Calcium binding and concomitant changes in the structure and heat stability of calprotectin (L1 protein)

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Abstract

Aim—To obtain further data on the structure and conformation of calprotectin, a prominent leucocyte protein found in many species.

Methods—The binding of Ca^{2+} to calprotectin was studied by means of equilibrium dialysis using ^{45}Ca as tracer. The thermal stability and denaturation kinetics of calprotectin were studied by means of differential scanning calorimetry. Concomitant alterations in optical activity resulting from different conditions were measured. A computer program calculated the parameters to fit different models of protein structure. Ultraviolet spectroscopy gave absorption spectra. Sedimentation velocity studies and molecular weight determinations by the low speed (sedimentation) equilibrium technique were performed.

Results—A maximum of six calcium ions were bound per calprotectin molecule at 0.7 mM calcium chloride. The apparent dissociation constants were calculated. Ca^{2+} ions increased the denaturation temperature by 26°K. The enthalpy of denaturation was also increased by Ca^{2+} . Addition of Ca^{2+} to the buffers caused a gradual change in the near UV circular dichroism spectrum, while only minor changes were seen at wavelengths of 210–240 nm. A gradual increase in the sedimentation coefficient was observed on addition of calcium chloride. The extinction coefficient at 279 nm was determined: $E_{279} = 2.53 \cdot 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

Conclusions—Calprotectin can bind six calcium ions. Upon binding, the protein shows distinct conformational changes and increased thermal stability. The former may be of importance for its function, while the biological significance of the latter is unknown.

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Keywords: Calcium binding, calprotectin, heat stability.

The name calprotectin¹ has recently been applied to the human leucocyte protein previously called L1,² an abbreviation which will be used here. Early studies³ suggested that L1 binds calcium. In recent years, it has become evident that the protein contains two types of poly-

peptide chain^{4,5} with molecular weights of about 8 and 14 kDa. The intact protein consists of two heavy chains (L1_H) and one light chain (L1_L). The molecular mass of L1 in both leucocytes and plasma is about 36.5 kDa unless denaturing agents are used.^{6,7} Their amino acid sequences have been determined,⁴ and each contains one classical and one modified calcium binding EF-hand.^{5,8}

On the basis of the amino acid sequences derived from cDNA studies⁵ the molecular weights of the light and heavy chains can be calculated to be 10 835 and 13 242 Da respectively, ignoring post-translational modifications. Applying SDS-PAGE, different investigators⁹ have shown bands corresponding to the L1 light and heavy chains and called them MRP-8 and MRP-14, respectively. In the studies presented here we have used 36 500 Da for all calculations. There seem to be no covalent bonds between the chains, since reducing agents are not needed for dissociation. They can, however, be separated in 6–8 M urea.⁷

L1 constitutes about 60% of the cytosol proteins or 5% of the total protein content in human neutrophil granulocytes and monocytes.² It may also be found in smaller amounts in the plasma membrane and the nucleus. It is found in mucosal squamous epithelium,¹⁰ but not in epidermis, except in several inflammatory skin diseases.¹¹ The protein is released from neutrophil granulocytes and monocytes during their activation or death, causing increased extracellular concentrations.

The plasma concentration of L1 may increase in various pathological conditions. In severe bacterial infections plasma L1 rises to 40–130 times the normal mean, while in viral infections normal or only slightly increased values are found.¹² The normal plasma concentration is 50–910 µg/l (mean 530 µg/l) for men and 80–880 µg/l (mean 440 µg/l) for women.

Recently it has been shown that L1 was present in increased concentrations in stools from patients with colorectal cancer. A test for faecal L1 determination may possibly be used as a diagnostic tool for gastrointestinal diseases.¹³

The aim of the present study was to obtain further data on the structure and conformation of L1 which may contribute to a better understanding of the structure and biology of this protein.

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Figure 1 Elution profile of proteins from a DEAE-Sephacel ionic exchange column (1.5×10 cm). The column was equilibrated with 20 mM barbitone buffer added 0.5 mM EDTA $_{2-}$, pH 8.5. L1 was eluted with a NaCl gradient (0–1 M). Total protein was determined by the Bio-Rad method and the L1 was determined by single radial immunodiffusion. The L1 protein appeared in fractions 40–50.

Methods

BUFFERS AND SALT SOLUTIONS

For dialysis experiments, distilled deionised water (Levatitt S100 resin; Bayer) stored in polypropylene bottles was used. Tris-maleate buffers were prepared as described by Gomori.¹⁴ Appropriate amounts of analytic quality Na $_2$ HPO $_4$, CaCl $_2$, and NaCl were added to Tris-HCl buffers (0.1 M, pH 8.1) for circular dichroism and differential scanning calorimetry (DSC) to obtain the required salt concentration and ionic strength.

PREPARATION OF CALPROTECTIN

Crude leucocyte extract³ was applied on a DEAE-Sephacel (Pharmacia) column 1.5×10 cm, equilibrated with 20 mM barbitone buffer with 0.5 mM EDTA $_{2-}$, pH 8.5. L1 was eluted with a 0 to 1 M sodium chloride gradient (see fig 1), concentrated by ultrafiltration using a Millipore® immiscible-CX molecular separator, and finally run on an Ultrogel AcA 54 (LKB) column 2.5×80 cm, equilibrated with 50 mM ammonium hydrogen carbonate buffer (pH 8.0). The L1 peak giving the typical bands on agarose gel electrophoresis purity test (see fig 2) was shell frozen, freeze dried, and stored in the cold until used. The quantification of L1 was done by single radial immunodiffusion as described earlier.³

Protein concentrations were determined by the Coomassie blue method using Bio-Rad protein assay kits.

DIALYSING CELLS

The dialysing cells used were made from acrylic, size 5 cm $\times$ 5 cm $\times$ 0.9 cm. Each of the cylindrical dialysing chambers had a diameter of 1.9 cm and a depth of 0.5 cm. The chambers were separated by a dialysing membrane.¹⁵ The dialysing membranes (Union Carbide) were stabilised by boiling with 1 mM EDTA and rinsed thoroughly in distilled water before use.¹⁶

EQUILIBRIUM DIALYSIS

45 Ca was delivered as calcium chloride in aqueous solution from Amersham International. The tracer was added to give a specific activity of about 2.5 GBq/mol Ca $^{2+}$ in the test solutions. The radioactivity was assayed with a Wallac 81000 liquid scintillation counter. Samples of 50 μ l volume were dissolved in 5 ml Opti-Flour scintillation fluid (Packard) and counted for 10 min.

The binding of Ca $^{2+}$ to L1 was studied by means of equilibrium dialysis using 45 Ca as tracer. In preliminary experiments optimal binding was found at about pH 7. Buffered protein solution (250 μ l) was instilled in one of the cell chambers and buffered solution of CaCl $_2$ and tracer (250 μ l) in the other chamber. The cells were sealed and shaken at 25°C for 16 h. Blank experiments showed that 16 h was sufficient time for Ca $^{2+}$ to equilibrate in the cells. At the end of dialysis, samples from each chamber were counted.

The Ca $^{2+}$ binding was investigated in two ways: (1) by keeping the L1 concentration constant at 0.02 mM and varying the Ca $^{2+}$ concentration (var Ca $^{2+}$) between 0.01 and 1.0 mM, and (2) by keeping the Ca $^{2+}$ concentration at 0.2 mM while varying the L1 concentration (var L1) between 0.001 and 0.04 mM.

DIFFERENTIAL SCANNING CALORIMETRY

The thermal stability and denaturation kinetics of the L1 protein was studied with a Perkin-Elmer DSC-2 calorimeter equipped with an intracooler. The DSC data were recorded at each degree increment in temperature using a computer on line with the DSC instrument. The data were treated interactively as described by Harbitz *et al.*¹⁷ The baseline is subtracted empirically¹⁷ as we used a calorimeter of intermediate sensitivity and therefore a high protein concentration.

Protein samples (60 mg/ml) were heated in aluminium pans (15 ml) from 7°C to 100°C (heating rate 10°C/min). The protein samples were dissolved in Tris buffers with increasing calcium concentrations until the protein was saturated with Ca $^{2+}$. Three measurements were recorded at each Ca $^{2+}$ to protein ratio. No modelling of the thermogram shapes to obtain information about the number of domains¹⁸ was attempted as insufficient replicats were made to validate properly the fitting of such a model. Thus the number of peaks reported are those which seem plausible by visual inspection of the thermograms, an approach used in reports on the DSC technique.¹⁹ The temperature when denaturation of the protein starts, T_{onset} , and the temperature when the heat flow is maximal, T_{max} , were determined with a standard error of $\pm 1.4^\circ\text{C}$.

CIRCULAR DICHROISM

Alterations in optical activity under different conditions were measured with a Jobin-Yvon dichrograph IV connected to a computer. The computer was programmed to subtract the

Figure 2 Agarose gel (1% w/v) electrophoresis, the buffer containing 75 mM barbitone/sodium barbitone, 2.5 mM EDTA $_{2-}$, and 0.2 mM merthiolate, adjusted to pH 8.6. Coomassie blue stained, showing typical patterns of (A) purified L1, (B) normal serum, and (C) crude leucocyte extract. The arrow indicates level of sample application.

Figure 3 Number of calcium ions bound per L1 molecule. (A) Number of Ca^{2+} ions bound per molecule as a function of the concentration of calcium on a logarithmic scale. Aliquots of Ca^{2+} , 0.2 mM including tracer, were dialysed against protein solutions. Concentrations varied from 0.001 to 0.04 mM. (B) Number of Ca^{2+} ions bound per molecule as a function of the concentration of L1 protein on a linear scale. Aliquots of L1 protein, 0.75 mg/ml, were dialysed against Ca^{2+} solutions with ^{45}Ca as a tracer. Total Ca^{2+} varied from 0.01 to 1.0 mM. Dashed line indicates extrapolation. The samples in (A) and (B) were dialysed for 16 h at 25°C in Tris-maleate buffer, pH 7.

baseline from the sample signals and calculate parameters to fit different models of protein structure. The spectra were recorded eight times at a scan rate of 20 nm/min. Each spectrum was recorded two or three times on independently prepared solutions. The statistical mean is given. Protein solutions for circular dichroism recordings (1.0 mg/ml) were prepared in Tris buffers with calcium concentrations from 0 to 0.60 mM. The solutions were passed through 0.45 μm filters before each run. Calibration of the dichrograph was performed periodically at 304 nm using epandrosterone (1.25 $\mu\text{g}/\text{ml}$, Roussel UCLAF OF 0269) in dioxane according to the specifications given by the manufacturer. To check the instrument, the daily starting up procedure included recording the spectrum of a reference lysozyme solution and comparing the resulting spectrum with previously recorded spectra stored in the software. Aliquots of lysozyme standards (Sigma, catalogue No 6876) were stored in liquid nitrogen. For recordings in the far UV range (185–265 nm) the light path in the cell was 0.1 mm; for the near UV (250–350 nm) it was 10 mm. The recordings were carried out at ambient temperature.

ULTRAVIOLET SPECTROSCOPY

Recordings of UV absorption were made with a Beckman model 35 spectrophotometer connected to a Beckman chart recorder (Beckman Scientific Instrument Division). The protein (1 mg/ml) was dissolved in Tris buffer containing 1 mM CaCl_2 . The lightpath in the quartz cells was 10 mm. Scans were made from 360 to 200 nm using scan speed 100 nm/min.

ANALYTICAL ULTRACENTRIFUGATION

Sedimentation velocity studies were performed using a Beckman Spinco model E analytical ultracentrifuge equipped with Schlieren optical systems. A titanium rotor (An-H) and a cell with a single sector charcoal filled centrepiece were run at 60 000 rpm for 2 h at 20°C. The cells were filled with 600 μl Tris-HCl buffer pH 8.1 containing 5 mg protein/ml and different concentrations of CaCl_2 . Pictures were taken automatically every 32 min unless otherwise stated. Molecular weight determinations were made by the low speed (sedimentation) equilibrium technique. The centrifuge was operated at 14 000 rpm, equilibrium was attained within 24 h in the cell with a liquid volume of 100 μl . The buffers and concentrations were identical to those in the sedimentation experiments.

Results

EQUILIBRIUM DIALYSIS

Figure 3A shows that the binding of Ca^{2+} to the protein increases with increasing concentration of the ligand, reaching a saturation value of six calcium ions per protein molecule at about 0.67 mM Ca^{2+} , corresponding to a Ca^{2+} to protein ratio of 30. The binding of Ca^{2+} started at a Ca^{2+} to protein ratio of about 5. Plotting the binding of Ca^{2+} against log Ca^{2+} concentration gives a non-symmetrical sigmoidal curve. The linear plot of occupied binding sites versus calcium concentration has the form of an adsorption isotherm (not shown). The maximum binding capacity of six calcium ions per L1 molecule was confirmed in dialysis experiments where different concentrations of L1 were dialysed against a constant (0.2 mM) concentration of CaCl_2 . Extrapolation of protein concentration to zero suggest the binding capacity of six calcium ions per protein molecule (fig 3B).

The apparent dissociation constants, $K_{d \text{ app}}$ calculated from the binding data are shown in table 1. A Scatchard plot of the parameters from the experiments gives a curve compatible with positive cooperative binding of Ca^{2+} to the protein (not shown).

DSC MEASUREMENT: EFFECTS OF DIFFERENT IONS AND CONCENTRATIONS ON THERMAL STABILITY

The series of thermograms in fig 4 show the effect of various calcium concentrations and different salts on the L1 denaturation enthalpy. The quantitative aspects of the effects are sum-

Figure 4 Effect of different cations and anions on the calorimetric thermograms of L1 (L1:60 mg/ml, 0.1 M Tris buffer, pH 8.1). The ionic strength was 0.1 M for the reference solutions and 0.28 M for all others. The thermograms were obtained with 0.9 mg of protein.

marised in table 2. The protein dissolved in Tris buffer at pH 8.1 had an apparent enthalpy of denaturation (ΔH) of 8.1 J/g. The denaturation started at 55.6°C, with the maximum value at 62.3°C. When calcium ions were added to increasing concentrations, the

Table 1 Apparent dissociation constants calculated from data obtained from equilibrium dialysis experiments, varying the calcium concentration $\text{var}(\text{Ca}^{2+})$, and the L1 concentration $\text{var}(\text{L1})$

Binding $\text{Ca}^{2+}/\text{L1}$	Dissociation constant ($K_{d,app} \times 10^{-4} \text{M}$)	
	$\text{Var}(\text{Ca}^{2+})$	$\text{Var}(\text{L1})$
1	2.75	7.80*
2	2.2	3.20
3	2.26	1.84
4	1.23	0.96
5	0.67	0.36*
6	0.040*	

* Extrapolated or approximated values.

enthalpy and T-onset increased. At the intermediate calcium concentrations, the denaturation had a stepwise or biphasic/multiphasic course. At the highest Ca^{2+} to protein ratio, the L1 molecule was significantly stabilised, with T_{onset} and ΔH_{app} equal to 84.3°C and 11.8 J/g, respectively. Using this technique a Ca^{2+} to protein ratio of >7 was needed in order to induce the transition in L1 that led to a more heat stable form of L1. The whole transition was complete at a Ca^{2+} to protein ratio of ≈ 13 . However, some further change in stability was seen upon increasing the Ca^{2+} to protein ratio even further (fig 4 and table 2). An increase in ionic strength (from 0.1 M to 0.28 M) did not alter T_{onset} or T_{max} using either NaCl or Na_2HPO_4 . A positive effect on ΔH for the L1-NaCl system is, however, possible. Phosphate ions had a minimal effect on all thermodynamic variables (fig 4).

EFFECT OF CALCIUM IONS ON SPECTRA OF L1

Figure 5A shows the far UV CD spectra of L1 with 0.6 mM (Ca^{2+} to protein ratio = 21) and without Ca^{2+} . The spectra were rather similar. Minor differences were detected for wavelengths between 210 and 240 nm; the lowest ellipticity was found in the presence of calcium at these wavelengths. Calculations of the secondary structure of L1 were attempted using Chang's model for protein structure.²⁰ This model is not ideally suited for the Ca^{2+} saturated molecule, and the calculations on secondary structure suggesting that the Ca^{2+} saturated form of L1 has a higher β sheet content should be interpreted with caution. Figure 5B shows the near-UV circular dichroic spectra (250–350 nm) of L1 with increasing concentrations of Ca^{2+} up to 0.60 mM (Ca^{2+} to protein ratio = 21). There are striking and significant differences in the spectra between 275 and 305 nm. The protein devoid of calcium has a positive peak value at ≈ 290 nm whereas the molecule saturated with calcium shows maximum negative ellipticity at ≈ 287 nm. The bundle of curves between these extremes shows a conformational alteration with increasing calcium concentration.

UV spectra of the L1 protein dissolved in 0.1 M Tris buffer (pH 8.1) confirmed that the molecule contains near-UV absorbing chromophores. The curves had a peak at 279 nm and a minimum value at 250 nm. The addition of CaCl_2 had no significant effect on the UV recordings (spectra not shown). At 279 nm the protein solution (1 mg/ml, 1 cm light path) absorbs 0.693. This gives an extinction coefficient $E_{279} = 2.53 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$.

ANALYTICAL ULTRACENTRIFUGATION

Figure 6 shows the variations in the sedimentation coefficient with various calcium concentrations. When the protein is devoid of calcium it has a sedimentation coefficient, $S_{20,w}$ of 2.04 S. As the calcium concentration increases, the curve describing the sedimentation coefficient versus calcium con-

Figure 5 Circular dichroism spectra of L1 in the presence and absence of calcium ions. (A) Far UV circular dichroic spectra of L1 (solid line) (L1:1.3 mg/ml, 0.1 M Tris buffer, pH 8.1) and L1 + Ca²⁺ (dashed line) (molar ratio L1:Ca²⁺ = 21). The inset shows the difference spectrum (—) and the "standard deviation" (four determinations) spectrum (hatched area) for the same two systems. (B) Near UV circular dichroic spectra of L1 (L1:1.3 mg/ml, 0.1 M Tris buffer, pH 8.1) with calcium concentration increasing up to 0.60 mM.

centration assumes a form of a sigmoid curve, reaching a maximum value of 3.47 S at 1.7 mM calcium (Ca²⁺ to protein ratio = 12.4). It can be seen from the figure that the transition using this technique started at a Ca²⁺ to protein ratio of 1.0 and seems to be finished at a ratio of 6. In 0.55 mM calcium (Ca²⁺ to protein ratio = 4) the analysis was also done at 68 000 rpm to increase the separation by reducing the diffusion. At 64 min two peaks were seen, suggesting inhomogeneity (results not shown). Thus the same protein solution was seen as homogeneous when devoid of calcium, as in-

homogeneous at a particular calcium concentration, and then again homogeneous when saturated with calcium.

MOLECULAR MASS DETERMINATIONS

Molecular weight determinations performed with and without calcium in the buffers showed substantial difference in molecular weight. The molecular weight calculated from data obtained without calcium was 34 kDa; when the calcium concentration was 1.5 mM (Ca²⁺ to protein ratio = 11) the obtained value was 73 kDa.

Table 2 Calorimetric data for the native to denatured transition for L1 at pH 8.1 (0.1 M Tris buffer) in the presence of different salts at constant ionic strength (0.28 M)

Salt added	Concentration (M)	ΔH (J/g)	Area ratio (low temp peak to high temp peak)	T_{melt} (°C)	T_{max} (°C)
None	0	8.1 (0.6)†	∞	55.6	62.3
CaCl ₂	0.075	8.1 (0.6)	∞	55.8	62.1
CaCl ₂	0.01	8.2 (0.8)	∞	56.7	62.1
CaCl ₂	0.012	8.2 (0.5)	∞	56.7	62.6
CaCl ₂	0.015	9.1 (0.5)*	1.4 ± 0.6	nd	75.1
CaCl ₂	0.0187	10.0 (0.4)*	0.2 ± 0.2	nd	79.1
CaCl ₂	0.0225	10.1 (0.5)*	0.2 ± 0.2	nd	82.2
CaCl ₂	0.03	10.8 (0.3)	0	80.0	84.1
CaCl ₂	0.06	11.8 (0.3)	0	84.3	88.2
Na ₂ HPO ₃	0.06	8.1	0	57.6	88.2
NaCl	0.18	9.3	0	56.9	63.4

* Encompasses a double peak.

† Mean (SEM).

nd = Not determined.

Figure 6 Variation in the sedimentation coefficient $S_{20,w}$ for L1 as function of calcium concentration. Protein concentration was 5 mg/ml in 0.1 M Tris-HCl buffer, pH 8.1. The rotor speed was 60 000 rpm. See the text for comments on results for 0.55 mM calcium.

Discussion

The results suggest a total of six calcium binding sites on each L1 molecule, which is in agreement with the presence of one classical and one modified EF-hand.⁵⁸ A positive co-operative binding of Ca^{2+} was found, which has also been reported for other calcium binding proteins.²¹ The small differences in $K_{d \text{ app}}$ determined by equilibrium dialysis with constant concentration of L1 or Ca^{2+} respectively are probably caused by different ionic strengths in the two systems. In our calculations we have used 36 500 Da as the molecular mass for the intact molecule. The differences between this value and molecular masses obtained by calculations from cDNA or other sources are about 2%, which seems insignificant. In preliminary experiments the protein showed optimal binding at pH 7, which is about 1 unit above its isoelectric point (6.3) when the protein will have a net charge. Tris-maleate buffers, 0.1 M, will, however, render the Donnan effect negligible.²² Generally, it is expected that the effect of the ligand is seen at lower ratios of ligand to macromolecule, when the total concentration of the ligand binding molecule is high. The observations given in figs 5B and 6 are compatible with the conclusion that the transition starts at a Ca^{2+} to protein ratio of about 2.

The gradual increase in sedimentation during analytical ultracentrifugation (fig 6) is most likely to be due to a change in molecular weight caused by association of L1 molecules. The values of the sedimentation coefficients, 2.04 and 3.47, seem plausible for compact, largely spherical structures of molecular weights 34 000 and 73 000, respectively, when the effect of protein concentration is taken into account.²³ However, the exact shape of L1 is presently unknown.

The doubling of molecular mass, as shown by the low speed equilibrium technique, is taken as evidence that L1 will dimerise in the presence of low concentrations of calcium. Gel filtration experiments have confirmed this finding (data not shown). Other proteins also dimerise when saturated with calcium,²⁴ but

the ubiquitous calmodulin is reported to exist as a monomer.²⁵

The true denaturation enthalpy reflects the breaking of various bonds in a protein molecule.²⁶ Since calorimetric methods are equally as sensitive to bonds formed between neighbouring amino acids as they are to bonds formed between more distant amino acids, the differential scanning calorimetry technique can be very sensitive to changes in the tertiary structure of a protein. Figure 4 and table 2 suggest changes in the tertiary structure of L1 upon binding of Ca^{2+} since the only substantial changes were found in the near-UV circular dichroism.

An important finding was that the presence of calcium ions improves the thermal stability of the molecule to a considerable degree. The stabilising effect is evidenced both by increasing enthalpy, ΔH , and higher denaturation temperature. At intermediate Ca^{2+} concentrations (7.5 mM to 23 mM) (Ca^{2+} to protein ratio = 4.7–14) the denaturation curves become quite complex, suggesting that different conformational L1 species may be present in the solution at the same time. The calorimetric technique also seems to respond late to the binding of Ca^{2+} when the effect of increased total protein concentration is considered. This suggests that the binding of Ca^{2+} is complete, or close to complete, before the protein is significantly stabilised. Our results are similar to those reported by Tsalkova and Privalov²⁷ on calmodulin and troponin C. They showed that a stepwise melting process reflects the fact that different intramolecular bonds are broken in sequence because of major structural differences. They reported up to a 7% increase in melting enthalpy and a 47° increase in denaturation temperature of calmodulin, while our figures were 26% and 31° for L1. Anteneodo *et al.*,¹⁸ who studied a sarcoplasmic reticulum calcium binding enzyme, reported a much smaller increase in stabilisation (13.5°C) and no significant increase in denaturation enthalpy. This suggest that although stabilisation by Ca^{2+} is to be expected, the magnitude of the change in denaturation temperature and enthalpy varies substantially between different calcium binding proteins.

The changes in the spectral region 275–305 nm (fig 5B) can be caused by changes in the positions of the aromatic amino acids tyrosine and tryptophan. To a lesser extent the side chains of phenylalanine and histidine also contribute to the spectra. There were strong indications that the ellipticity of tryptophan residues may be sensitive to Ca^{2+} concentration because of the presence of the peaks at 284 and 292 nm²⁸ in the L1- Ca^{2+} system (fig 5B). It is also possible that tyrosine residues of L1 were sensitive to Ca^{2+} .²⁹ The stepwise alteration of the different spectra may be due to gradual alterations in the groups or residues involved in, or affected by, the binding of calcium ions.

In conclusion, the data in fig 5B suggest that the binding of Ca^{2+} affects tryptophan and tyrosine residues. These amino acids are present in, or close to, the EF-hands in both the

light and the heavy L1 chains. It should finally be pointed out that L1 differs from other calcium binding proteins with respect to changes in secondary structure; calmodulin and troponin C show an increase in the α helix content of 10% and 21% respectively,³⁰ whereas L1 decreases its α helix content by about 5% upon exposure to Ca^{2+} .

The protein concentrations used to obtain figs 5A and 5B are close to the protein concentrations used for the binding studies used in fig 3A. The data in figs 5A and B are therefore interpreted as reflecting Ca^{2+} binding. The association process does not seem to lead to large changes in secondary structure (fig 5A).

Our findings suggest that L1 will associate to form a dimer, bind up to six calcium ions per molecule without severe structural changes, and thereby obtain a considerable increase in thermal stability.

All physical measurements reported here (analytical ultracentrifugation, circular dichroism, differential scanning calorimetry) point to a specific effect of Ca^{2+} on L1, in agreement with the data from equilibrium dialysis.

To comply with the demands of the different techniques, we used different protein-ligand concentrations. The resulting Ca^{2+} to protein ratios are, however, found under physiological conditions. The effects shown in this study are seen within the normal physiological range of calcium concentrations and may well be important in vivo. Others have suggested that different modifications of a protein molecule may have different functions. It is speculated that calmodulin may be recognised, in theory, as 4² different conformations,⁸ a reasoning which also may be applied to L1. One must expect that the protein will change its conformation when moving from the cytoplasm to the extracellular milieu where it will be saturated with calcium.

The biological significance of increased temperature stability in the presence of calcium is unknown.

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