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Binding of lead to collagen type I and V and α_2(I) CNBr (3,5) fragment by a modified Hummel–Dreyer method

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Abstract

Binding of lead (as lead acetate) to collagen type I α_1 and α_2 chains, collagen type V and a large cyanogen bromide fragment of type I collagen [α_2(I)$CB_{3,5}$] was investigated by the large-zone Hummel–Dreyer method. It was demonstrated that two categories of binding sites exist in the collagen molecule, the number of which correlates rather well with the available aspartic and glutamic acid residues. Similar results were obtained for all collagen chains (fragments) used. The number of sites thus obtained was compared with the cross-striation pattern (reflecting areas where lead is bound) of the SLS form of collagen type I (α_1 chain); it is suggested that the number of bands seen in the SLS form reflects primarily the number of available aspartic acid residues in the molecule. The association constants obtained are comparable with the low affinity interactions seen e.g., between Cu and bovine serum albumin.

Keywords: Hummel–Dreyer method; Protein–lead interactions; Lead–protein interactions; Collagen; Lead; Proteins

1. Introduction

Lead has been shown to be a toxic element in most of its chemical forms no matter whether it is inhaled or ingested in water or food [1]. It was also shown that the total bodily amount of lead does not affect lead absorption as long as lead does not possess a feedback mechanism which would set absorption limits. On the other hand it was demonstrated that lead absorption in the intestine is affected both by the active and passive transport [2].

In the animal (laboratory rat) and/or human body a three-compartmental pool for lead metabolism is widely recognised, namely blood, soft tissue (hair, nails, connective tissue, salivary, gastric, pancreatic and biliary secretions) and skeleton. Most of the attention, both from the biological and analytical views, has been paid to bone-deposited lead [3] where its toxic effect upon the body appears to be rather low. Regarding soft tissues and blood the most studied toxic effect is apparently directed to hematopoiesis where lead interferes with two steps in the heme synthetic pathway [1]; interferences are based on a specific interaction with δ-aminolevulinic acid dehydrase and ferrochelatase [4–6]. This interference is interpreted as a result of direct interaction of these two enzymes with lead by which the enzymatic activity is decreased. Because the activity of δ-aminolevulinic acid dehydrase could be restored by Zn^{2+} addition (in vitro), it is widely accepted that Zn has a preventive effect against lead adverse effects [7].

As we have shown in a preliminary report, lead

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accumulates in diverse soft tissues upon oral administration [8]. However very little is known about what kinds of protein–lead interactions are involved. Because collagen is the most common protein in animal body and because it was shown to be capable of storing a number of heavy metals (particularly if administered in the chelate form [9]), it appeared a suitable candidate for model studies.

There are generally two main categories of methods allowing one to characterize metal–protein complexes, namely those requiring a separation of the free and bonded species and those in which this separation is not needed. The non-separation requiring methods are either spectroscopic [UV, nuclear magnetic resonance (NMR), IR, fluorescence and electron spin resonance (ESR)] [10–17] or electrochemical procedures like amperometry and polarography [18–21].

Of the methods which require separation of the bound and unbound species, equilibrium dialysis is, perhaps the most common approach [22]. However, chromatographic and electrophoretic methods can be used as well [23,24], of which immobilized metal affinity chromatography enjoyed considerable development over the past decade.

Regarding chromatographic methods, gel permeation chromatography appears well suited for studying diverse (namely protein–protein) interactions. For small ligands binding this approach is known as the Hummel–Dreyer method for revealing drug–protein interactions [25]. The main problem when this method is applied to protein–metal binding appears the accurate measurement of peak areas corresponding to the bound metal and metal free zone. In order to avoid this problem Sandier et al. [26] elaborated a method in which the peak heights are measured rather than the peak areas. The method involves successively a gel permeation technique by which the complexed and free metallic ions are separated and the eluate is evaluated off-line with inductively coupled plasma atomic emission spectrometry (ICP-AES). Applying the so called large-zone Hummel–Dreyer method and collecting small fractions during the chromatographic separation allows the evaluation of the chromatogram (by ICP-AES) at constant and known protein concentration. The applicability of this technique has been demonstrated by the authors [26] on the serum albumin–copper interactions, a model situation in which the number of protein–metal interactions per molecule and their nature can be compared with the results of methods based on different physical principles.

In this paper we used the method of Sandier et al. [26] to reveal the number and types of interactions between collagen α-chains (and their fragments) and lead. Because of the reasons explained above we also attempted to compare the number and location of Pb binding sites with the cross-striation pattern of lead stained collagen SLS forms (for collagen nomenclature see Ref. [27]).

2. Experimental

2.1. Preparation of acid soluble collagen type I

Tendons dissected from tails of three-month-old rats were cut into ~1 cm pieces and washed with phosphate buffered saline; this material was extracted with 5% acetic acid (100-fold mass excess) under stirring overnight [28]. The undissolved portion was spun off at 5000 *g* and the supernatant containing α_1 and α_2 collagen polypeptide chains was used for binding studies. The purity of the product was tested by polyacrylamide gel electrophoresis, amino acid analysis and CNBr peptide pattern. In polyacrylamide gel electrophoresis about 25% of the released material was present as chain dimers. Collagen samples were then dialyzed against 2000-fold excess of double-distilled water with three changes overnight and freeze–dried.

2.2. Preparation of collagen type I α_1 and α_2 chains

This was done by two subsequent chromatographic steps. First, denatured type I collagen (50 mg) was removed contaminating chain polymers by gel permeation chromatography; 50 mg of crude collagen sample was loaded on a 155×1.5 cm Bio-Gel A5 cm column which was eluted with 2 *M* guanidine–HCl, 0.05 mol/l Tris (pH 7.5) at a flow-rate of 15 ml/h; α-chains of type I collagen emerged in the fraction corresponding to 190–210 ml of the eluate. This fraction was accumulated from 30 runs, dialyzed against 0.5 mol/l acetic acid and freeze–dried.

Separation of the α_1 and α_2 chains was done on a 7×0.9 cm column packed with carboxymethylcellulose; for a single run 30 mg of the freeze–dried sample was used. The sample was dissolved in a minimum volume of 0.1 *M* NaCl in 0.02 *M* sodium acetate containing 1 mol/l urea to ensure complete solubility of the collagen sample[1]. The total gradient volume was 250 ml. In both steps detection was done by monitoring the UV absorbance at 230 nm. Under these conditions the α_1(I) chain eluted between 40–60 ml of the eluate while the α_2(I) chain was collected in the fraction representing 80–125 ml of the eluate.

2.3. Preparation of the α_2(I)$CB_{3,5}$ peptide

The title peptide was isolated from crude collagen type I preparation. Treatment with CNBr was done in 70% formic acid as described by Scott and Veis [29]. In order to maximize the CNBr cleavage the reaction time was increased to 24 h. Under these conditions we obtained about 80% of the theoretical amount of the α_2(I)$CB_{3,5}$ peptide [the remaining 20% were apparently cleaved into α_2(I)CB_3 and α_2(I)CB_5 peptides]. Isolation of the α_2(I)$CB_{3,5}$ peptide was done with a Waters automated gradient controller (Millipore, Milford, MA, USA) equipped with Waters Model 510 pumps. The steel column was packed with macroporous C_4 resin, Supelcosil LC 309 (250×4.6 mm I.D., 5 μm, 300 Å pores, Supelco, Bellefonte, PA, USA) and connected to a 2 cm guard column packed with the same material. Crude CNBr peptide mixture (30–40 mg) was dissolved in 0.5 ml 2% heptafluorobutyric acid and 20 μl was injected onto the column. Elution was done by a linear gradient between water (A) and acetonitrile (B), both containing 0.1% heptafluorobutyric acid from 30% B to 100% B over 60 min followed by 10 min of isocratic washing with 100% B. The flow-rate was 1 ml/min and the column temperature was held at 60°C. The eluate was monitored at 210 mm using a Waters 490 E detector. The fraction emerging between 35–40 min was accumulated from five preparative experiments each comprising 40 runs. The separation columns were exchanged after each set of preparations, pre-columns were replaced routinely after every 10 runs. (The isolation/purification followed the procedure described in Ref. [30]). Because the source material was acid soluble collagen, the product was not contaminated with α_1(III)CB_9 peptide.

[1]Collagens are exceptional in exhibiting better solubility in the denatured than in the native state. They can be fully renatured after removing the denaturing agent e.g., by dialysis.

2.4. Separation of collagen type V

Collagen type V was obtained by pepsin solubilization from human placenta by an established method [28] and purified by carboxymethylcellulose chromatography; 25 mg of crude collagen type V sample was chromatographed at 4°C on a 10×2.5 cm column at a flow-rate 150 ml/h in 0.02 *M* Tris containing 6 *M* urea, pH 8 with a linear gradient from 0.0 to 0.25 mol/l NaCl over a total volume of 400 ml. Collagen type V was obtained from the eluate fraction between 60–140 ml. Gradient was started after all type I collagen was eluted, i.e., after the recording came back to zero after the first peak on the chromatogram. The procedure was essentially identical to that described by Miller and Rhodes [28].

2.5. Analysis of lead in eluted fractions

Measurements were performed on a Perkin-Elmer Elan 6000 (Perkin-Elmer, Norwalk, CT, USA) ICP-MS instrument; sample decomposition was accomplished in a microwave mineralizer with a focused field BM-1S/II (Plazmotronika, Wroclaw, Poland) according to a procedure described previously [31]. Lead stock standard solution 1000 mg/l (Analytika, Prague, Czech Republic) was used for preparation of working standards. Nitric acid and hydrogen peroxide used for sample decomposition was of Suprapur grade (Merck, Darmstadt, Germany). Deionized Milli-Q water (Millipore, Bedford, MA, USA) was used for preparation of all solutions. Lead was quantified as a sum of signals of ^{206}Pb, ^{207}Pb and ^{208}Pb isotopes and ^{209}Bi was used as internal standard. The optimized ICP-MS conditions are summarized in Table 1.

Table 1
Optimum ICP-MS operating conditions

Radio frequency power	1000 W
Dwell time	50 ms
Sweeps/replicate	20
No. of replicates	4
Total acquisition time per a.m.u.	4 s
Acquisition mode	Peak hopping
Ar nebulizer flow	0.80 l/min
Ar plasma flow	17 l/min
Ar auxiliary flow	1.2 l/min
Lens voltage	Autolens mode, optimized before each measurement
Sample uptake rate	1 ml/min

2.6. Binding experiments

For all binding experiments a 200×5 mm column (Pharmacia, Uppsala, Sweden) packed with Sephadex G-15 (Pharmacia) was used. The preparation of the column followed the procedure described by Sandier et al. [26]. Briefly, the gel was allowed to swell for 3 h at room temperature in the acetate buffer (pH 3, 0.1 *M*). After this the gel was poured into the column and elution was started at 0.5 ml/min. Three bed volumes of the buffer were allowed to pass through the column to stabilize it. Protein concentration in the eluate was detected by UV absorbance detection at 210 nm.

A Gilson peristaltic pump (Miniplus 2, Gilson, Villiers-le-Bel, France) and Altex-Beckman injector (Gagny, France) and an ISCO UV detector (ISCO, Lincoln, NV, USA) were used along with the above-mentioned Pharmacia column to materialize the experiments. The fraction collector used was from Laboratorní Prístroje, Prague, Czech Republic.

The binding assays were done by following the Hummel–Dreyer method [25] using the modification described by Sandier et al. [26]. Before each experiment the column was equilibrated with the protein solution (2 g/l) in 10^{-2} mol/l sodium acetate, pH 4.5. An appropriate volume of protein sample dissolved at the same concentration along with lead acetate (at a concentration required by the experiment) in the equilibration buffer was introduced as a sample. The chromatogram showed a positive and negative peak representing metal–protein complex (at the retention time of the protein) followed by a local decrease of the ligand concentration in the eluent. The bound ligand quantity can be deduced from the area of the negative peak (the second one). In Sandier et al.'s method [26] the main alteration is in using a large (4.5 ml) volume of the sample, which creates a plateau on the chromatogram rather than a peak. The amount of the bound metal was measured directly on the protein plateau rather than by evaluating the deficit in the ligand concentration (negative peak area), though this deficit can be clearly revealed on the chromatograms obtained. Maintaining the originally introduced flow-rate (0.4 ml/min) the metal concentration in the positive plateau was assayed by ICP-MS.

2.7. Electron microscopy

All electron microscopic studies were done with Siemens Elmiskop I at 80 kV. Collagen and collagen fragments samples (2 mg/ml) were dialyzed against 50 mmol/l ATP in two steps overnight (twice). In the second dialysis the dialyzing solution (ATP) was made 50 mmol/l with respect to $Pb(NO_3)_2$. Densitometric evaluation was done using Umax Astra densitometer (Umax, Fremont, CA, USA) and custom made evaluation programme (2nd Medical Faculty, Charles University, Prague, Czech Republic).

3. Results and discussion

At the very beginning of our experiments we have checked the difference between the elution volumes of α_1(I) collagen chain and Pb^{2+}. This turned out to be 10 and 17 ml, respectively, which appeared quite satisfactory for the binding experiments.

In the next stage we applied the large zone Hummel–Dreyer method (in the Sandier et al. modification, Ref. [26]) to assess the proportion between bound and free lead using collagen type I α_1 and α_2 chains, collagen type V and a large cyanogen bromide fragment of collagen type I ($\alpha_2CB_{3,5}$). A typical chromatogram is shown in Fig. 1. Plots of bound (%) vs. free (mol/l) lead for the different collagen samples are summarized in Fig. 2. Scatchard plots (r/[Pb free] vs. r where r is defined as the ratio of bound Pb concentration to the concentration of protein species) for all protein entities investigated are shown in Fig. 3. These graphs represent lead binding capacity at constant protein concentration (2 g/l). This naturally varies with different proteins/protein fragments used. It is documented here that the binding capacity of both collagen type I α chains, collagen type V and the $\alpha_2CB_{3,5}$ fragment of collagen type I increases with decreasing concentration of lead in the surrounding buffer.

To prove that our system was giving real numbers of binding sites and real values of the binding constants we reproduced the bovine serum albumin–Cu experiments [26] and determined values of one high and 18 low affinity sites with complexation constants of $K_1=3.2\cdot10^6\ M^{-1}$ and $K_2=2.8\cdot10^3\ M^{-1}$ for the high and low affinity sites, respectively, which is in good agreement with the published data (Ref. [26] reports 1 high affinity and 17 low affinity binding sites in bovine serum albumin with complexation constants of $3.5\cdot10^6\ M^{-1}$ and $3.0\cdot10^3\ M^{-1}$, respectively).

The Scatchard plots shown in Fig. 2 can be interpreted as two linear relationships indicating the presence of two categories of binding sites along all types of collagen molecules used. It has to be emphasized that all experiments were done under denaturing conditions and, consequently, complexation owing to higher structural arrangement of collagen molecules was unlikely to be revealed in our experiments. As documented in Table 2 the number of interactions along the collagen polypeptide chain correlates well with the number of aspartic and glutamic residues though other possibilities, namely binding to lysine and arginine should be also taken into consideration [9]. The complexation constants are well distinguishible except for the low affinity sites in collagen type V where no sufficient data were available to us because of difficulties of

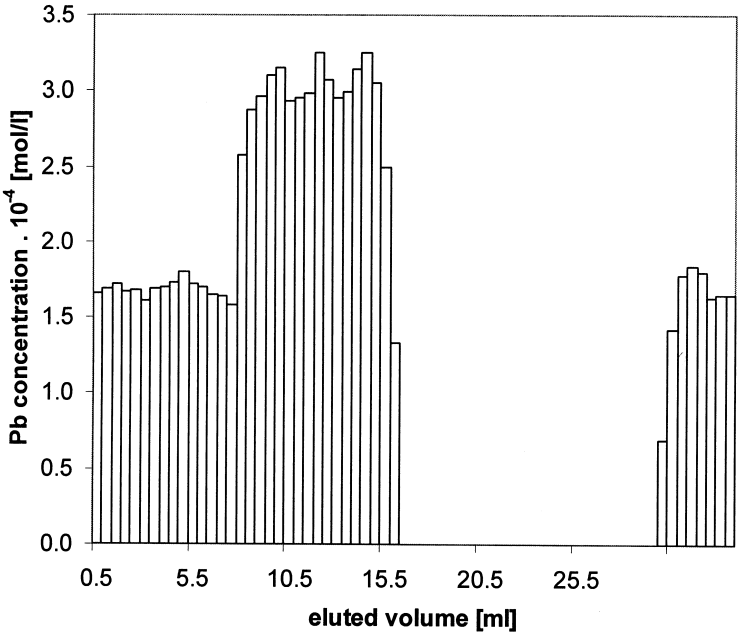

Fig. 1. ICP-MS analysis of fractions (0.5 ml) collected from Sephadex column. For details see Experimental.

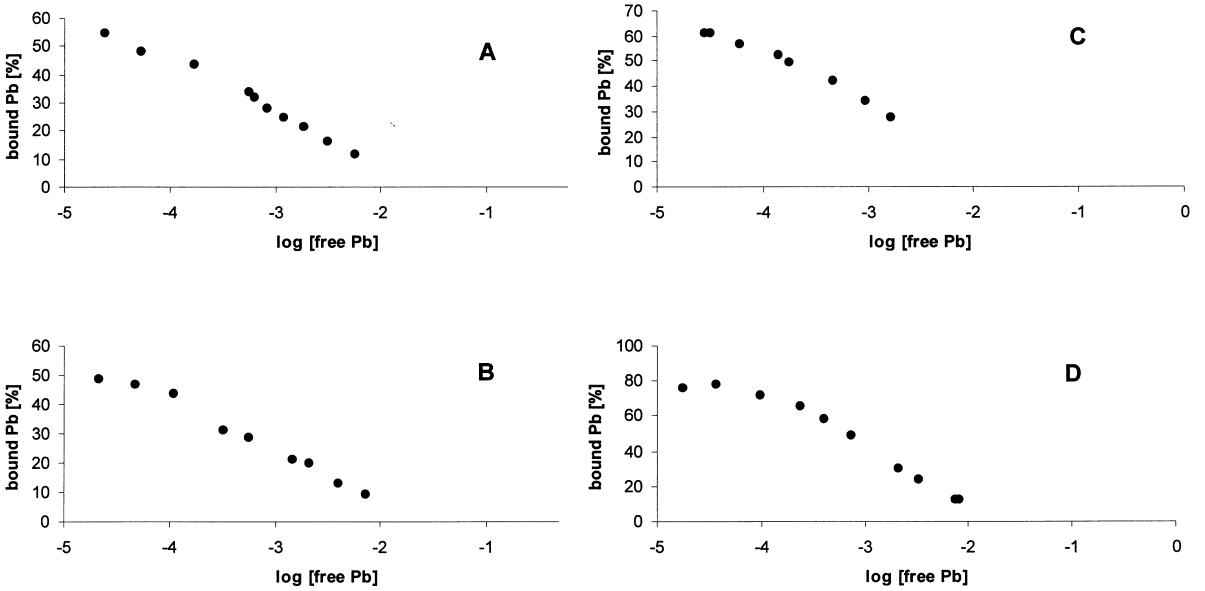

Fig. 2. Percentage of bound lead versus log of concentration of the free lead. (A) Collagen type I α_1 chain, (B) collagen type I α_2 chain, (C) cyanogen bromide fragment of collagen type I ($\alpha_2CB_{3,5}$), (D) collagen type V. For details see Experimental.

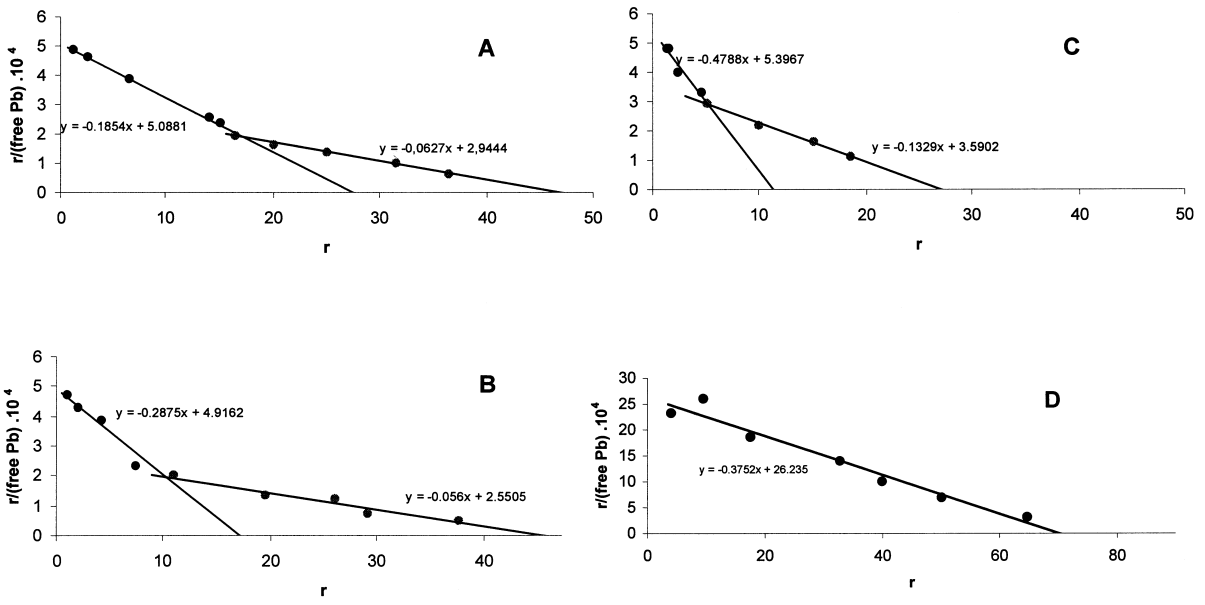

Fig. 3. Scatchard plot of lead binding to collagens (r/[free Pb] versus r), where r is the binding ratio. (A) Collagen type I α_1 chain, (B) collagen type I α_2 chain, (C) cyanogen bromide fragment of collagen type I ($\alpha_2CB_{3,5}$), (D) collagen type V. For details see Experimental.

Table 2
Correlation between the number of the two categories (low and high affinity) binding sites and glutamic and aspartic acid residues for different collagen types (chains and respective binding constants)

Collagen type/chain	No. of binding sites		No. of residues				Binding constant (M^{-1})	
	n_1 (low affinity)	n_2 (high affinity)	E (Glu)	D (Asp)	K (Lys)	R (Arg)	Low affinity	High affinity
Type I, α_1	46.96	27.44	48	28	58	71	$0.62 \cdot 10^3$	$1.85 \cdot 10^3$
Type I, α_2	45.54	17.1	45	17	67	72	$0.55 \cdot 10^3$	$2.87 \cdot 10^3$
$\alpha_2 CB_{3,5}$	27.01	11.27	28	12	–	–	$3.18 \cdot 10^3$	$4.78 \cdot 10^3$
Type V[a] $(\alpha_1)_2(\alpha_2)$	ND[b]	69.9	73.2	87.78	63	72	ND[b]	$3.75 \cdot 10^3$

[a] Calculated to averaged α-chain composition.
[b] ND=Not determined.

accumulating sufficient amount of adequately pure collagen type V. Such interactions were reported for binding of toxic metals to anionic sites available in membrane lipids and lipoproteins obtained from diverse sources (bacterial, plant, animal tissue materials) (see Cowan [32]). As far as binding to cationic sites is concerned, this appears unlikely because the number of binding sites (estimated by the Scatchard plot), the number of cross-striation bands and the number of aspartic residues correlate rather well. On the contrary the total number of lysine (K) and arginine (R) residues is much higher than the number of acidic residues (Table 2). Were it so that binding occurs at the positively charged amino acids, it would be necessary to assume that a considerable proportion of lysine and/or arginine is not involved in lead binding. Also it is fair to say that in the quoted paper [9] binding of heavy metals (gold, bismuth and mercury) to positively charged sites was a proposed possibility supported by direct evidence; however, it maybe also argued that these metals and Pb have different binding sites in the collagen molecule.

On the other hand it has been reported already in the sixties that upon staining of collagen fibrils with heavy metals (mainly gold) phosphotungstic acid and uranyl acetate about 14 cross-striating bands can be distinguished in one identity period (the intensity of individual bands varies considerably) [33,34]. It is to be emphasized that such staining can be achieved also in vivo [35–37]. In the so-called SLS form of collagen (an assembly of laterally aggregated collagen molecules the length of which corresponds to a single collagen molecule[2]) numerous bands can be seen.

We felt that this approach would represent supporting evidence for the number of interacting sites revealed by the Scatchard plots. As shown in Fig. 4, the densitometric profile of the SLS form of type I collagen incubated with lead acetate showed a rich banding pattern. Regarding band intensity three categories of bands can be seen in the profile: very intense bands, bands of intermediate intensity and a number of bands the intensity of which is very low. The number of bands of large and medium intensity is 24. This corresponds well with the aspartic acid residues present and the number of the high affinity binding sites (28 and 27.44, respectively). The number of low intensity bands is 38 in comparison to the low affinity sites which turned to be 46.96 (there are 48 glutamic acid residues per molecule). The lower number of both high and low intensity bands seen in the electromicrographs as compared to the number of binding sites revealed by the evaluation of chromatographic data by Scatchard plots appears quite acceptable as there are two reasons for it. First it is likely that not all association capable residues are equally accessible to lead ions as some of them may not be located in sterically advantageous positions (sterical restrictions). Second, the profile shown

[2] SLS collagen forms are composed of the parent collagen molecules formed by $(\alpha_1)_2(\alpha_2)$ collagen α chains or represent trimers of α_1 or α_2 chains if pure parent α_1 chains are treated with ATP.

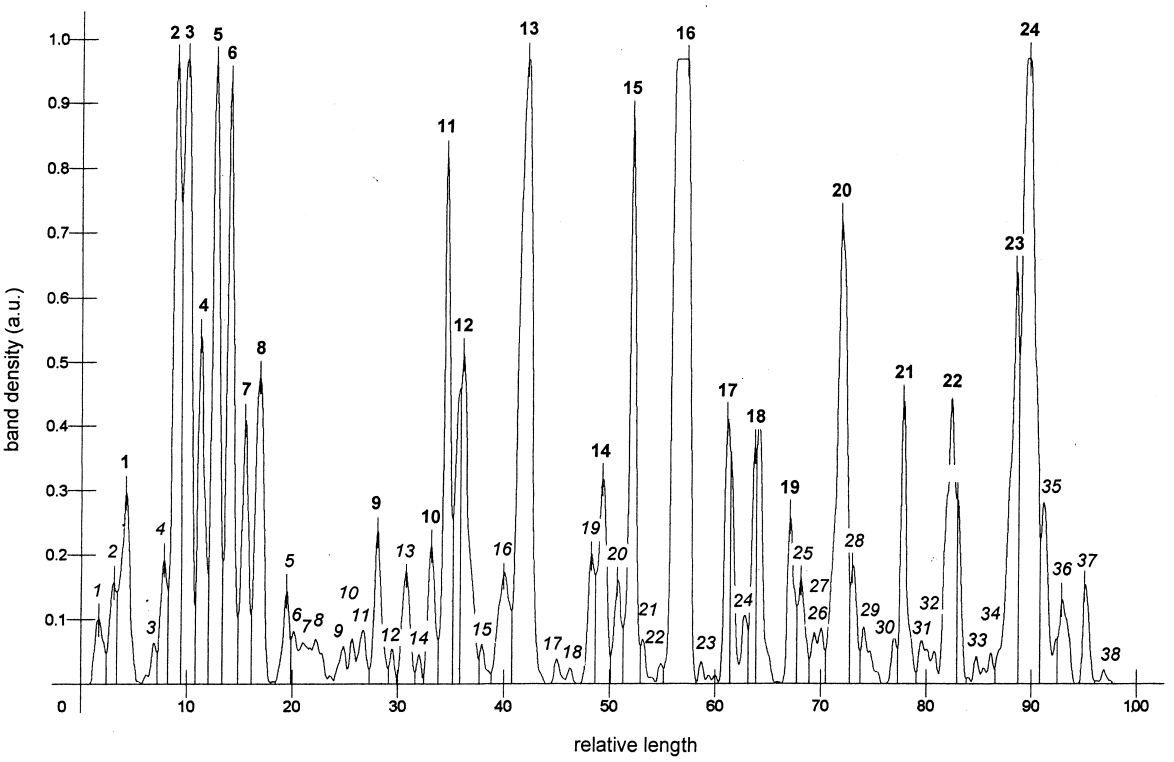

Fig. 4. Densitometric profile of the SLS form of type I collagen incubated with lead acetate. Bold numbers mark bands of large and medium intensity and italic numbers mark low intensity bands.

corresponds to the whole length of the collagen type I α_1 chain, i.e., roughly 1000 amino acid residues. As some of the asp and glu residues are are positioned in clusters (only a few amino acid residues apart) they may not be distinguishible in the electromicrograph (see examples in Table 3). Taking into account these two facts the coincidence between the number of binding sites and the number of acid residues appears quite acceptable.

Table 3
Clustered aspartic acid residues in collagen type I α_1 chain

231–240	DDGEAGKPGR
271–280	SGLDGAKGDA
700–720	GAPGNDGAKGDAGAPGAPGS
741–750	PKGDRGDAGP
751–760	KGADGSPGKD
831–840	DAGAKGDAGP
1021–1040	EGSPGRDGSPGAKGDRGETG

4. Conclusions

Using the Hummel–Dreyer method with the Sandier et al. modification it was possible to show a correlation between the aspartic acid residues in the collagen type I α_1 and α_2 chains, collagen type V and a large CNBr peptide collagen type I [α_2(I)CB$_{3,5}$] molecules and analytically determined number of binding sites. Binding that involves the glutamic acid residues, appears to be weaker.

The results were compared with the cross-striation pattern of the SLS form of collagen type I α_1 chain. A reasonable agreement between the number of

cross-striation bands and the number of the stronger binding sites (aspartic acid residues) was obtained.

Acknowledgements

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