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Short Communication

Determination of calcium ions tightly bound to proteins

Katsutoshi Nitta* and Akihiro Watanabe

Department of Polymer Science, Faculty of Science, Hokkaido University, N10 W8 Kita-ku, Sapporo, Hokkaido 060 (Japan)

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ABSTRACT

A rapid and sensitive procedure is described for the determination of calcium ions tightly bound to proteins using high-performance gel filtration chromatography, followed by the destabilization of the protein conformation and fluorimetric analysis with Quin-2. With this method, contaminating, unbound calcium can be eliminated simultaneously and one can determine the content of a calcium ion in a protein utilizing less than 200 pmol of the protein.

INTRODUCTION

In order to investigate a calcium-binding protein, one must first determine the number of calcium ions bound to that protein by means of, for example, atomic absorption spectrometry. However, using this method contamination by calcium ions from labware is frequently a source of trouble; one must eliminate contaminating non-bound calcium ions carefully, and additionally with this method, samples once burned are not recoverable. Thompson *et al.* [1] reported a suitable method for comparing the migration rates of a protein in polyacrylamide gel electrophoresis with and without ethylene glycol tetraacetate for the purpose of detecting calcium-binding proteins. However, the specificity of their method is considered to be insufficient. Metalloproteins binding metals other than calcium ions would be equally detected.

To overcome such disadvantages, a microanalytical method has been developed in this study, using high-performance gel-filtration chromatography together with the use of a calcium-specific fluorescent reagent. With this method, one can determine the number of bound calcium ions with less than 200 pmol of the protein. The analysis and elimination of the contaminating calcium ions are performed simultaneously and one can recover the protein because the sample need not be burned.

EXPERIMENTAL

Materials

Quin-2 was purchased from Wako (Osaka, Japan). Special-grade urea for biochemical use was obtained from Nacalai Tesque (Kyoto, Japan). Other reagents were all of guaranteed grade from Nacalai Tesque.

Bovine α-lactalbumin [2] and equine lysozyme [3] were prepared previously. Carp parvalbumin III was prepared from carp white muscle according to the literature [4]. Absorptivities, $A^{1\%}$, of these proteins are known to be 20.1 at 280 nm for bovine α-lactalbumin [5], 23.5 at 280 nm for equine lysozyme [44] and 1.76 at 259 nm for carp parvalbumin III [6].

Methods

A schematic diagram of the aparatus is shown in Fig. 1. A Biofine PO-4K gel filtration column (Ja-

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pan Spectroscopic) (300 mm × 7.5 mm I.D., exclusion limit 4000 dalton) was equilibrated with 0.01 *M* 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid (HEPES) buffer (pH 7.5, 0.1 *M* KCl), which had been decalcified with a Chelex A-100 ion-exchange column. The same buffer was introduced to the column at a flow-rate of 1 ml/min by a high-performance liquid chromatographic pump (1) (Model 885 PU; Japan Spectroscopic). A volume of 10 μl of sample or standard solution was applied from a sample injector with a microsyringe (Hamilton, Reno, NV, USA). With this column, macromolecular proteins and low-molecular-weight ions were separated. From reservoir 2, 8 *M* aqueous urea containing 20 μM Quin-2 [6], which had also been decalcified, was transmitted with another pump (2) (Model 880 PU; Japan Spectroscopic) at a flow-rate of 1 ml/min and combined with the eluate from the column. After passing through a 150-cm mixing coil, the solution was introduced to the flow cell of a spectrofluorimeter (Model FP 210; Japan Spectroscopic), with excitation at 335 nm and emission at 492 nm. The resulting fluorescence intensity was recorded with a Chromatocoder 12 (System Instruments, Tokyo, Japan) and integrated with time coordinates by an internal computer. Bound and contaminating calcium ions were detected at *ca.* 5 and 13 min after injection, respectively (Fig. 2).

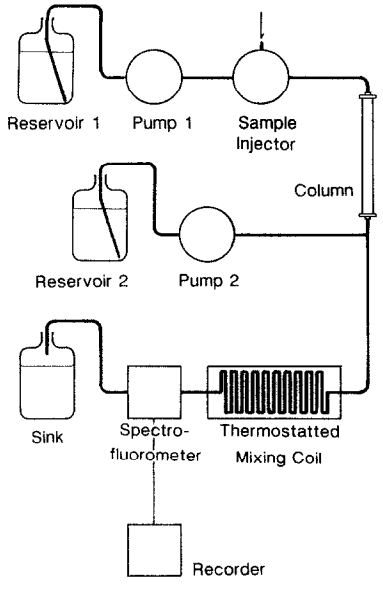

Fig. 1. Schematic diagram of the aparatus.

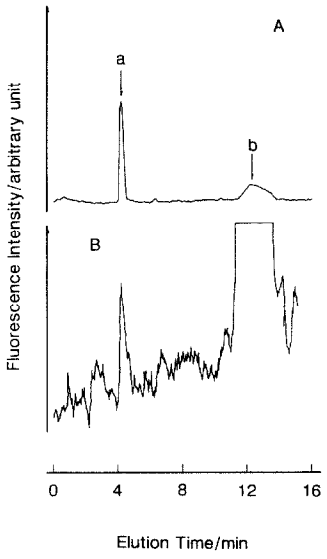

Fig. 2. Elution profiles of calcium bound to α-lactalbumin. amount of α-lactalbumin loaded: (A) 10 μl of 100 μM; (B) 10 μl of 1 μM. Peak 1, elution peak of calcium bound to α-lactalbumin; peak 2, non-bound, contaminating calcium.

RESULTS AND DISCUSSION

The binding strengths of calcium-binding proteins are high and in many instances exceed those of typical chelating reagents. For example, the first binding constant of carp parvalbumin III has been reported to be $6.3 \cdot 10^8$ l mol^{-1} [7]. On the other hand, among many reported fluorescent calcium indicators [8–10], Quin-2 binds calcium ions the most tightly, but the binding constant is $10^{7.1}$ l mol^{-1}, one order of magnitude less than that of carp parvalbumin III.

Calcium-binding protein binds a calcium ion only when it is in the native conformation. Unfolded protein binds the ion with a far lower binding strength, if it binds at al.

Accordingly, binding equilibrium may be expressed as follows:

$$N + Ca \overset{K_{Ca}}{\rightarrow} NCa \qquad (1)$$

$$N \xrightarrow{K_U} U \qquad (2)$$

where N, NCa and U represent apoprotein in the native conformation holoprotein and unfolded apoprotein, respectively, K_{Ca} is the binding constant of native apoprotein and K_U is the equilibrium constant of the unfolding of the apoprotein. From eqns. 1 and 2, one can deduce an apparent binding constant (K_{app}) of the protein as follows:

$$K_{app} = \frac{[NCa]}{([N] + [U])[Ca]} = \frac{K_{Ca}}{1 + K_U} \qquad (3)$$

One can decrease the binding strength by increasing the value of the denominator on the right-hand side of eqn. 3. The reduction in the conformational stability of apoprotein results in the reduction of the binding strength of the protein. In this study, aqueous urea, used for destabilization, and Quin-2, which binds calcium ions the most tightly among the fluorescent calcium ion indicators [8–10], were used. It should be noted that the sample protein need not be denatured. It is sufficient if the apparent binding constant is decreased to be sufficiently lower than that of Quin-2 for this study.

Calcium chloride solution (100 μM) was used as a standard, and the concentration of bound calcium was determined by the comparison of the areas under the peaks of the chromatograms. The results are shown in Fig. 3. Without the urea solution, calcium was not detected quantitatively (data not shown). With bovine α-lactalbumin and equine lysozyme, heating of the mixing coil was unnecessary. Bound calcium was analysed quantitatively at room temperature. However, with carp parvalbumin III, bound calcium was quantified only to 90% at room temperature. For more accurate quantification, the mixing coil had to be heated to 40°C. The results for carp parvalbumin III in Fig. 3 were obtained by heating to 40°C.

The linear least-squares method was applied to the data in Fig. 3 with the equation

$$C_{Ca} = n\,C_p + c \qquad (4)$$

where C_{Ca} is the observed concentration of bound calcium, n is the number of bound calcium ions per protein molecule, C_P is the concentration of the protein of the injected solution and c is a constant. The values of n obtained (± standard deviations) were 1.07 ± 0.03, 0.94 ± 0.07 and 1.93 ± 0.15 for bovine α-lactalbumin, equine lysozyme and carp parvalbumin III, respectively. The results were quite satisfactory, as these proteins bind one [11], one [3], and two calcium ions [7], respectively. The values of c were obtained as −1.2 ± 1.4, 1.6 ± 4.3, and 2.1 ± 5.3 μM, respectively, which were equal to 0 μM within standard errors.

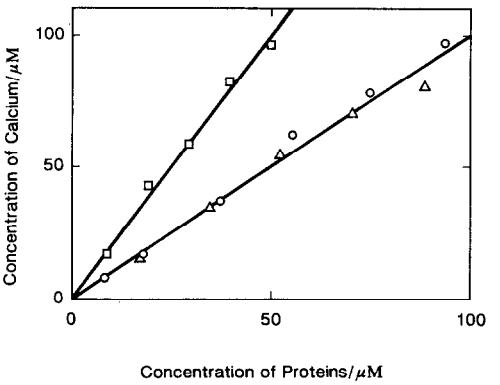

Fig. 3. Relationships between the concentrations of applied calcium-binding proteins and the observed calcium concentrations. ○ = Bovine α-lactalbumin; △ = equine milk lysozyme; □ = carp parvalbumin III. The upper and lower straight lines show the relationships for proteins containing two and one calcium ions, respectively.

To examine the limit of detection of calcium proteins, dilute solutions of α-lactalbumin were applied. The result utilizing 1 μM α-lactalbumin is shown in Fig. 2B. A peak for bound calcium ions can be seen above the noise level and is well separated from a very large contaminating, non-bound calcium ion.

After decreasing the binding strength with urea, bound calcium ions react quantitatively with Quin-2. One can employ other denaturants such as guanidinium hydrochloride or sodium dodecyl sulphate equally for the same purpose. Quin-2 was employed in this study because it binds calcium ions the most tightly, and it is the cheapest among the commercially available fluorescent calcium indicators. Other indicators, such as Fura-2 and Indo-1, are 30 times more fluorescent than Quin-2 [9]. Their use may improve the sensitivity, but they are 100

times more expensive than Quin-2. Such indicators may be used for very precious proteins.

A gel filtration column with a low exclusion limit was employed in this study. Another gel filtration column with a higher exclusion limit or another column with a different separation mechanism could be employed, assuming that the contamination by calcium could be sufficiently supressed and calcium-binding proteins and non-bound calcium can be separated. With this method, one can determine bound calcium with sufficient accuracy by using less than 200 pmol of a protein. Moreover, this method is doubly specific for calcium; not only does Quin-2 show a high stability constant for calcium against magnesium, but also the emission spectrum of the calcium complex of Quin-2 is totally different from that of the magnesium complex [8].

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