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MERCURY-BINDING PROTEINS OF MYTILUS EDULIS

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INTRODUCTION

Low molecular weight, metal-binding proteins were first reported for a marine invertebrate species by Casterline and Yip (1975) who described an oyster cadmium-binding protein with some properties similar to metallothionein. Such proteins have now been identified in a number of marine species (reviewed by Roesijadi, 1981). Although the function(s) of these proteins is still to be completely elucidated, recent evidence is supportive of a role in trace metal detoxification, a subject of practical concern in light of anthropogenic metal contamination of the marine environment.

Metal-binding proteins of Mytilus edulis have been studied by a number of investigators (Noel-Lambot, 1976; Talbot and Magee, 1978; George et al., 1979; Marshall and Talbot, 1979; Frankenne et al., 1980; Viarengo et al., 1981; Roesijadi and Hall, 1981; Roesijadi et al., 1981; Roesijadi, in press; Roesijadi and Drum, in press). Past work by one of us (G.R.) has dealt with the role of low molecular weight, metal-binding proteins in the sequestration of mercury by M. edulis. The objective of the present paper is to summarize the findings reported to date and to describe more recent and preliminary work on development of an immunoassay for these proteins and their occurrence in early developmental stages of mussels when parent organisms are exposed to mercury.

SOME PROPERTIES OF MERCURY-BINDING PROTEINS OF MYTILUS EDULIS

While it has been known for some time that Mytilus edulis possesses low molecular weight proteins capable of binding cadmium, copper, and zinc (Noel-Lambot, 1976), the binding and storage of mercury by similar proteins has only recently been reported (Roesijadi et al., 1981). That study and subsequent ones have shown that inducible, mercury-binding proteins are present in M. edulis and that these proteins are probably involved in the detoxification of inorganic mercury. The gills of M. edulis are capable of concentrating high levels of mercury, and much of the work described here deals with the mercury-binding proteins of the gills.

Typical separations of cytosol fractions of gill tissue on Sephadex G-75 (equilibrated in 20 mM tris-HCl, pH 8.6) result in elution profiles such as that in Figure 1. Mercury occurs in three peaks associated with the void of the column (I) and at positions which correspond to about 20,000 (II) and 10,000 (III) daltons molecular weight. The latter two peaks (II and III) represent the low molecular weight, mercury-binding proteins while peak I consists of high molecular weight proteins of > 70,000 daltons. The dual peaks II and III are characteristic of separations carried out under non-reducing conditions (Roesijadi and Drum, in press) and most likely represent dimer and monomer forms, respectively, of the same or similar mercury-binding proteins. Under the usual conditions of extraction and isolation, peak II or the apparent dimer is the predominant mercury-binding peak (see for example Roesijadi and Hall, 1981). In the presence of 2-mercaptoethanol, however, this peak appears to dissociate, with mercury eluting as a single peak of lower molecular weight (Roesijadi and Drum, in press). Routine use of mercaptoethanol to prevent aggregation of mercury-binding proteins, however, was not recommended since it was found that

mercaptoethanol competes with the protein for the binding of mercury and causes a shift in the mercury distribution to form a new peak which probably represents a mercaptoethanol-mercury complex (Roesijadi and Drum, in press).

Peak II, the predominant low molecular weight mercury-binding protein, has been further purified as described by Roesijadi and Hall (1981). Determination of its amino acid composition indicates a similarity with cadmium-binding proteins isolated from oysters (Ridlington and Fowler, 1979) and mussels (Marshall and Talbot, 1979; George et al., 1979) but not with metallothionein (Table 1). These results show that, in addition to metallothionein which has been identified in mussels (George et al., 1979; Franken et al., 1980), other metal-binding proteins are also present and are involved in binding mercury.

POSSIBLE ROLE IN MERCURY DETOXIFICATION

Kagi and Vallee (1960; 1961), who were among the first to study metallothionein, posed the possibility that these proteins may serve to protect against the toxic effects of cadmium by binding large quantities of the metal. Subsequent work has shown that detoxification of metals such as cadmium, copper, zinc, and mercury may, indeed, be one of the functions of metallothionein and other low molecular weight, metal-binding proteins (reviewed by Webb, 1979; Roesijadi, 1981). The recent work with mercury in Mytilus edulis is supportive of a role for mercury-binding proteins as a compartment for the storage and detoxification of mercury.

The levels of mercury in the tissues of mussels not previously exposed to elevated mercury concentrations are very low, about 10 to 20 ng Hg/g or ppb in wet tissue (Roesijadi et al., 1981; Roesijadi, in press). Mercury-binding proteins are barely detectable in digestive glands and non-detectable in gills

Table 1. Amino acid compositions of mussel and oyster metal-binding proteins and equine metallothionein.

Amino acid	<u>Mytilus edulis</u> Hg-PB II ¹	<u>Mytilus edulis</u> Cd-BP MT III ²	<u>Crassostrea virginica</u> Cd-BP ³	Equine metallothionein ⁴
½ Cystine	7.8	6.3	7.6	32.6
Aspartic acid	12.2	12.3	12.9	5.0
Threonine	7.0	8.1	5.8	3.9
Serine	7.3	8.2	7.3	11.6
Glutamic acid	12.2	7.8	9.7	4.5
Proline	5.6	5.2	4.4	5.1
Glycine	9.4	10.8	9.5	10.1
Alanine	5.8	5.0	6.6	9.4
Cysteine	-	-	-	-
Valine	5.4	3.5	6.1	2.6
Methionine	1.2	tr	1.5	1.5
Isoleucine	4.7	2.7	3.9	0.6
Leucine	5.7	2.8	4.5	0.6
Tyrosine	2.3	0.6	2.6	-
Phenylalanine	2.8	1.0	3.5	-
Histidine	0.8	3.4	1.2	-
Lysine	6.5	9.8	8.9	10.4
Arginine	2.5	1.6	2.9	2.2
Tryptophan	0.3	-	tr	-

¹ This paper² George *et al.* (1979)³ Ridlington and Fowler (1979)⁴ Kagi *et al.* (1974)

of such mussels (Roesijadi et al., 1981). Following exposure to mercury, mercury-binding proteins are induced and bind large amounts of mercury. Experiments which followed the incorporation of mercury into these proteins showed that induction of the major protein peak II (Fig. 1) occurs between four and seven days of an exposure to 5 $\mu\text{g Hg/l}$ (Roesijadi, in press). Once the proteins are induced, their incorporation of mercury is relatively rapid and linear for up to 21 days of exposure (Fig. 2). After 21 days, the proteins are saturated with mercury.

Prior to induction of mercury-binding proteins, the mercury incorporated into the soluble fraction of tissues was bound to proteins in the high molecular weight pool (peak I). The levels of mercury in the high molecular weight pool stabilized between days 7 and 21, then began to increase again after saturation of the mercury-binding proteins at day 21. This sequence of events (summarized in Fig. 3) suggests that the high molecular weight proteins (which may be considered a sensitive subcellular compartment) were spared from the effects of mercury by the mercury-binding proteins. Saturation of the latter and coincident increase of mercury on the high molecular weight proteins was consistent with the concept of "spillover" described by Brown and Parsons (1978).

Additionally, it was observed that mussels which had been exposed for 28 days to low levels of mercury (0.5 $\mu\text{g Hg/l}$ and less) possessed an enhanced tolerance to toxic concentrations (Fig. 4), whereas mussels exposed to a high concentration of 5 $\mu\text{g Hg/l}$ for 28 days did not exhibit this enhanced tolerance. Since this latter exposure concentration and duration, 5 $\mu\text{g/l}$ and 28 days, respectively, corresponded to the "spillover" described above, it appears that saturation of mercury-binding proteins was also associated with a disruption of the protective effects of mercury pre-exposure. Exposures to

the low mercury concentrations resulted in induction of the proteins, but were either not high enough or of insufficient duration to result in the spillover condition. A logical extension of these above observations would suggest that enhanced tolerance was a result of prior induction of mercury-binding proteins and that protective effects were conferred by the binding of mercury to these proteins instead of other subcellular compartments.

DEVELOPMENT OF IMMUNOASSAYS FOR METAL-BINDING PROTEINS

Although the importance of metal-binding proteins in metal storage is recognized and a role in detoxification exists as described above, direct assays for the proteins for routine use have not yet been established. Identification of the proteins in, for example, Sephadex separations is based on the occurrence of a metal peak in a characteristic position of the chromatogram. While this procedure provides quantitative information on the metal content of the protein peaks, similar information for the protein itself is not forthcoming by this technique. In order to address this problem, efforts have been directed toward development of an immunoassay for the gill mercury-binding protein of Mytilus edulis which was partially characterized by Roesijadi and Hall (1981).

Antiserum to the mercury-binding protein was prepared from goats injected with a suspension of 1 mg protein in Freund's complete adjuvant. Pre-immunization bleedings were taken for control serum, and booster injections of 0.5 mg protein were given after 1, 2, 4, and 8 months. Interfacial ring tests and Ouchterlony double gel diffusion assays were employed as assays for the presence of antibodies to the mercury-binding proteins. As can be seen in Figure 5 which shows the results of Ouchterlony tests, precipitation reactions occurred between immunized goat serum and the

mercury-binding protein and indicated the induction of antibodies against the protein. No reaction was observed in the pre-immunization serum sample. The anti-(mercury-binding protein) fraction of goat serum immunoglobulins was purified by ammonium sulfate precipitation and chromatography of the precipitate on DEAE-cellulose (Whatman DE-52) anion exchange resin. The purified antibodies are presently being used for development of radioimmunoassays and immunocytochemical assays for the mercury-binding proteins.

MERCURY-BINDING PROTEINS IN MUSSEL LARVAE

To date, the existence of low molecular weight, metal-binding proteins in the early developmental stages of marine animals has not been reported. We have initiated studies to determine whether such proteins occur in larvae of Mytilus edulis and present preliminary evidence which shows that mercury in M. edulis larvae can occur in a region of Sephadex G-75 profiles where mercury-binding proteins would be expected to elute.

Adult mussels were exposed to 5 or 10 $\mu\text{g Hg/l}$ for two weeks. Exposed mussels were spawned and larvae were reared for 48 hours in clean seawater. Controls consisted of larvae from unexposed adult mussels. The cytosol fraction was extracted from each of the three groups of larvae as previously described (Roesijadi and Hall, 1981) and fractionated on Sephadex G-75. In all cases, the levels of mercury in the soluble fraction were very low, indicating that very little mercury occurred in this compartment in larvae although the exposed adult mussels would have possessed very high tissue mercury concentrations.

Examination of mercury elution profiles showed that all of the detectable mercury in the soluble fraction of control larvae was associated with high

molecular weight proteins in the column void ($> 70,000$ daltons) (Fig. 6). On the other hand, in larvae from mussels exposed to 5 or 10 $\mu\text{g Hg/l}$, mercury was detected in the region of the low molecular weight, mercury-binding proteins although no peaks were observed (V_e/V_o equals 1.65 for major peak II of Fig. 1). The much higher concentrations which occurred on high molecular weight proteins indicated that this latter compartment was more significant in binding mercury when larvae were treated as described above. No major differences were observed between larvae from the 5 or 10 $\mu\text{g Hg/l}$ exposure of adults. The data shown here suggests that mercury-binding proteins may occur, on the basis that mercury elutes in the proper position during Sephadex G-75 separations. However, the results demand that further experimentation be conducted to clarify the existence of such proteins in mussel larvae.

SUMMARY

Mytilus edulis possesses low molecular weight, mercury-binding proteins. The predominant protein isolated from gill tissue is enriched in cysteinyl residues (8%) and possesses an amino acid composition similar to cadmium-binding proteins of mussels and oysters. Continuous exposure of mussels to 5 $\mu\text{g/l}$ mercury results in "spillover" of mercury from these proteins to high molecular weight proteins. Antibodies to these proteins have been isolated, and development of immunoassays is presently underway. Preliminary studies to determine whether exposure of adult mussels to mercury will result in induction of mercury-binding proteins in offspring suggest that such proteins occur in larvae although additional studies are indicated for a conclusive demonstration.

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Figure Legends

Figure 1. Sephadex G-75 fractionation of the soluble fraction of gills from mussels exposed to 5 $\mu\text{g Hg}/\ell$ for 28 days. Gills were homogenized in 0.02 M tris-HCl pH 8.6, 0.275 M NaCl, 0.1 mM phenylmethylsulphonyl fluoride. The column was equilibrated in 0.02 M tris-HCl pH 8.6.

Figure 2. Incorporation of mercury into the predominant, low molecular weight, mercury-binding protein (peak II) as a function of time.

Figure 3. Relative amounts of mercury in high molecular weight proteins (peak I) and mercury-binding proteins (peaks II and III) as a function of time.

Figure 4. Cumulative percent survival of mussels exposed to 75 $\mu\text{g Hg}/\ell$ in a 7-day bioassay. Mussels were pre-exposed to control seawater (0.006 $\mu\text{g Hg}/\ell$) or to elevated concentrations of 0.5 or 5.0 $\mu\text{g Hg}/\ell$ prior to the bioassay.

Figure 5. Ouchterlony double gel diffusion assays for mercury-binding proteins (A) run against immune goat serum (B) and non-immune goat serum (C). Arrow indicates precipitation reaction between A and B.

Figure 6. Sephadex G-75 fractionation of the soluble fraction of mussel larvae whose parents had been treated with control seawater or 5 and 10 $\mu\text{g Hg}/\ell$ for two weeks. Extraction and column conditions as described in legend of Figure 1.

Fig. 1

Fig. 1

Fig. 4

Fig. 5

Fig. 6