 min restored the latent period which dialysis had abolished and reduced oxidation rates of both extracts by over 90%. It is also important to note that when lysates from other animals were examined, the effects of chelation and divalent cations varied considerably and in one case, chelation and divalent cations had virtually no effect on catechol oxidase activity in the nidamental gland lysate.

The absolute period of oxidase latency tended to be fairly consistent when right and left nidamental gland extracts from the same animals were compared. Intraorganismal variation was typically on the order of ±1 min or less. However, interorganismal differences in latent periods varied from about 1.5 to 12 min. We also found that the concentration of 4-methylcatechol substrate used in assays had a dramatic impact on enzyme latency, as shown in Fig. 1. Increasing substrate concentration caused a corresponding rise in oxidase activity with a concomitant decrease in latency. A ten-fold increase in substrate concentration resulted in a 60% decrease in the latent period. Further increases in substrate caused diminution of latency in smaller increments.



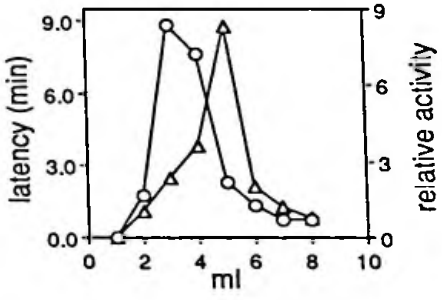


Fig. 1 (left) - Effect of substrate concentration on catechol oxidase latency (Δ) and activity (O). Aliquots of lysate (0.05 ml) were equilibrated for 2 min in 10 mM HEPES, 0.5 M NaCl, pH 7 at ambient temperature. Reactions were initiated by the addition of substrate.

Fig. 2 (right) - Gel filtration of nidamental gland lysate. The column contained a 2 X 0.5 cm bed of Sephadex G-25. 0.5 ml of lysate with an endogenous latency of 12.1 ± 1.2 min was applied and eluted with 10 mM HEPES, 0.5 M NaCl, pH 7. 1 ml fractions were collected and assayed for oxidase activity as above. Latent period (Δ) and relative activity (O) for each fraction are shown, where one unit of relative activity equals 0.001 O.D. units/min at 400 nm.

Extracts prepared in 10 mM HEPES responded to dialysis in a similar manner to those above. For example, dialysis to a dilution factor of 4000 resulted in a 70% decrease in latency (from 2.2 ± 0.2 min to 0.7 ± 0.1 min) while a dilution factor of 10^6 resulted in complete loss of the latent period. In addition, dialysis caused a doubling in the initial rate of substrate oxidation. Because latency was reduced or eliminated by dialysis, extracts were subjected to gel filtration to determine whether the latency factor could be separated from catechol oxidase. Prior results have shown that when an aliquot of nidamental gland lysate was centrifuged through a bed of Sephadex G-25, a reduction in latency resulted (Straus & Cox, 1991, op cit.). A short (2 cm) Sephadex G-25 column was used to minimize dilution and separation time for chromatographic

fractionation. As shown in Fig. 2, when an extract was subjected to gel filtration, the oxidase and the latency factor separated into two distinct but overlapping peaks. The first three fractions containing oxidase activity (collected at 2, 3, and 4 ml respectively) exhibited highly reduced latency while the subsequent fraction (collected at 5 ml) retained a nine minute latent period.

Glutathione, a thiol reducing reagent, proved to be a potent mimic of the endogenous latency factor. As shown in Fig. 3A, addition of reduced glutathione to nidamental gland lysate resulted in a concentration dependent increase in the latent period. Reduced glutathione had no apparent effect on initial rates of substrate oxidation. In contrast, oxidized glutathione had no effect on either latency or activity (Fig. 3B). Another thiol reductant, dithiothreitol, also increased latency in a similar manner without affecting activity.

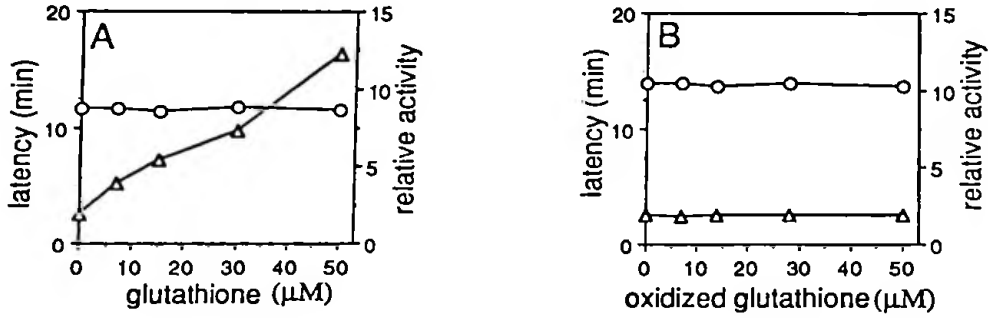


Fig. 3. Effect of reduced (A) and oxidized (B) glutathione on catechol oxidase latency (Δ) and activity (O). Aliquots of extract were equilibrated for 2 min with the indicated concentrations of glutathione at ambient temperature. Reactions were initiated by the addition of substrate and monitored for oxidase activity. For relative activity see Fig. 2.

The regulation of catechol oxidase is essential for egg capsule formation and hence, reproduction in the little skate. The results presented above provide intriguing information on the behavior of nidamental catechol oxidase. We have established that lysates contain an endogenous factor which induces temporary enzyme latency in a concentration dependent manner (Straus & Cox, 1991, op cit.). The factor is resistant to heat denaturation, destroyed by acid hydrolysis, and removable by dialysis and gel filtration. These data indicate that the latency factor has a low molecular weight (< 12, 000) and suggest that the factor could be a peptide since it was susceptible to acid hydrolysis. The ability of glutathione and dithiothreitol to induce temporary latency in a manner similar to the endogenous factor suggest that the factor might function as an antioxidant. The effects of chelation and divalent cations are difficult to interpret since there was extensive interorganismal variation. Clearly, metals have profound effects on catechol oxidase latency and activity in some, but not all nidamental gland lysates. The ability of calcium to dramatically increase latency and diminish oxidase activity in certain extracts indicates that enzyme sensitivity to divalent cations might depend on the stage of the reproductive cycle. Similarly, the considerable interorganismal variation in catechol oxidase latency and activity might arise from reproductive stage differences. A thorough understanding of this system will require biochemical characterization of purified catechol oxidase and regulatory components, as well as an examination of the physiological parameters and reproductive stage effects pertinent to enzyme secretion and activation.

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