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Ferritin and hemocyanin: ^{210}Po molecular traps in marine fish, oyster and lobster

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ABSTRACT: The relative degree of binding of ^{210}Po with fish, mollusc and crustacean ferritins was investigated. Comparison of ^{210}Po concentrations in the purified ferritins from liver of the Atlantic mackerel *Scomber scombrus* and from the visceral mass of oysters *Crassostrea gigas* confirmed the high affinity of polonium for these iron-containing proteins. The ferritin fraction in lobster *Homarus gammarus* hepatopancreas contained an order of magnitude more ^{210}Po than pure ferritin from fish and oyster; however, the hepatopancreatic ferritin fraction was not pure and it also contained the respiratory protein hemocyanin. A high performance size-exclusion chromatography analysis further revealed the important contribution of hemocyanin to ^{210}Po fixation in lobster. The combined ^{210}Po binding capacity of ferritin and hemocyanin in lobster hepatopancreas most probably accounts for the very high ^{210}Po concentrations found in the hepatopancreas of many higher crustaceans.

KEY WORDS: Polonium · Fish · Oyster · Lobster · Ferritin · Hemocyanin

INTRODUCTION

Polonium-210, a high-energy α-particle emitter in the ^{238}U decay chain, is accumulated to very high levels in marine organisms and in fact is responsible for the majority of the internal radiation dose in these species (Cherry & Heyraud 1982, Carvalho 1988). Furthermore, because of these high concentrations of ^{210}Po, which occur in the edible portions of marine organisms, ^{210}Po is considered to be the most important contributor to the radiation dose received by humans via fish and shellfish consumption (Aarkrog et al. 1997).

The accumulation of ^{210}Po in marine organisms is generally derived from the food chain (Carvalho & Fowler 1993, 1994), and significant differences in the amount of polonium have been noted in various marine species. For example, 2.4 Bq kg^{-1} wet wt in fish, 15 Bq kg^{-1} wet wt in molluscs and 6 Bq kg^{-1} wet wt in crustaceans have been computed as 'global' average concentrations of ^{210}Po in the edible fraction of these types of organisms (Aarkrog et al. 1997). Especially high levels of ^{210}Po have been observed in the individual tissues and organs of these species such as the digestive tract of fish, the digestive gland of molluscs and the hepatopancreas of crustaceans. These levels of ^{210}Po are generally 1 to 2 orders of magnitude higher than in muscle in these species (Cherry et al. 1983, Skwarzec 1988, McDonald et al. 1993, Stepnowski & Skwarzec 2000a,b).

In a previous work in which the subcellular localization of ^{210}Po in fish liver of *Scomber scombrus* was investigated, we identified 2 molecular components that play a major role in ^{210}Po accumulation, i.e. metallothioneins and ferritin (Durand et al. 1999). The principal aim of this study was to compare the ability of ferritin from different marine organisms to accumulate ^{210}Po. To do this, the ferritins from fish liver of *Scomber scombrus*, the visceral mass of oysters *Crassostrea gigas* and the hepatopancreas of lobster *Homarus gammarus*

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were purified by chomatographic methods and analyzed for ^{210}Po content.

MATERIALS AND METHODS

Thirty-nine specimens of mackerel *Scomber scombrus*, 28 cm mean length and 158 g mean body wt, were collected during the spring of 2000 off the Atlantic coast of France (Le Croisic). Livers were removed, weighed (89.3 g) and homogenized in 100 ml of 150 mM NaCl buffered to pH 7.5 with 10 mM HEPES and containing 0.1 mM phenyl methyl sulfonyl fluoride (PMSF) to inhibit digestive proteases (buffer A). Homogenization was performed with a Waring blender (20 s bursts at high speed) and then in a Potter-Elvehjem homogenizer with a motor-driven Teflon pestle (3 strokes at 200 rpm). The homogenized tissue was heated at 65°C for 10 min in a water bath with continuous stirring. After centrifuging the homogenate at 10 000 × *g* for 10 min, the pellets were discarded and the supernatant was brought to 75% saturation with ammonium sulfate. The suspension was further centrifuged at 10 000 × *g* for 10 min and the pellet was dissolved in 30 ml of 1 mM HEPES, 2 mM 2-mercaptoethanol and 0.1 mM PMSF at pH 7.5 (buffer B). This protein solution was dialyzed overnight against the same buffer to eliminate residual ammonium sulfate. The solution was then applied to a column of diethylaminocellulose DE 52 Whatman (25 × 120 mm) equilibrated with buffer B. The elution was performed initially by passing through 300 ml of buffer B at a flow rate of 82.5 ml h^{-1} and then using a linear gradient of 0 to 0.3 M NaCl in buffer B (400 ml) at the same flow rate. The elution was monitored for protein and iron concentration. The fractions containing the crude ferritin were pooled and brought to 60% saturation with ammonium sulfate. After centrifugation at 10 000 × *g* for 10 min, the pellets were solubilized in 4.5 ml of 1 mM HEPES buffer at pH 7.5. This solution was centrifuged again at 10 000 × *g* for 10 min, and the supernatant was applied to a column of Sephacryl S300 (30 × 900 mm) eluted by 1 mM HEPES (pH 7.5) at a flow rate of 35.5 ml h^{-1}. This gel filtration gave a single peak of protein corresponding to an iron peak.

The ferritin from the visceral mass of oysters *Crassostrea gigas* was purified from 30 adult specimens collected from the Atlantic coast of France (Baie de Bourgneuf, Port du Bec). Oysters were dissected, and 101 g of visceral tissues was pooled and homogenized in 100 ml of buffer A. The ferritin was purified as described above for mackerel ferritin (DE 52 ion exchange chromatography followed by Sephacryl S300 gel filtration).

The ferritin of lobster *Homarus gammarus* collected from the Atlantic coast of France (Quiberon) was purified from 5 specimens with an average weight of 692 ± 120 g. The hepatopancreases were removed, weighed (total weight = 103 g) and homogenized in 100 ml of buffer A. The ferritin was isolated according to the method used for the other species but a further purification step was performed after Sephacryl S300 gel filtration. An additional size-exclusion chromatography on a TSK G4000 SW column (HPLC) was performed to improve the separation between ferritin and hemocyanin.

Lobster hemocyanin was prepared from hemolymph collected by puncturing the base of one leg. This hemolymph was allowed to clot, and the clotted hemolymph was then homogenized and centrifuged at 12 000 × *g* for 10 min. The supernatant was used for sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) identification of hemocyanin. SDS-PAGE of the protein fractions was performed in a 15% gel along with commercial Bio-Rad low molecular weight markers (phosphorylase b, 97.4 kDa; bovine serum albumin, 66.2 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 31 kDa; soybean trypsin inhibitor, 21.5 kDa; lysozyme, 14.4 kDa) according to the method of Laemmli (1970). Gels were stained for the detection of protein using 0.25% Coomassie brilliant blue R-250 in methanol (40%), acetic acid (7%) and water followed by destaining in methanol (5%), acetic acid (7%) and water.

The molecular weights of ferritins were estimated by chromatography on a HPLC TSK G4000SW column (21.5 × 600 mm) previously calibrated with Pharmacia Biotech high molecular mass markers (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; aldolase, 158 kDa). The elution was performed at 20°C with 1 mM HEPES and 100 mM NaCl (pH 7.5) at a flow rate of 1 ml min^{-1}. The eluate was monitored at 280 nm, the maximum wavelength of the strong light absorption band of the proteins. The amino acid compositions of the purified ferritins were determined by hydrolysis of the proteins under acidic vapours HCl + TFA (trifluoracetic acid) for 45 min at 150°C. The amino acids in the hydrolysates were analyzed on a Beckman 6300 amino acid analyzer. Methionine and cysteine contents were determined after performic acid oxidation and 6M HCl hydrolysis by HPLC using a Pico-Tag system (Waters Associates). Protein concentrations were determined by the method of Lowry et al. (1951), using bovine serum albumin as a standard. Iron was quantified by the absorption at 560 nm of the Fe (II)-ferrozine [3(2-pyridyl)-5-6bis(4-phenylsulfonic acid)-1, 2, 4 triazine] complex formed after reduction of ferritin iron with thioglycolic acid (Carter 1971).

^{210}Po was determined using ^{209}Po as an internal tracer for radiochemical yield determination and measured on low background silicon surface barrier detectors (EG&G ORTEC) (Carvalho & Fowler 1994).

RESULTS

The ferritins were purified by salt fractionation and ion exchange chromatography followed by Sephacryl S300 gel filtration. Following purification, the ferritins of mackerel and oyster showed only 1 peak, and they displayed only 1 or 2 bands in SDS-PAGE migrating at the level of ferritin subunits (18.5 to 21 kDa) (Fig. 1). The same purified fractions were used for ^{210}Po determinations and amino acid analyses.

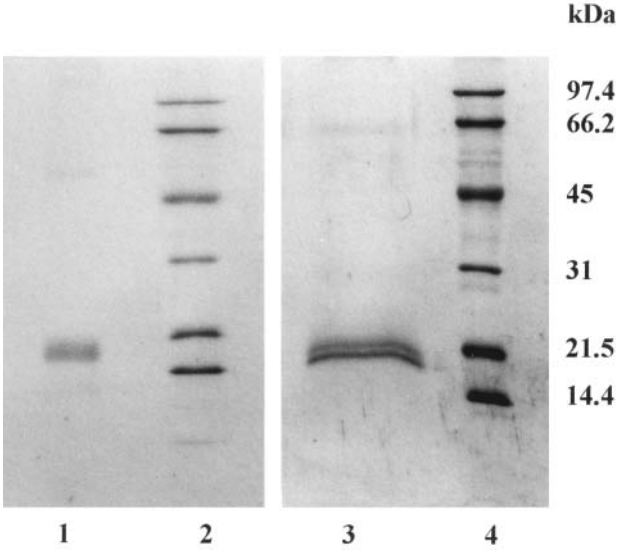

Fig. 1. Sodium dodecyl sulfate (SDS) gel electrophoretic pattern of purified ferritins. Lane 1: oyster *Crassostrea gigas* ferritin; Lane 2: low molecular weight markers (Bio-Rad); Lane 3: mackerel *Scomber scombrus* ferritin; Lane 4: low molecular weight markers (Bio-Rad)

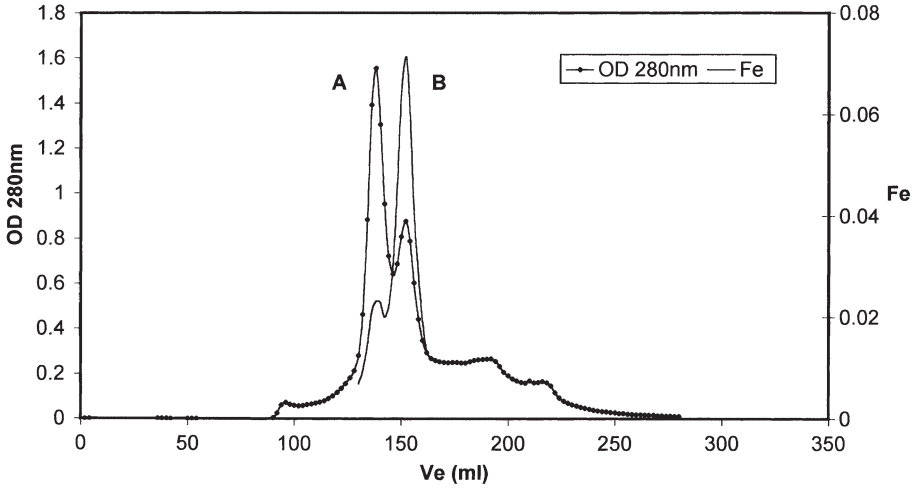

Fig. 2. Chromatography of lobster *Homarus gammarus* ferritin (Sephacryl S300 fraction) on a HPLC TSK G4000 SW column. Distribution of proteins by absorbance at 280 nm (optical density [OD]). Distribution of iron by spectrophotometric determination according to Carter (1971), in arbitrary units. Ve: elution volume. Iron elutes in peak B containing ferritin and hemocyanin. Peak A contains predominantly hemocyanin

Table 1. Amino acid composition (mole%) of mackerel, oyster and horse ferritin

Amino acid	Mackerel ferritin	Oyster ferritin	Horse ferritin[a]
Cys	3.3	0	1.7
Asx	11	13	10.7
Glx	12.6	19.1	14.8
Ser	6.3	6.9	5.6
Gly	7.6	10.8	6.1
His	2.5	2.9	3.6
Arg	4.2	4.5	5.9
Thr	4.9	2.9	3.4
Ala	7.8	7.3	8.7
Pro	6.3	1.7	1.7
Tyr	2.3	0.3	3.1
Val	5.1	4.2	4.2
Met	2.7	0.5	1.7
Ileu	3.6	3	2.1
leu	9.6	9.6	15.5
Phe	4.5	4.1	4.5
Lys	4.9	6.7	5.4
Total acidic	23.6	32.1	25.5
Total basic	11.6	14.1	14.9

[a]Crichton et al. (1973)

The Sephacryl S300 chromatographic separation of lobster ferritin revealed the presence of contaminants. The ferritin present in the main peak was heterogeneous electrophoretically. This fraction was further purified by HPLC on a TSK G4000 SW column (Fig. 2). Two peaks of proteins were obtained; however, the second peak (B), which corresponded to the ferritin elution volume and showed the presence of iron, was also heterogeneous electrophoretically. The first peak (A) was identified as hemocyanin using SDS-PAGE and by comparison with the electrophoretic mobility of lobster hemocyanin subunits (molecular masses: 70 to 80 kDa) obtained from lobster hemolymph (Fig. 3). These 2 fractions (A and B) were used for the ^{210}Po analysis.

The molecular weights of mackerel and oyster ferritin (410 kDa and 420 kDa, respectively) were determined by chromatography on a calibrated HPLC TSK G4000 SW column. The amino acid composition of the oyster and mackerel ferritin is given in Table 1 and is compared with horse spleen ferritin. Both oyster and mackerel ferritin contain 10% or more aspartic and glutamic acid, and these compositions are very similar to that of

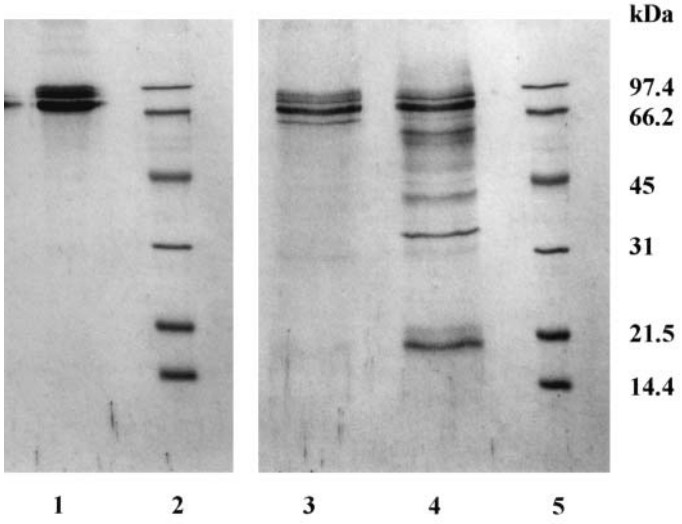

Fig. 3. SDS gel electrophoretic pattern of HPLC lobster ferritin fractions. Lane 1: lobster hemocyanin; Lane 2: low molecular weight markers (Bio-Rad); Lane 3: HPLC fraction peak A; Lane 4: HPLC fraction peak B; Lane 5: low molecular weight markers (Bio-Rad)

horse spleen ferritin. Very low quantities of sulfur-containing amino acids were found in the ferritin of the 2 species.

The ^{210}Po concentrations in the whole organs are presented in Table 2. The ^{210}Po concentrations in Sephacryl S300 purified oyster, mackerel and lobster ferritin are shown in Table 3. The concentration of ^{210}Po in the lobster ferritin fraction is 13 times greater than in oyster ferritin and 18 times higher than in that of mackerel; however, electrophoretic and chromatographic analyses showed that the lobster ferritin fraction was not pure. HPLC analysis of this fraction indicated the presence of hemocyanin (peak A), which also contributed to the ^{210}Po binding.

DISCUSSION

The method of ferritin purification essentially followed those of Crichton et al. (1973), Cetinkaya et al. (1985) and Geetha & Deshpande (1999) but with several modifications. The major modification was the use of a lower temperature for the thermal denaturation (65°C instead of 75°C). This lower temperature enhances the conservation of ferritin. The purification was also improved by an additional ion exchange step before Sephacryl S300 gel filtration chromatography to eliminate a greater quantity of contaminating proteins. The purified mackerel ferritin was very similar to marine fish ferritins previously described by Geetha & Deshpande (1999). The molecular mass of 410 kDa determined by HPLC analysis is in good agreement with the values reported for ferritin of different fish species. SDS-PAGE showed a double protein band of 18.5 to 21 kDa, reflecting a certain degree of subunit heterogeneity observed for fishes and various other species (Andersen et al. 1995, Theil 1987).

We reported here the purification of oyster ferritin, a protein that was first identified in the tropical rock oyster *Saccostrea cucullata* (Webb et al. 1985). Its molecular mass of 420 kDa is in the range of values determined for the other species. Following SDS-PAGE, unlike with mackerel, a double protein band was not observed; nevertheless, the polypeptide obtained migrated at a similar molecular weight of 18.5 to 21 kDa.

The amino acid composition of both mackerel and oyster ferritin was comparable to that of the other species. As with horse spleen ferritin, mackerel and oyster ferritin contained high concentrations of aspartic and glutamic acids. Different authors have shown the fixation of monoatomic metal ions on apoferritin, e.g. Cd(II), Zn(II), Mn(II), Tb(II), UO(III) and VO(IV). Some of these metals alter the rate of iron core formation and probably bind to protein sites that play a role in accumulation of iron in the core of ferritin. Carboxylate residues (Asp and Glu) also appear to be involved in these binding sites (Theil 1987, Theil et al. 2000), and such sites may also be important in ^{210}Po fixation on ferritin.

Table 2. ^{210}Po concentrations (µBq mg^{-1} wet weight) in mackerel liver, oyster digestive gland and lobster hepatopancreas

	Mackerel liver	Oyster digestive gland	Lobster hepatopancreas
^{210}Po	36 ± 3	261 ± 65	332 ± 10

Table 3. ^{210}Po concentrations (µBq mg^{-1} of protein) in mackerel, oyster and lobster ferritins obtained from Sephacryl S300 chromatography and ^{210}Po concentrations in peak A (hemocyanin) and in peak B (ferritin/hemocyanin) obtained from TSK G4000SW chromatography of lobster ferritin (Fig. 2)

Sephacryl S300 fractions

	Mackerel ferritin	Oyster ferritin	Lobster ferritin[a]
^{210}Po	206 ± 14	280 ± 18	3678 ± 413

TSK G4000SW fractions of lobster ferritin[a]

	Peak A	Peak B
^{210}Po	1179 ± 90	704 ± 78

[a]Ferritin separation was not pure and contained hemocyanin as a contaminant

Lobster ferritin purification proved to be more difficult because of the abundance of hemocyanin present in lobster hepatopancreas, a protein that is not found in fish or in lamellibranch molluscs (George 1982). Furthermore, hemocyanin behavior is very similar to that of ferritin in ion exchange chromatography. The 2 molecules, which probably have very similar isoelectric points, eluted at almost the same level of the NaCl gradient and therefore could not be separated efficiently by this method. The remaining hemocyanin present in the ferritin sample applied on the size-exclusion chromatographic columns caused an overlapping of peaks of the 2 proteins that eluted in a narrow range. It was noted in preliminary experiments that the chromatographic columns had a relatively low resolution power for these high molecular weights (400 to 800 kDa). Thus, these chromatographic purifications (Sephacryl S300 and TSK G4000SW, successively) did not allow a complete separation of ferritin and hemocyanin, as shown by the SDS-PAGE results. Nevertheless, our experiments demonstrated a double protein band at 18.5 to 21 kDa corresponding to ferritin subunits, an observation that corroborates ferritin subunit heterogeneity reported for other crustaceans species (Huang et al. 1996).

Several authors have demonstrated the presence of ^{210}Po in whole fish, molluscs and crustaceans, but fewer works exist on the distribution of the radionuclide in the different organs of these species. In the case of fish, detailed studies of mackerel have shown the following specific activities of ^{210}Po: muscle, 3.5 Bq kg^{-1}; liver, 203 Bq kg^{-1}; gonad, 94 Bq kg^{-1}; stomach, 105 Bq kg^{-1}; caeca, 529 Bq kg^{-1}; and intestine, 803 Bq kg^{-1}wet wt (Carvalho 1990). In other fish species, reported ^{210}Po contents of internal organs lie within a broad range, from 2 Bq kg^{-1} wet wt in muscle to 204 Bq kg^{-1} wet wt in intestine. Skwarzec (1988) has reported differing ^{210}Po concentrations in fish liver, e.g. 138 Bq kg^{-1} wet wt (*Gadus morrhua*), 9 Bq kg^{-1} wet wt (*Clupea harengus*) and 13 Bq kg^{-1} wet wt (*Pleuronectes flesus*). Far less information is available on the tissue distribution of this radionuclide in bivalve molluscs. For example, Gouvea et al. (1987) reported the following distribution in *Perna perna*: visceral mass, 1090 mBq g^{-1}; muscle, gill and mantle, 905 mBq g^{-1}; byssus, 79 mBq g^{-1}; and shell 3 mBq g^{-1} dry wt. In another mussel species *Mytilus trossulus*, the hepatopancreas contained 1026 Bq kg^{-1}; gills, 232 Bq kg^{-1}; muscle, 56.5 Bq kg^{-1}; shell, 0.9 Bq kg^{-1}; and byssal threads, 30 Bq kg^{-1} dry wt (Stepnowski & Skwarzec 2000b). Likewise, in a species of clam *Mya arenaria*, ^{210}Po in hepatopancreas was 87 Bq kg^{-1}; gills, 29.2 Bq kg^{-1}; muscle, 4.9 Bq kg^{-1}; and shell, 0.4 Bq kg^{-1} dry wt (Stepnowski & Skwarzec 2000b). For crustaceans, Heyraud & Cherry (1979) determined a polonium concentration in the euphausiid *Meganyctiphanes norvegica* and the crab *Carcinus maenas* that was 10 times and 32 times higher in hepatopancreas than in muscle, respectively. It is evident from all the species studied that the digestive organs are important accumulation sites for polonium.

The concentrations of polonium in the liver of mackerel, in the digestive gland of oyster and in lobster hepatopancreas (Table 2) are in accordance with the levels found by other authors in similar species (McDonald et al. 1993, Bellamy & Hunter 1997, Stepnowski & Skwarzec 2000a,b). Concentrations of ^{210}Po observed in ferritin fractions (Table 3) show that ^{210}Po is measurable in ferritin (or in ferritin/hemocyanin) fractions. However, at present, it is very difficult to estimate what percentage of the ^{210}Po in the organ is associated with ferritin or hemocyanin. We do not know the actual concentration of ferritin or hemocyanin in the different organs of these marine species, and it is difficult to assess the actual yield of our preparations of ferritin. This could be achieved by iron concentration evaluation, but the iron storage in ferritin is very variable. Ferritins may contain just a few iron atoms or up to about 4500 (Harrison & Arosio 1996). Furthermore, The concentrations of ^{210}Po measured on extracted ferritin fractions are not necessarily the actual amount of ^{210}Po associated with ferritin in the intact cell. The extraction method was adjusted to obtain a pure ferritin, and different steps involve very high ionic strengths (ammonium sulfate precipitations), which can dissociate a part of the polonium from the ferritin. This partial dissociation can also occur during the different successive chromatographic separations, as was observed in previous experiments with other radionuclides. The salient fact is that a significant amount of ^{210}Po was still present on ferritin, hemocyanin or both after the purification process. This observation indicates that ^{210}Po is sequestered by some high affinity sites in ferritin, and this is even more evident in the case of hemocyanin. However, in previous works on subcellular distribution, we found 80% of polonium in the cytosolic fraction of *Scomber scombrus* liver. This distribution may be different in crustaceans; other authors have shown an important fixation of ^{210}Po in the microsomal fraction in the lobster hepatopancreas (Heyraud et al. 1987). On the other hand, in mackerel liver at the cytosolic level, the distribution of ^{210}Po is approximately equivalent between metallothioneins and ferritin (28 and 30% of fixation, respectively), so obviously ferritin is not the only binding site at the cellular level (Durand et al. 1999).

Other authors have found significant correlations between iron and ^{210}Po uptake in the digestive gland of squid *Nototodarus gouldi* (Smith et al. 1984), and subcellular fractionation of the abalone *Haliotis rubra*

digestive gland demonstrated a high ^{210}Po content in iron-containing granules (Hyne et al. 1992). Our results have shown that iron-containing ferritin is also significantly involved in the fixation of ^{210}Po in molluscs and crustaceans. These facts may indicate a common metabolic pathway between iron and polonium, and may help explain the high level of ^{210}Po observed in the red muscle of teleost fish or in marine mammal muscle, which contain iron in the heme protein myoglobin (Cherry et al. 1994). Ferritin, an iron-storage protein, consists of an apoprotein shell of 24 subunits around a core of iron in the form of ferric-hydroxyphosphate. It can bind various metals on different types of sites: within the cavity, near the inner channel openings or the outside of apoferritin (Price & Joshi 1983, Pead et al. 1995). Ferritin can also bind transuranium elements (Goudard et al. 1991, Milcent et al. 1996). ^{210}Po is probably able to bind to these different sites present on ferritin and perhaps also to the iron core of the protein. Amino acid analysis showed very small amounts of cysteinyl residues in the ferritins of mackerel and oyster. Thus, the mechanisms of polonium fixation to ferritin are probably quite different from those involved in metallothioneins, in which cysteines are very abundant. Sulfhydryl groups (-SH) have long been suspected as important binding sites for ^{210}Po on cytoplasmic proteins (Hill 1965, Lanzola et al. 1973, Heyraud et al. 1987, Wildgust et al. 1999); however, this does not seem to be the case for ferritin. According to Wildgust et al. (1998), ^{210}Po seems to behave like a class B metal but shows positive correlation with borderline metals and some class A metals. This special physico-chemical character allows it to bind to -SH like those present in metallothioneins, but also to groups with oxygen as the donor atom (phosphate, carboxilic acid and carbonyl); this may explain why polonium shows a strong affinity for other types of molecules with other chemical characteristics like ferritin and hemocyanin. On ferritin, different sites of fixation are possible, e.g., on the protein shell (amino and carboxylic functional groups) or on the iron(III) hydroxide core, which also contains phosphate groups.

For the lobster, the ^{210}Po concentrations in the fractions isolated by TSK G4000 SW HPLC analysis indicated a significant contribution of hemocyanin to the binding of ^{210}Po in the hepatopancreas of this crustacean. The SDS-PAGE isolation of this fraction revealed several protein bands (molecular weight 70 to 80 kDa), which correspond to those obtained with hemocyanin from lobster hemolymph (Klarman & Daniel 1981, Mangum 1993, Stoeva et al. 1999). This oligomeric copper-containing metalloprotein, synthesized in the hepatopancreas (Senkbeil & Wriston 1981), is able to bind different metal ions (Brouwer et al. 1983), and the active site of metal fixation may be able to bind polonium. The binding of ^{210}Po on hemocyanin has not been previously demonstrated, but a role for this protein has been suggested in the internal transfer of some radionuclides (Guary & Fowler 1990). Although the HPLC ferritin fraction was not pure, it seems clear that the degree of ^{210}Po binding to lobster ferritin is probably similar to that of the other species of fish and molluscs, and in the case of lobster ^{210}Po appears predominantly associated with hemocyanin.

We conclude that ferritin effectively plays a significant role in polonium accumulation in the different species studied. However, in crustaceans other specific vectors, especially respiratory pigments like hemocyanin, are important binding molecules for ^{210}Po. This fact may explain the very high levels of polonium found in crustacean hepatopancreas, which is the site of hemocyanin synthesis; thus, further work is needed to improve our knowledge on the mechanisms of ^{210}Po binding to hemocyanin.

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