Short Communication

Zinc Chelates Bind Human Hemopexin

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Hemopexin is a serum β-glycoprotein (M_w = 63 000) that consists of a single polypeptide chain containing 439 amino acid residues and six oligosaccharide chains.[1] It has one high affinity binding site for heme, which binds in a 1:1 molar ratio to hemopexin after transfer from ferrihemoglobin.[2] The heme–hemopexin complex is taken up by specific receptors on the hepatic cell surface.[3] After catabolism of the heme, iron is bound by ferritin and the apohemopexin returns to the circulation.[3,4] Hemopexin plays a physiological role in preventing the loss or deficiency of iron[4] and prevents the toxic effects of heme following tissue damage by acting as an extracellular antioxidant.[5]

It has been shown that hemopexin can bind other metalloporphyrins, such as coproporphyrin I and III (k_d of 0.5 μM) and protoporphyrin IX (k_d of 2 μM), in a 1:1 molar ratio with the same binding site as heme.[6] Zinc porphyrin IX is also bound by hemopexin (k_d = 0.5 M) but in a molar ratio of 2:1.[6] The physiological significance of porphyrins bound to hemopexin is a matter of discussion, since albumin is regarded as the major porphyrin-binding protein in human serum.[6] To our knowledge it has not been reported that hemopexin has an affinity towards the binding of zinc ions. By use of a zinc Sepharose 4B column prepared by the method of Porath *et al.*,[7] it was found that hemopexin at low pH binds with higher affinity than other proteins to the zinc Sepharose. Both α_2-macroglobulin, transferrin, ceruloplasmin and haptoglobin are known to bind to zinc Sepharose.[7]

Experimental

The preparation of α_2-macroglobulin from 200 ml of human plasma was carried out in accordance with the procedure described by Imber and Pizzo.[8] The preparation of pregnancy zone protein (PZP) from human pregnancy serum was by the method of Sand *et al.*[9] Heme–hemopexin was a gift from Dr. Haupt, Marburg, Germany. Elution of hemopexin from the zinc Sepharose 4B (100 ml) was effected by adding EDTA to a final concentration of 20 mM to the normal elution buffer consisting of 0.01 M sodium acetate, 0.15 M NaCl, pH 5.0.[8] After the eluate had been concentrated to 1 ml, it was loaded on a Sephacryl S 300 (26×1.5 cm) column in 0.1 M sodium phosphate, pH 8.0 at 4 °C, and a gel filtration was performed at 10 ml h^{-1} and fractions of 1.4 ml were collected. Binding studies of hemopexin to α_2-macroglobulin were performed by filtration on Sephacryl S-200 and by SDS/non-SDS PAGE Laemmli.[11] These binding studies were performed at 5 min, 30 min, and 4 h incubations at both 36 and 24 °C by use of ^{125}I-labeled hemopexin. [^{125}I]hemopexin was made by use of iodobeads from Pierce. Further techniques used were immunodiffusion[10] and SDS/non-SDS PAGE Laemmli.[11] Absorbance was measured on a Beckman DU-7 Spectrophotometer.

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Results and discussion

During the preparation of α_2-macroglobulin the elution of proteins bound to the zinc Sepharose 4B was effected by adding EDTA to the normal elution buffer. Gel filtration on Sephacryl S-300 of the eluted proteins gave two peaks as seen in Fig. 1. By use of double immunodiffusion, SDS PAGE and protein sequencing (not shown) the first peak (P.1) was found to contain α_2-macroglobulin and the second peak (P.2) was hemopexin. In the preparation of the pregnancy zone protein (PZP) from human pregnancy serum, hemopexin was similarly detected in the elution step from the zinc Sepharose together with PZP (not shown).

As judged by spectrophotometer scans of the eluted hemopexin fractions the absence of absorbance at 412 nm indicates that the hemopexin is in the apohemopexin form.

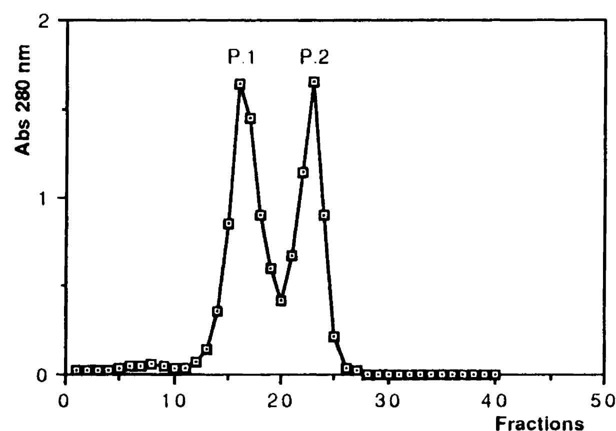

Fig. 1. Gel filtration on Sephacryl S-300 of the eluate from the zinc Sepharose 4B column after addition of EDTA to the elution buffer. P.1 and P.2 represent α_2-macroglobulin and hemopexin, respectively.

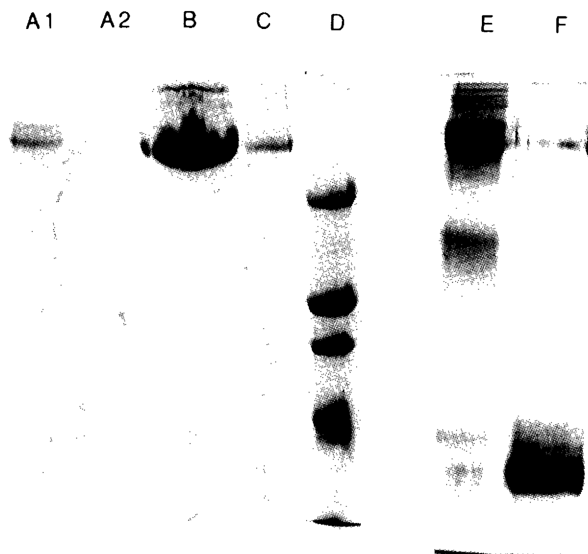

Fig. 2. SDS-polyacrylamide (7.5 %) gel electrophoresis performed on: A1, sodium acetate elution; A2, EDTA elution from zinc Sepharose column after elution with sodium acetate (A1); B, EDTA elution without sodium acetate elution; C, dimers of purified α_2-macroglobulin (360 000 Da); D, marker proteins: myosin (200 000 Da), β-galactosidase (116 250 Da), phosphorylase b (97 400 Da), bovine serum albumin (66 200 Da). The lanes A1, A2, B, C and D were stained with Coomassie Brilliant Blue G 250. E and F are the same as in A1 and A2, respectively, but silver-stained.[13]

Binding studies of apohemopexin and heme–hemopexin to the zinc Sepharose 4B indicated no difference in binding ability. This suggests that hemopexin does not bind to the zinc Sepharose at the heme binding site, an observation which is in accordance with that of Lönnerdal and Keen,[12] who noted that several proteins are able to bind to zinc Sepharose and be recovered in their initial metal-saturated form. Furthermore, they suggest that the binding is, in part, due to the propensity of the chelate metal to bind to surface-exposed groups of the protein, properties which a metal in its 'free' form does not have.

A change in the buffer composition used for equilibration and elution from the zinc Sepharose step indicated that almost pure hemopexin was eluted from the column. Human plasma (30 ml) was dialyzed against distilled water for 3 days (with three exchanges of water) at 4 °C. After centrifugation for 20 min at 15 000×*g*, zinc Sepharose 4B (10 ml equilibrated with 0.1 M sodium phosphate, pH 8.0) was added to the supernatant at room temperature. After thorough washing with 0.1 M sodium phosphate, pH 8.0, the bound proteins were eluted by use of 0.25 M sodium acetate, 0.15 M NaCl, pH 5.0. α_2-macroglobulin was the major eluent with trace amounts of ceruloplasmin, haptoglobin and transferrin. Further elution was then carried out by adding EDTA to a concentration of 20 mM to the sodium acetate buffer and almost pure hemopexin was eluted with trace amounts of α_2-macroglobulin (Fig. 2). One gel filtration on Sephacryl S-300 yields pure hemopexin. A similar procedure was performed, but omiting the sodium acetate elution step, and all the above-mentioned proteins were eluted by the EDTA (Fig. 2).

To ascertain that the binding of hemopexin in the zinc Sepharose column was not correlated with binding of hemopexin to α_2-macroglobulin, binding studies were performed to test whether hemopexin binds to α_2-macroglobulin. The conclusive result was that neither apohemopexin nor hemopexin binds to native or the complexed form of α_2-macroglobulin. Furthermore, hemopexin does not bind to the free thiol group liberated from the thiol ester in α_2-macroglobulin during incubation with the nucleophile methylamine, which is known to transform the native α_2-macroglobulin into the receptor-recognized form.

The binding profiles suggest that hemopexin binds more strongly to zinc Sepharose, at low pH, under the conditions given, compared with haptoglobin, transferrin, ceruloplasmin and α_2-macroglobulin; this is in accordance with the acid-stable properties described for hemopexin.[14]

We suggest that the amount of plasma hemopexin which binds to zinc Sepharose is dependent on the saturation of the column by α_2-macroglobulin – the higher the ratio of zinc Sepharose to plasma (or α_2-macroglobulin), the more the proteins will bind, owing to the presence of more non-saturated zinc Sepharose.

The use of metal chelate affinity chromatography can be used as a new method of purifying hemopexin from plasma. Since no binding of zinc ions to hemopexin has been reported, it is questionable whether hemopexin plays any physiological role in the binding of zinc ions *in vivo*. It is possible that hemopexin is unable to bind 'free' zinc in the plasma, but this remains to be tested.

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