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Novel heme-binding component in the serum of the channel catfish (*Ictalurus punctatus*)

George Massad, Jean E. L. Arceneaux & B. Rowe Byers

Department of Microbiology, University of Mississippi Medical Center, Jackson, MS, USA

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The serum of the channel catfish (*Ictalurus punctatus*) was examined for heme- and hemoglobin-binding proteins. Electrophoretic mobility retardation assays failed to detect a hemoglobin-binding material similar to mammalian haptoglobin; however, a heme-binding component (not previously described) was identified in catfish serum. The heme-binding component was purified by gel filtration chromatography; electrophoretic analyses suggested it to be composed of two polypeptide subunits of molecular masses about 115 and 98 kDa. This composition is inconsistent with hemopexin, the known heme-binding serum protein of mammals. Although it was not fully saturated with heme, the catfish component contained detectable heme in normal sera. When complexed by the binding material, heme was used as an iron source by isolates of the bacterial Gram-negative genus *Aeromonas*; the capacity of other bacteria to use the complex was not tested. The physiological function of the catfish heme-binding serum protein is presently not clear.

Keywords: heme-binding serum protein, hemopexin, haptoglobin, *Ictalurus punctatus, Aeromonas* species

Introduction

Iron is an indispensable cofactor for several fundamental metabolic processes, but the undisciplined presence of the metal can be fatal for biological systems (Byers 1987). Too much iron in a vertebrate predisposes the animal to infection by circumventing its iron-withholding defenses and promoting microbial growth (Weinberg 1984, Byers 1987). Iron also generates the universally malefic oxygen radicals, inducing membrane lipid peroxidation, as well as strand breaks and other damage to DNA. An increased body iron level correlates with elevated risk of cancer (Weinberg 1984, Stevens *et al.* 1986, Shelby & Friedman 1988, Stevens *et al.* 1988) and iron bound by asbestos has been implicated as the agent responsible for the toxicity and carcinogenicity of asbestos (Lund & Aust 1991). To surmount some of these problems, mammals produce three plasma proteins (transferrin, haptoglobin and hemopexin) whose purposes are the safe management and movement of iron and heme-containing molecules (Putnam 1975). In the present paper, heme is used as a general term referring to iron–protoporphyrin complexes. Transferrin binds the iron atom itself, while haptoglobin and hemopexin associate with hemoglobin and heme, respectively.

Transferrin is a β-globulin of vertebrate plasma with the outstanding property of reversible binding of ferric iron (de Jong *et al.* 1990). The protein consists of a single polypeptide chain (with two *N*-linked carbohydrate moieties) which contains two single atom iron-binding centers; the molecular mass of human transferrin is about 79 kDa (de Jong *et al.* 1990). Transport and delivery of iron to cells is a major function of transferrin, and it may be essential for growth and differentiation of some cells (de Jong *et al.* 1990). Transferrin is also a notable member of the iron-withholding defenses allied against microbial invasion of a vertebrate host, although pathogenic microorganisms can obtain iron from Fe-transferrin either with an appropriate siderophore or by synthesizing an external transferrin receptor (reviewed in Weinberg 1984, Byers 1987).

Mammalian hemopexin is a plasma β-glycoprotein, of molecular mass about 60 kDa, which binds heme in stoichiometric amounts (Hrkal & Muller-

Address for correspondence: B. R. Byers, Department of Microbiology, University of Mississippi Medical Center, 2500 North State Street, Jackson, MS 39216-4505, USA. Fax: (601) 984-1708.

Eberhard 1971). It transports heme to cells, including the liver, where the iron is separated for reuse (Sears 1970). Cellular uptake of hemopexin involves receptor-mediated endocytosis with recycling of the intact protein, similar to recycling of transferrin after its delivery of iron (Smith & Hunt 1990, Smith *et al.* 1991). Also, like transferrin, hemopexin functions as an antioxidant, inhibiting deleterious reactions such as oxidative degradation of unsaturated lipids (Gutteridge & Smith 1988). Hemopexin may assist in iron-withholding defense against microbes. Binding of heme by hemopexin prevents use of its iron by some *Neisseria* species (Dyer *et al.* 1987) but not by *Haemophilus influenzae* (Stull 1987).

The haptoglobin of mammals is an α_2-globulin that forms a stable complex with hemoglobin. This complex is rapidly cleared by the reticuloendothelial system but, unlike transferrin and hemopexin, the entire hemoglobin–haptoglobin complex is metabolized and haptoglobin is not recycled (Krauss & Sarcione 1966, Chancone *et al.* 1968, Putnam 1975). There are three major human haptoglobin phenotypes, one being monomeric (composed of α and β chains) while the others are polymeric of quite different molecular masses (Sutton 1970, Putnam 1975). A major biological function of haptoglobin is to prevent loss of iron through urinary excretion and to protect the kidney from damage by hemoglobin (Putnam 1975). A defence function against microbial infection has been suggested for haptoglobin because a pathogenic strain of *Escherichia coli* is unable to obtain iron from the hemoglobin–haptoglobin complex (Eaton *et al.* 1982). However, other bacteria use such complexes as a sole iron source (Francis *et al.* 1985, Dyer *et al.* 1987, Stull 1987, Zakaria-Meehan *et al.* 1988, Massad *et al.* 1991).

Although transferrin-like proteins have been identified in both vertebrates and invertebrates (de Jong *et al.* 1990), less is known about the occurrence of hemopexin and haptoglobin outside of mammals. In fish, hemoglobin-binding serum proteins similar to haptoglobin exist in carp (Creyssel *et al.* 1964) but they are absent from trout (Perrier *et al.* 1974). Fish transferrins have significant iron-withholding bacteriostatic activity. At least two (*Vibrio anguillarum* and the *Aeromonas* species) Gram-negative bacterial pathogens of fish must produce certain siderophores in order to acquire iron from Fe-transferrin (Crosa 1984, Massad *et al.* 1991). For example, those isolates of the *Aeromonas* species synthesizing the siderophore amonabactin use Fe-transferrin in catfish serum as an iron source while other strains producing the siderophore enterobactin fail to use Fe-transferrin in serum. Regardless of the siderophore synthesized, all isolates of the *Aeromonas* species grow with either heme or hemoglobin as a sole iron supply (Massad *et al.* 1991). Whether or not the ability to acquire iron from heme and hemoglobin is important in aeromonad infections of fish may depend on the presence of hemopexin-like and haptoglobin-like proteins in fish plasma that could restrict utilization of these iron depots by the bacteria. These possibilities were examined in the channel catfish (*Ictalurus punctatus*).

Materials and methods

Analyses of hemoglobin- and heme-binding capacities of channel catfish serum

Whole sera were prepared from catfish blood obtained by venipuncture of fish kept in the laboratory animal facility or at commercial processing plants. Catfish were maintained in the laboratory in plastic tanks equipped with recirculating water that was passed through a biological filter. The laboratory fish were fed commercial catfish food and were exposed to a photoperiod of 12-h light, 12-h dark. They were acclimated to the laboratory for at least 3 weeks before use. Whole sera (or fractions of sera obtained by gel filtration as described below) were analyzed for hemoglobin- and heme-binding capacities by an electrophoretic mobility retardation assay. Either hematin (Sigma, St Louis, MO, USA) or catfish hemoglobin, prepared from freshly collected blood as described by Massad *et al.* (1991), was added to the samples which then were analyzed by non-denaturing polyacrylamide gel electrophoresis (NPAGE) with 6% gels and NPAGE buffer consisting of 150 mM Tris and 40 mM glycine (pH 9.2). After electrophoresis, protein bands were demonstrated by staining with Coomassie blue G-250 (Sigma) and heme-containing components in the gels were stained with dimethoxybenzidine (Sigma) by the method of Sutton (1970). Binding of heme and hemoglobin by serum components should alter electrophoretic mobility of these substances in the NPAGE system. If desired, the bands corresponding to those stained by dimethoxybenzidine were electroeluted, according to the manufacturer's instructions, using a model 422 Electro-eluter apparatus (Bio-Rad Instruments, Richmond, CA, USA) from NPAGE gel slices into NPAGE buffer. The eluted material was examined under reducing conditions by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) with 7.5% gels in the discontinuous buffer system of Laemmli (1970).

^{55}Fe labeling and fractionation of channel catfish blood proteins

Three channel catfish (weighing 200 g each) were injected (intraperitoneal) with 120 μCi of [^{55}Fe]ferric citrate (Dupont NEN Research Products, Wilmington, DE, USA) in 0.5 ml 10 mM potassium phosphate pH 7.4 containing

150 mM NaCl. After 2 weeks, blood was collected from each animal and pooled. The ^{55}Fe-labeled hemoglobin was prepared from erythrocytes by the method of Massad *et al.* (1991) and the serum was fractionated by gel filtration on a column (1.5 × 100 cm) of agarose A5M (Bio-Rad Laboratories) that was eluted with Tris buffer (10 mM Tris/HCl, 150 mM NaCl and 5 mM EDTA, pH 7.4). Fractions (3.75 ml) were collected, their protein concentrations estimated by determining A_{280}, and the ^{55}Fe content of each fraction was determined by liquid scintillation counting with Aquasol-2 (Dupont NEN Research Products) and a Tracor Analytic Delta 300 counter. The gel filtration column was calibrated with molecular size markers obtained from Sigma. As a control, unlabeled catfish serum was similarly fractionated. To detect ^{55}Fe-containing components in electrophoretic gels, X-Omat XRP5 film (Eastman Kodak, Rochester, NY, USA) was exposed to the gels for 7 days at −70 °C.

Utilization of catfish heme-binding components by Aeromonas *species*

The assay employed by Massad *et al.* (1991) for bacterial utilization of heme and heme compounds as iron sources by bacteria was used to determine if isolates of the *Aeromonas* species could utilize the catfish heme-binding serum component as a sole source of iron. The test assesses the capacity of a bacterial strain to use heme compounds to reverse growth inhibition caused by the chelating agent ethylenediamine di(*o*-hydroxyphenylacetic acid) (EDDA). A total of 21 isolates of *A. hydrophila,* nine of *A. sobria* and three of *A. caviae* (described by Massad *et al.* 1991) were used in this study.

Results and discussion

Lack of a haptoglobin-like protein in catfish serum

The possible existence of hemoglobin-binding proteins (functionally similar to mammalian haptoglobin) in the serum of the channel catfish was investigated. The electrophoretic mobility of catfish hemoglobin (which appears as four or five bands in the nondenaturing gel electrophoresis system used here) was unaltered by adding catfish hemoglobin to catfish serum (Figure 1). If the hemolglobin had strongly associated with a serum component, its mobility in the gel should have been retarded; lack of an effect on hemoglobin movement means that the catfish probably did not produce a hemoglobin-binding protein like haptoglobin.

Catfish heme-binding serum protein

Although a hemoglobin-binding material was not apparent in catfish sera, the electrophoretograms revealed a heme-containing protein band (detected with the dimethoxybenzidine stain for heme and with the Coomassie reagent for proteins) that migrated more slowly than hemoglobin (Figure 1). This material was evident in sera to which neither hemoglobin nor heme had been added (Figure 1) and it was seen in all serum samples tested, regardless of the source (either fish at commercial plants or laboratory fish).

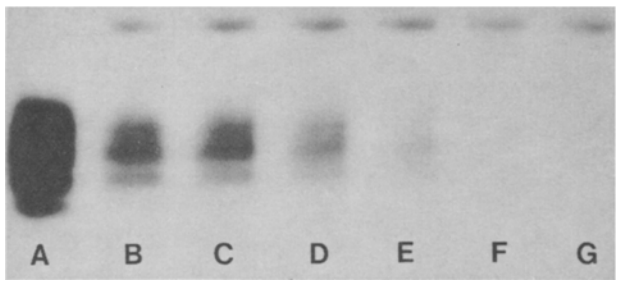

Figure 1. Heme-containing component in channel catfish serum (with and without added catfish hemoglobin) demonstrated by NPAGE and dimethoxybenzidine staining. Lanes: A, catfish hemoglobin only; B–F, catfish hemoglobin added (to catfish serum) in concentrations (mg/ml) of 1, 0.5, 0.25, 0.125 and 0.05, respectively; G, catfish serum only.

In vivo *labeling, purification, and partial characterization of the heme-binding serum component.*

To confirm that the putative heme-binding serum protein contained iron, it was labeled *in vivo* by injecting fish with ^{55}Fe. After allowing 2 weeks for assimilation of the metal, the fish were bled and the sera collected and pooled. It then was fractionated by gel filtration on agarose A5M (Figure 2). Most of the ^{55}Fe was not associated with the bulk of the serum proteins but was found in three radioactive peaks (peaks A, B and C in Figure 2) that corresponded to molecular mass ranges of 200–400, 70–100 and 45–70 kDa, respectively. The respective ^{55}Fe contents of the peaks represented 22, 39 and 38% of the total radioactive iron in the serum. Electrophoretic analyses in non-denaturing polyacrylamide gels and subsequent autoradiographic examinations of the gels (Figure 3) showed a single ^{55}Fe- and heme-containing band in peak A with mobility similar to the heme-binding component noted in whole serum. This material was not fully saturated with heme; adding heme before electrophoresis markedly intensified its dimethoxybenzidine staining reaction (Figure 4). Adding heme to whole serum similarly increased the staining reaction of the component (data not shown). The heme-binding component may be a protein composed of two

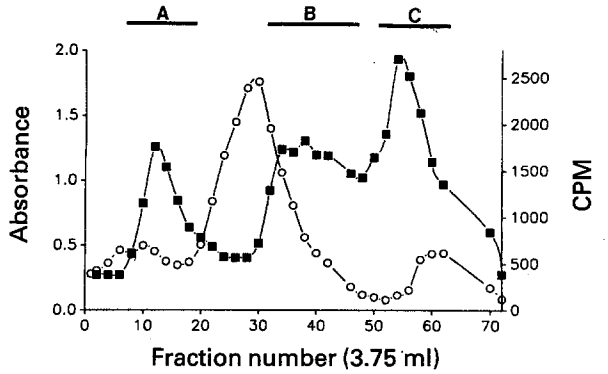

Figure 2. [^{55}Fe]Iron (cpm/50 μl) and protein (A_{280}) elution profiles of channel catfish serum (labeled *in vivo* with ^{55}Fe) fractionated on agarose A5M. Fractions (3.75 ml each) 9–16, 29–42 and 43–62 were pooled to give radioactive peaks A, B and C, respectively. (○—○ = Absorbance (280); ■—■ = cpm).

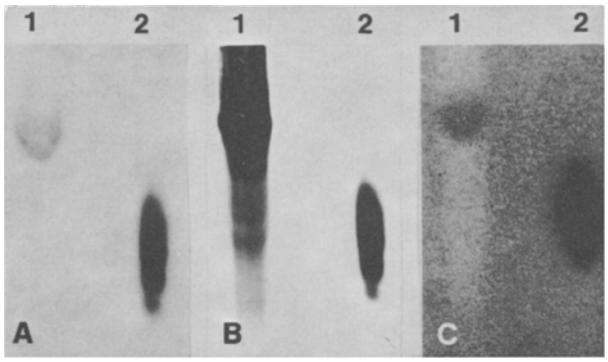

Figure 3. Non-denaturing PAGE of ^{55}Fe-labeled serum proteins. Lanes: 1, [^{55}Fe]iron peak A shown in Figure 2 (50 μg protein); 2, ^{55}Fe-labeled catfish hemoglobin (5 μg protein). (**A**) Gels stained for heme with dimethoxybenzidine; (**B**) gels stained for proteins with Coomassie blue; (**C**) autoradiogram of gels showing ^{55}Fe.

subunits because SDS–PAGE under reducing conditions of material (obtained by electroelution from a non-denaturing electrophoretic gel) showed two polypeptides of molecular masses about 115 and 98 kDa (Figure 5).

Although dimethoxybenzidine staining of electrophoretic gels failed to detect heme in the broad radioactive iron peak B (Figure 2), this peak probably contained a low level of ^{55}Fe-labeled hemoglobin. When unlabeled catfish serum that contained hemoglobin was fractionated on the A5M column, the electrophoretic bands of catfish hemoglobin were present in the fractions corresponding to peak B in Figure 2 (data not shown). [^{55}Fe]Iron

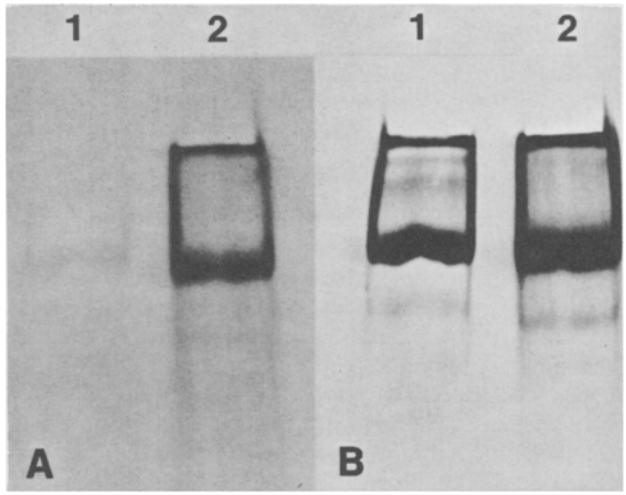

Figure 4. Increased heme binding demonstrated by NPAGE of [^{55}Fe]iron peak A (10 μg protein) shown in Figure 2 with no addition (lanes 1) and 0.83 μg heme added (lanes 2). Gels were stained (**A**) with dimethoxybenzidine for heme and (**B**) with Coomassie blue for protein.

peak C (Figure 2), comprised of material of molecular mass range approximately 45–70 kDa, contained no heme compounds detectable by reaction with dimethoxybenzidine. Fractions equivalent to peak C that were obtained by A5M gel filtration of unlabeled catfish serum contained no heme. Peak C may include ^{55}Fe-labeled catfish transferrin but this was not determined.

Utilization of the serum heme-binding component by Aeromonas *species*

Previous assays (Massad *et al.* 1991) showed that most, if not all, strains of the *Aeromonas* species can use heme as a sole source of iron. The same assay was used to determine if binding of heme to the catfish serum component might hinder use of this material by *Aeromonas* species. All of the 33 isolates tested used heme bound by the serum component to reverse inhibition by the chelating agent EDDA; therefore, formation of the complex did not impede use of iron from heme by the *Aeromonas* species.

Conclusions

We conclude that the channel catfish (*I. punctatus*) does not produce a serum hemoglobin-binding protein (similar to mammalian haptoglobin) that can be demonstrated by the methods used here. Rather, we identified a serum heme-binding component that

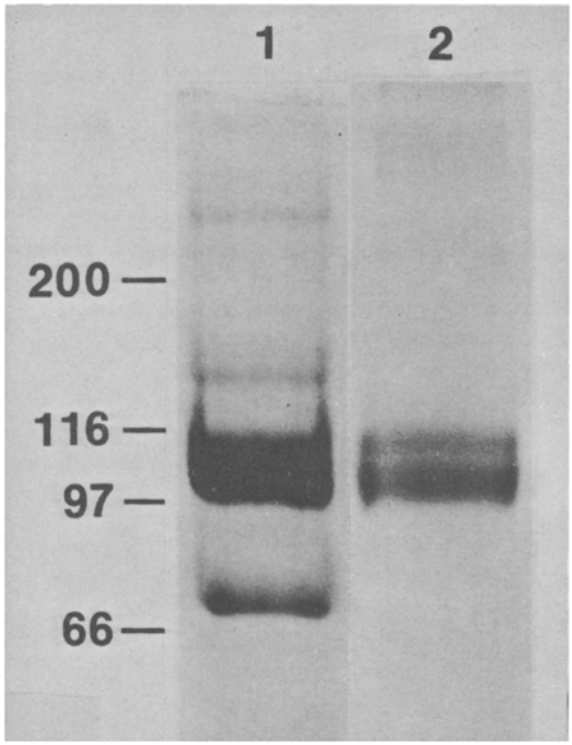

Figure 5. Possible subunits of catfish heme-binding serum component demonstrated by SDS–PAGE under reducing conditions of total polypeptides (10 μg protein) in peak A shown in Figure 2 (lane 1) and the heme-binding component obtained by electroelution of a slice (containing the component) from a non-denaturing electrophoretic gel (lane 2). Positions of molecular mass markers are shown (in kDa) on the left.

(in normal sera) contained detectable heme, but which was not fully saturated. Preliminary characterization suggested a heme-binding protein composed of two polypeptide units of molecular masses about 115 and 98 kDa. This size and composition are inconsistent with the mammalian serum heme-binding protein hemopexin. It is possible that the heme-binding serum protein of the channel catfish is functionally similar to mammalian hemopexin. These possibilities, as well as the heme-binding capacity of the material, should be examined.

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