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# Divalent cation binding to ceruloplasmin

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**Binding of calcium to human and sheep ceruloplasmin was investigated by metal substitution with manganese and competitive displacement of bound manganese by calcium monitored by electron paramagnetic resonance spectroscopy. The $K_d$ for calcium was found to be 1.4 mM. Magnesium also bound to ceruloplasmin, with $K_d = 0.3$ and 0.7 mM for the human and sheep protein, respectively. The thermal stability of ceruloplasmin, as studied by differential scanning calorimetry, was affected by calcium but not by magnesium. A considerable increase of the $T_m$ value, from 73.8 to 83.1°C, was observed for sheep ceruloplasmin in the presence of calcium. The $T_m$ value of the human protein was only slightly altered by calcium (from 85.1 to 87°C). The interaction of ceruloplasmin with the chromatographic material used for its isolation, Sepharose 4B derivatized with chloroethylamine, was weakened by calcium. This allowed us to set up a novel purification scheme that made it possible to efficiently isolate ceruloplasmin and prothrombin from plasma with the same single-step chromatography.**

**Keywords:** calcium, ceruloplasmin, prothrombin

## Introduction

Ceruloplasmin is a blue copper-containing protein present in the plasma of vertebrates. The protein bears five to six copper atoms strongly associated to sites with different spectroscopic and functional properties. Ceruloplasmin exhibits the enzymatic activity typical of multinuclear copper oxidases, with the prosthetic copper atoms acting as electron shuttles from a variety of substrates to achieve the complete reduction of dioxygen to water. The physiological substrate of ceruloplasmin is still unknown. *In vitro*, the substrate which shows the lowest $K_m$ and highest $V_{max}$ appears to be Fe(II) (Frieden 1980). A role for ceruloplasmin in iron metabolism, mediated by its ferroxidase activity, has recently received a confirmation (Harris *et al.* 1995). Ceruloplasmin is also involved in copper homeostasis and has repeatedly been shown to be able to exchange $^{67}Cu$ with tissues (Campbell *et al.* 1981) and copper enzymes (Dameron & Harris 1987). Studies with the human erythroleukemic K562 cell line have demonstrated that copper exchange appears to be a receptor-mediated process in which only the metal enters the cell (Percival & Harris 1990). Receptors for ceruloplasmin have been identified on various cell types, including erythrocytes (Barnes & Frieden 1984), monocytes, granulocytes and lymphocytes (Kataoka & Tavassoli 1985), liver endothelium (Omoto & Tavassoli 1990), and Kupffer cells (Dini *et al.* 1990).

Additional copper atoms can interact with non-prosthetic sites on ceruloplasmin, two to six sites with $K_d = 10\ \mu M$ have been reported for different mammalian ceruloplasmins (Zgirski & Frieden 1990). Transition metal ions, such as zinc, nickel and cobalt, have been shown to exhert an inhibitory effect on the oxidase activity of the protein (McKee & Frieden 1971). Thus, the presence on ceruloplasmin of multiple metal-binding sites other than the prosthetic sites has been repeatedly suggested. Different experimental evidence indicates that divalent cations other than transition metals might be able to bind to ceruloplasmin. As a matter of fact, the interaction of ceruloplasmin with its receptor on red blood cells appears to be mediated by calcium ions, which markedly increase binding of the human protein to cells (Saenko & Yaropolov, 1990). However, this finding has not been further circumstantiated, and no report exists in the literature on a possible interaction between ceruloplasmin and calcium.

Direct measurement of the binding of calcium to proteins

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can be obtained only by using particular isotopes of this metal ion, such as radioactive $^{45}Ca$ (Deerfield *et al.* 1987) or $^{43}Ca$, which has a nuclear spin 7/2 and is therefore amenable for NMR studies (Forsen *et al.* 1993). However, unless the chemical exchange rate between bound and free $Ca^{2+}$ is very slow or very fast, the appearance of the $^{43}Ca$-NMR spectrum strongly depends in a complex way on the exchange rate, the $Ca^{2+}$ binding constant and the relaxation rate. The analysis is therefore not straightforward, and needs to be supported by independent methods for the exchange rate assessment. On the other hand, equilibrium binding experiments with $^{45}Ca$ often require prolonged incubation times and/or high concentrations of the protein, depending on the affinity for calcium. Ceruloplasmin is an extremely labile protein and manipulations like these might be too challenging. In order to spectroscopically probe the calcium-binding sites in several systems, it has been found to be convenient to perform metal substitution studies, as calcium has few spectral properties which can be used to investigate its environment. Typical examples are those involving terbium and other lanthanides as fluorescent probes (Brittain *et al.* 1976, Cierniewski *et al.* 1994), and paramagnetic metal probes to be used both in electron paramagnetic resonance (EPR) and NMR studies (Mildvan & Cohn 1970, Kemple *et al.* 1990). Manganese is particularly well suited for these studies, as the EPR signal of the metal bound to large molecules is virtually undetectable, allowing the direct measure of the free metal ion concentration from the intensity of the EPR signal. Thus, titration of a protein with manganese has the advantage of yielding both the binding constants and the number of binding sites in a rapid and reliable manner (Jaffe *et al.* 1995).

In this paper, the binding of calcium to ceruloplasmin has been investigated by competition with manganese followed by EPR spectroscopy. Then the effect of the metal on the conformation of the protein has been studied.

## Materials and methods

All reagents were of maximum purity available and were used as such, except chloroethylamine hydrochloride (Carlo Erba-Farmitalia, Milan, Italy) which was recrystallized before use. Sepharose 4B was from Pharmacia-LKB (Uppsala, Sweden). Ecarin was from Sigma (St Louis, MO).

Ceruloplasmin was purified from human or sheep plasma as previously described (Calabrese *et al.* 1989, Musci *et al.* 1993).

SDS–PAGE was performed according to Laemmli (1970) with the Biorad Mini Protean II system. The gel slabs were stained with Coomassie Brilliant Blue.

X-band EPR spectra were recorded on a Varian E-9 spectrometer interfaced to a Stelar Prometheus Data System for computer analysis and handling of the spectra. Optical spectra were recorded on a Perkin Elmer 330 spectrophotometer equipped with a Hoefer RCB 300 temperature controller. The oxidase activity of ceruloplasmin was measured as described (Calabrese *et al.* 1989). Activation of prothrombin by ecarin was carried out according to Wu *et al.* (1992) and measurement of the amidolytic activity of the activated protein toward the thrombin synthetic substrate S-2238 (Kabi, Stockholm, Sweden) was performed according to the manufacturer's instructions.

Differential scanning calorimetry (DSC) runs were performed on a Microcal MC-2 calorimeter equipped with the DA-2 acquisition system. All traces were corrected for the instrumental baseline obtained by filling both cells with the buffer. Deconvolution in two-state transitions was performed with software provided by Microcal, as already described (Bonaccorsi di Patti *et al.* 1990).

N-terminal sequence analysis was carried out using an Applied Biosystem model 473A pulsed liquid sequencer with on-line PTH-amino acid analysis. The proteins were loaded onto prewashed, polybrene-coated trifluoroacetic acid-treated glass fibre filters.

## Results and discussion

The binding of calcium to ceruloplasmin was investigated by EPR spectroscopy by substituting this metal ion with the paramagnetic ion Mn(II). Following the intensity decrease of its EPR signal upon binding, a Scatchard plot was obtained by titration of human ceruloplasmin with Mn(II) (Figure 1A), which indicated the presence of three to four high-affinity binding sites with $K_d \cong 25\ \mu M$ and more than 10 binding sites with $K_d$ at least one order of magnitude lower. Evidence that calcium is able to bind to the high-affinity sites was obtained by competition studies in which bound manganese was progressively displaced by increasing concentrations of calcium. In order to calculate the $K_d$ for calcium, ceruloplasmin was titrated with manganese at fixed calcium concentrations. The obtained $K_{app}$ was then used to calculate a $K_d$ for calcium of about 1.4 mM (Figure 1B). These metal binding sites appeared not to be specific for calcium since it was found that also magnesium was able to compete with manganese with an affinity about 5 times higher than that exhibited by calcium, as $K_d \cong 0.3$ mM (data not shown). The metal-binding properties of sheep ceruloplasmin were found to be analogous to those of the human protein. Sheep ceruloplasmin bound three to five Mn(II) ions with $K_d \cong 23\ \mu M$ and showed more than 20 low-affinity binding sites (Figure 2A). Also in this case calcium displaced bound manganese (Figure 2B), with $K_d$ about 1.4 mM and magnesium exhibited a higher affinity than calcium, with $K_d$ about 0.7 mM.

The low specificity of the calcium-binding sites is a feature common to many other proteins which bind this metal with low affinity ($K_d > 10^{-5}$ M), possibly due to the geometric flexibility of these sites. Proteases, such as trypsin, are able to bind calcium and many other different cations which have a stabilizing effect on the protein (McPhalen *et al.* 1991). Blood clotting factors V and VIII are formed by subunits held together by divalent metal ions (Mann *et al.* 1988). Reassociation of isolated subunits and restoration of cofactor activity can be achieved *in vitro* in the presence of

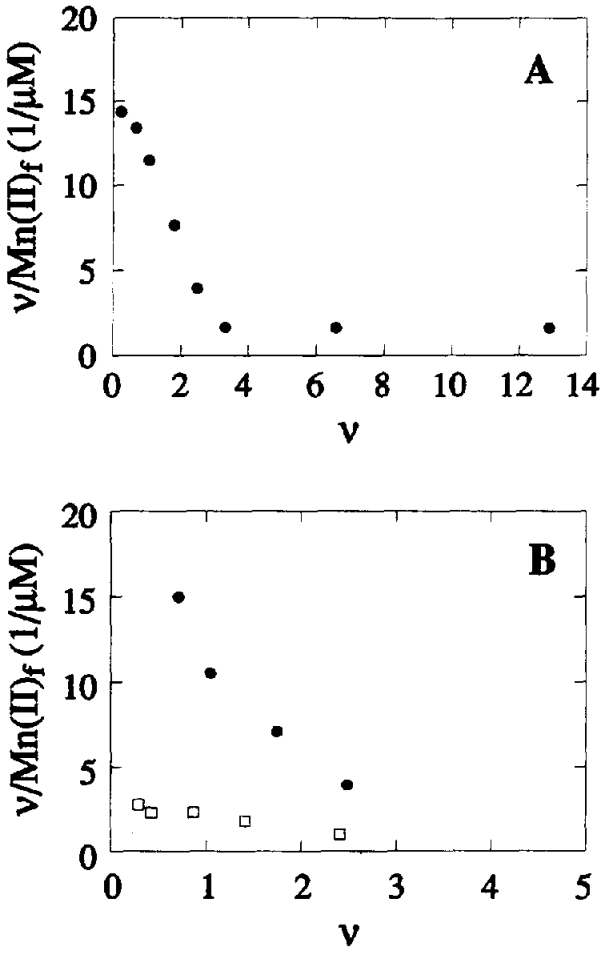


**Figure 1.** (A) Scatchard plot of Mn(II) binding to human ceruloplasmin. Protein (15 $\mu$M) in 50 mM MOPS, 100 mM KCl, pH 7.4, was titrated with $MnCl_2$ (5 $\mu$M to 1 mM). (B) Scatchard plot of Mn(II) binding to the high-affinity sites of human ceruloplasmin in the absence (●) and presence (□) of 5 mM $CaCl_2$. Protein (15 $\mu$M) was titrated with Mn(II) solutions from 15 to 200 $\mu$M. EPR experimental settings: frequency 9.4 GHz; power 100 mW; modulation amplitude 10 G; $T$=25°C.

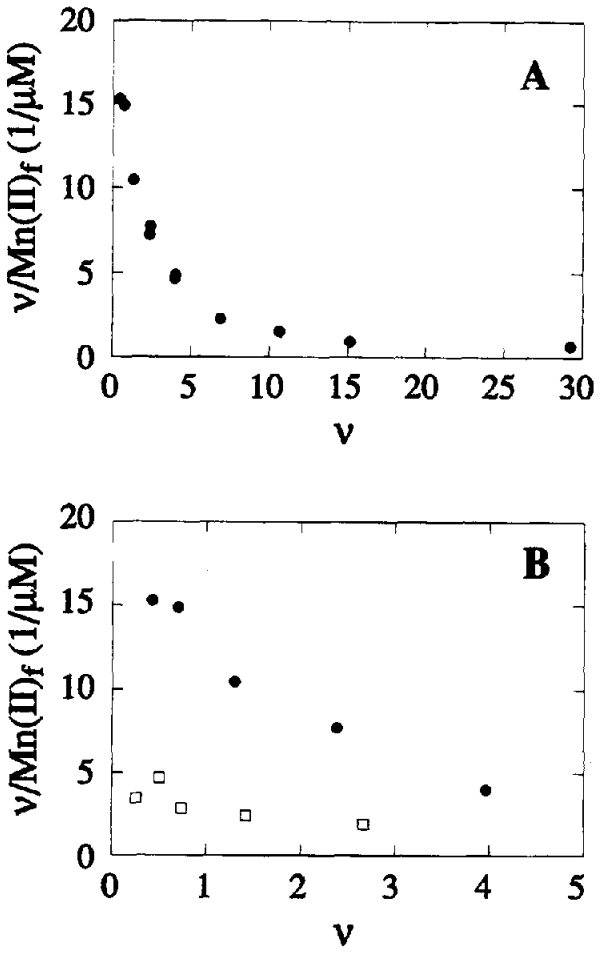


**Figure 2.** (A) Scatchard plot of Mn(II) binding to sheep ceruloplasmin. Protein (29 $\mu$M) in 50 mM MOPS, 100 mM KCl, pH 7.4, was titrated with $MnCl_2$ (15 $\mu$M to 5 mM). (B) Scatchard plot of Mn(II) binding to the high affinity sites of sheep ceruloplasmin in the absence (●) and presence (□) of 5 mM $CaCl_2$. Protein (29 $\mu$M) was titrated with Mn(II) solutions from 15 to 200 $\mu$M. Experimental settings as in Figure 1.

both manganese or calcium (Esmon 1979, Nordfang & Ezban 1988). One of the most studied calcium-binding proteins is prothrombin. Although the physiologically relevant metal is calcium, the protein is able to accommodate in the same sites many other metal ions, including magnesium and manganese. While magnesium shows an affinity lower than that of calcium, $K_d$ = 1.12 versus 0.2 mM (Deerfield *et al.* 1987), manganese binds more tightly with $K_d$ = 12 $\mu$M (Bajaj *et al.* 1976). In the case of ceruloplasmin, it appears that both manganese and magnesium have a higher affinity for the protein than calcium. However, while manganese is virtually absent from plasma, the $K_d$ values obtained for calcium and magnesium are both in the range of their physiological concentrations in plasma, 2.4 and 0.9 mM, respectively (Iyengan *et al.* 1978).

The spectroscopic properties, both optical and EPR, of ceruloplasmin were not altered in the presence of calcium or magnesium, suggesting that no gross changes involving the prosthetic copper atoms take place upon binding. In agreement with this finding, the catalytic parameters for the oxidase activity of ceruloplasmin towards *p*-phenylenediamine were not altered when 10 mM calcium or magnesium was present in the assay (data not shown).

Possible changes in the overall structural organization of ceruloplasmin induced by calcium binding were further investigated by DSC. The curves obtained for human or sheep ceruloplasmin heated in the presence or absence of 5 mM $CaCl_2$ are shown in Figure 3 and the corresponding parameters are tabulated in Table 1. At higher calcium concentrations precipitation of the protein occurred. In all cases a single peak was evident and denaturation was found to be irreversible, consistent with data already reported (Bonaccorsi di Patti *et al.* 1990). Deconvolution of the DSC peak of sheep ceruloplasmin in the absence of calcium

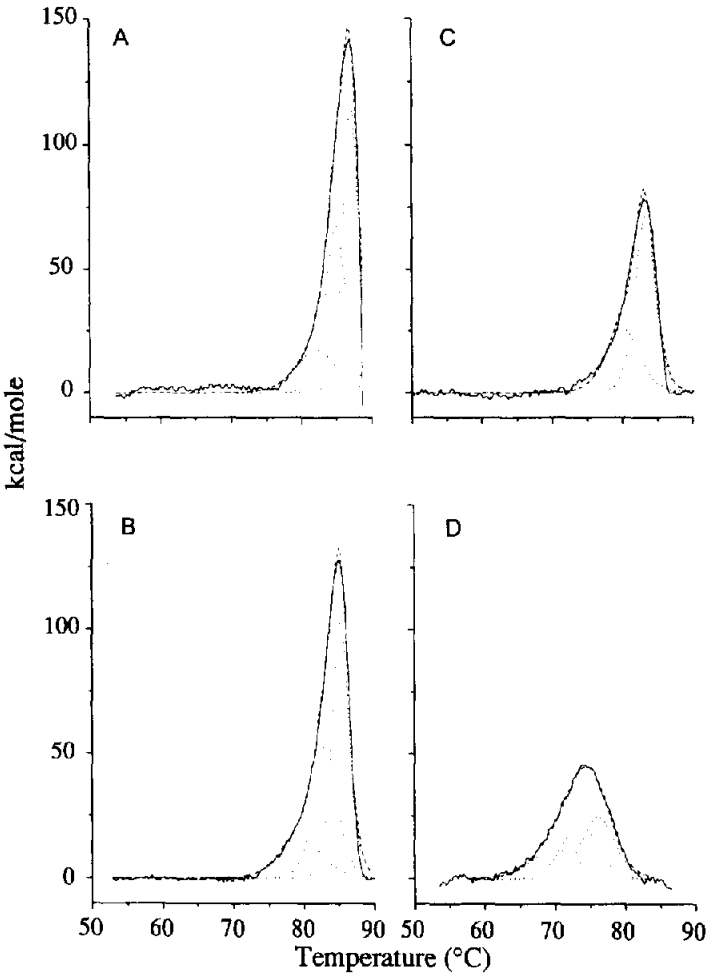


**Figure 3.** DSC traces of human ceruloplasmin with (**A**) or without (**B**) calcium and of sheep ceruloplasmin with (**C**) and without (**D**) calcium. Protein (15 $\mu$M) was heated at a rate of 1°C min$^{-1}$ in 50 mM MOPS, 100 mM KCl, pH 7, in the presence or absence of 5 mM $CaCl_2$. The broken lines represent the best fit obtained by deconvolution of the curves in two-state components (dotted lines).

**Table 1.** Calorimetric parameters for human and sheep ceruloplasmin

| Protein | Experimental | | Deconvolution[a] | |
|---|---|---|---|---|
| | $T_m$ (°C) | $\Delta H_{cal}$ (kcal mol$^{-1}$) | $T_m$ (°C) | $\Delta H$ (kcal mol$^{-1}$) |
| Human | 85.1 | 667 | 79.5 | 130 |
| | | | 83.1 | 234 |
| | | | 85.2 | 328 |
| Human + Ca(II) | 87.0 | 715 | 82.1 | 132 |
| | | | 84.9 | 263 |
| | | | 87.1 | 346 |
| Sheep | 73.8 | 408 | 69.9 | 107 |
| | | | 73.4 | 149 |
| | | | 76.1 | 155 |
| Sheep + Ca(II) | 83.1 | 419 | 80.1 | 161 |
| | | | 83.2 | 270 |

[a] Assuming $\Delta H_{cal} = \Delta H_{vH}$, where $\Delta H_{cal}$ and $\Delta H_{vH}$ are the calorimetric and the van't Hoff enthalpy, respectively.

showed the presence of three two-state components corresponding to the three calorimetric domains of the protein. The thermal stability of sheep ceruloplasmin was markedly affected by calcium with a $T_m$ value 9°C higher (Figure 3). Deconvolution of the DSC peak evidenced only two two-state components (Table 1), suggesting that a conformational transition with rearrangement of one or more subdomains takes place upon calcium binding to the protein. The presence of the prosthetic copper atoms was required in order to observe the effects of calcium, as the thermal stability of the apoprotein was not affected by this ion (data not shown). Human ceruloplasmin showed a particularly high thermostability, with a $T_m$ value almost 10°C higher than that of the sheep protein. Deconvolution of the DSC trace revealed the presence of three two-state components (Table 1), analogous to those found for sheep and other ceruloplasmins (Bonaccorsi di Patti *et al.* 1990). The $T_m$ value in the presence of calcium appeared only

slightly affected ($T_m$ = 87.0 versus 85.1°C). However, the metal ion even at 5 mM concentration strongly favoured aggregation of the protein at high temperature, evidenced by the exotherm which distorts the denaturational endotherm and by a massive precipitation of the protein at the end of the DSC scan (Figure 3). Due to the presence of the exotherm which partially overlaps the DSC peak, the thermodynamic parameters $T_m$ and $\Delta H_{cal}$ should be considered only minimal estimates. For this reason, deconvolution of the DSC curve should be regarded as only tentative. Magnesium, 5 mM, had no effect on the thermal stability of both ceruloplasmins as the DSC curves were superimposable to those obtained for the proteins in the absence of this cation (data not shown).

Calcium appears to act as a stabilizing factor in many proteins, protecting against thermal denaturation or proteolytic degradation (McPhalen *et al.* 1991). DSC studies on proteins such as troponin C have demonstrated an increase of the $T_m$ value and of the reversibility of unfolding in the presence of calcium (Jacobson *et al.* 1981). The thermal stability of the $Ca^{2+}$-ATPase of the sarcoplasmic reticulum is only slightly increased by calcium; however, DSC reveals that the protein unfolds through two steps in the presence of the metal and through a single step in its absence (Lepock *et al.* 1990). Calcium also stabilizes α-lactalbumin against thermal denaturation and modulates the interaction of this regulatory protein within the 'lactose' synthase complex (Musci & Berliner 1985). Variations of the stability of these proteins have been attributed to the conformational change elicited by calcium binding. The stabilizing effect of calcium on ceruloplasmin might be physiologically relevant, as the binding of the protein to its receptor on red blood cells has been demonstrated to be mediated by calcium but not by magnesium (Saenko & Yaropolov 1990). The DSC results are consistent with this finding, allowing us to speculate that calcium but not magnesium binding to ceruloplasmin favours a conformation of the protein which has a higher affinity for its receptor.

Calcium was also found to affect the interaction of ceruloplasmin with the chromatographic material routinely used to purify the protein from plasma. The isolation of ceruloplasmin is usually achieved by chromatography on Sepharose 4B derivatized with chloroethylamine (Calabrese *et al.* 1988, 1989), with the human protein being recovered by washing with potassium phosphate buffer at ionic strength $I \sim 0.5$. When MES buffer containing increasing concentrations of $CaCl_2$ was instead used, ceruloplasmin began to elute at 60 mM $CaCl_2$ and was completely recovered with 100 mM $CaCl_2$ (Figure 4). The corresponding ionic strengths were 0.18 and 0.3, considerably lower than that needed with phosphate buffer. The mechanism which governs the selectivity of derivatized Sepharose toward specific proteins is not clear. It has been suggested that the derivatization procedure of the resin introduces positively charged polyethyleneimine 'tentacles' on the Sepharose matrix, so that specific charge patterns on a protein would be required to fit in the positive cavities formed by the 'tentacles' for efficient binding to occur (Stern *et al.* 1992). Our results indicate that binding of calcium to ceruloplasmin

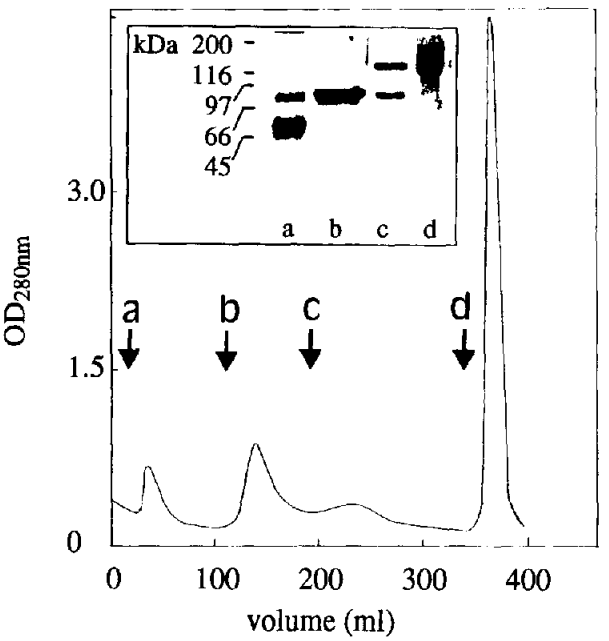


**Figure 4.** Elution profile of human plasma chromatographied on Sepharose 4B derivatized with chloroethylamine. Human plasma (200 ml) supplemented with 20 mM ε-aminocaproic acid and 100 μM phenylmethylsulphonyl fluoride were applied to a column containing 20 ml of the derivatized Sepharose equilibrated with 150 mM MES, pH 7. After passage of the plasma, the resin was washed with 150 mM MES, 100 mM ε-aminocaproic acid, 100 mM NaCl, pH 7, to remove loosely bound proteins. The arrows mark the addition of 150 mM MES, pH 7, containing 20 mM (a), 40 mM (b), 60 mM (c) and 100 mM $CaCl_2$ (d). The inset shows the SDS–PAGE patterns of the corresponding pooled fractions.

results in a modified charge pattern on the protein surface, possibly reflecting a conformational rearrangement of the protein.

When phosphate is used as the elution buffer, a good recovery of human ceruloplasmin is prevented by coelution from the resin of a contaminant with $M_r$ = 72 000 (Musci *et al.* 1993). The sequence obtained by N-terminal analysis allowed us to identify this protein as prothrombin. When calcium was used, prothrombin was recovered separately from ceruloplasmin, at lower ionic strengths (Figure 4). Prothrombin fractions were judged to be highly pure by SDS–PAGE. Moreover, Western blot for clotting factors VII and X, the most relevant contaminants of this protein, confirmed the high purity of prothrombin isolated by this method (data not shown). The protein exhibited clotting activity and amidolytic activity toward the thrombin synthetic substrate S-2238 after activation by ecarin, demonstrating its functional integrity. A third component, eluting at even lower calcium concentrations, was identified as $\alpha_2$-HS glycoprotein by N-terminal analysis. It is worth noting that $\alpha_2$-HS glycoprotein, as prothrombin (Mann *et al.* 1988), is also a calcium-binding protein (Triffit *et al.* 1976).

The results presented in this paper have shown that ceruloplasmin is able to bind calcium and magnesium. The

affinity of ceruloplasmin for these two metal ions is such that binding may occur also *in vivo* and have a physiological role in processes involving this protein. While the affinity of ceruloplasmin for magnesium has been revealed for the first time in this work and its physiological relevance remains to be established, a clear involvement of calcium in the interaction of ceruloplasmin with its receptor on red blood cells has been shown (Saenko & Yaropolov 1990). Our data suggest that binding of calcium modifies the conformation of ceruloplasmin. Whether this is the mechanism by which this metal ion is able to enhance the interaction between the two proteins or whether also the receptor binds calcium and is modified remains to be estalished. The affinity of ceruloplasmin for calcium is also intriguing in light of the role of this metal ion in the blood clotting cascade. It should be recalled that ceruloplasmin shares high sequence homology with factors V and VIII (Church *et al.* 1984), which are involved in the activation of prothrombin and factor X, respectively, and bind calcium and other divalent cations. Moreover, an interaction between ceruloplasmin and protein C, an anticoagulant protease which inactivates factors V and VIII, has been demonstrated (Walker & Fay 1990).

The procedure reported in this paper allows isolation of both ceruloplasmin and prothrombin from human plasma with high purity and high yield in a very rapid and simple fashion by taking advantage of the calcium-binding properties of these two proteins. The method also allows the purification of ceruloplasmin samples from prothrombin, which can often be a minor contaminant of this protein, even in commercial preparations (unpublished observation).

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