SPECTROSCOPIC STUDIES ON Cd^{2+} BINDING TO HUMAN ERYTHROCYTE SPECTRIN

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Abstract

The binding of Cd^{2+} to the human erythrocyte spectrin tetramer (SPT) and the consequent conformation change were studied as a function of the Cd^{2+} /SPD mole ratio (R) by fluorescence and circular dichroism (CD) spectra. The conformation dependence on R has a biphasic feature: in the range less than 60, the Cd^{2+} ions are likely bound to a small fraction of the thiol groups and the non-thiol groups on the surface of SPT and the conformational changes are not significant as characterized by the labeled N-(1-pyrenyl)maleimide (NPM) fluorescence and CD spectra. When R reaches 60, as a result of a conformational change, the SH-related strong binding sites are exposed and bind with Cd^{2+} . The biphasic conformational change at this turning point might be interpreted on the basis of thiol groups as critically important for maintaining the secondary structure of SPT.

Introduction

The membrane skeleton of erythrocyte plays an important role for the maintenance of the cell activity.^[1] Spectrin, as a major component of the skeleton network, is composed of heterodimeric subunits α [250KD] β [230KD]. The dimer associates in a head-to-head mode to form 200nm long tetramer (SPT), which is the predominant state of spectrin in erythrocyte cells^[2]. It is known that metal ions such as Ca^{2+} , Mg^{2+} and Tb^{3+} tend to bind to spectrin and alter its self-association state. The metal ions thus studied are all hard acid ions.^[3-11] Li et al studied the effect of platinum complexes on the conformation of spectrin.^[12] Yang et al

reported the interactions between SeO_3^{2-} and spectrin.^[13-15] It might be expected that the binding with soft cations and the consequent conformation change are different from hard ones act. For this sake, we studied the Cd^{2+} -SPT interactions by measurement of the fluorescence spectra, both intrinsic and SH-labeled SPT and the CD spectra.

Methods and materials

Materials. Fresh human blood was obtained from the Beijing Blood Central Bank. Other reagents are all of analytical grade.

Purification of spectrin

Human erythrocyte ghosts were prepared according to Dodge^[16] and spectrin was purified according to the Gratzer method^[17] with some modifications. After extraction with a very low ionic strength buffer (of 0.3mM phosphate, 0.1mM EDTA and 0.1mM PMSF pH 7.5), the suspension was dialyzed against the same buffer at 4°C. The suspension was then centrifuged at 17,000rpm for 30 min. and the supernatant was centrifuged again at the same condition to remove the vesiculated membranes completely. The supernatant was concentrated by dialysis against polyethylene glycol (Mr.20KD) and then applied to a column of Sepharose 4B 2.5×80cm. A pH 7.4 buffer consisting 130mM KCl, 20mM NaCl, 1mM EDTA, 10μM DTT and 0.5mM NaN_3 was used for elution. The SPT peak was pooled down and reapplied to the column to collect the purified spectrin. The spectrin solution was dialyzed exhaustively against a pH8.0 buffer containing 2mM EDTA and the procedure was repeated three times and then dialyzed against the deionized water to remove the EDTA. Finally, the spectrin solution was immediately dialyzed against the appropriate buffer. The spectrin concentration were determined spectrophotometrically ($\text{E1\%1cm}=10.7$) and checked with Lowry's method. They are calculated and expressed as those of SPD. CdCl_2 stock solution is 1.6mM. Without specification, all the buffers used are composed of 0.1M NaCl, 0.05M Tris-HCl and pH 7.4.

Fluorescence measurements

All the steady state fluorescence measurements were conducted with a Hitachi 3000 spectrophotofluorometer at 22°C. Slits for both excitation and emission were set at 5nm.

Intrinsic fluorescence

In typical experiments, SPT(0.26μM) solutions were titrated with CdCl_2 stock solution. The final volume change was less than 5%. The Cd^{2+} concentration is in the range of 1.48×10^{-6} - $1.48 \times 10^{-5}\text{M}$, and the samples were excited at 280nm and emission was measured at 340nm.

NPM labeled fluorescence

The Cd²⁺-SPT complexes labeled with NPM Various volumes of the CdCl₂ stock solution were added to a series of SPT (0.26μM) solution. After standing at room temperature for 4hr, 10 μl 3mM NPM stock solution was added to the solutions, and set them overnight at 4°C. The samples were excited at 342nm and the emission fluorescence was measured at 375nm.

The titration of NPM-SPT with Cd²⁺ 200μl 3mM NPM solution was added to 50 ml 0.47 μM SPT solution. After standing overnight at 4°C and dialyzing against the same buffer to remove the free NPM, the solution was centrifuged at 18,000rpm at 4°C for 1hr. The NPM-SPT was titrated with CdCl₂ by adding various volumes of CdCl₂ stock solution to 2.5ml of the NPM-SPT solution. After standing for 4hr, the fluorescence spectra were recorded.

Circular dichroism measurements Measurements were performed on an Jasco-20 Polarizing Spectrophotometer. SPT concentration was diluted to 0.125μM using the buffer and the spectra were scanned from 260 to 190nm. Cd²⁺ concentration in the samples varied from 0 to 1.4×10⁵M.

Results

(1) **Intrinsic fluorescence** As shown in Fig.1, the fluorescence intensity was reduced in the course of the Cd²⁺ titration. Since the fluorescence of SPT is mainly due to tryptophane residues, this result indicates that the microenvironment of tryptophane in SPT was changed by the binding of Cd²⁺, which makes the tryptophane residues exposed to the aqueous media.

(2) **NPM labeled Cd²⁺-SPT complexes** When the SPT treated previously with Cd²⁺ was labeled with NPM, NPM reacts with the thiol groups unoccupied by Cd²⁺. The results revealed that the fluorescence intensity decreases slightly in the range of R less than 60, but the intensity decreased significantly with R higher than 60 (Fig. 2). NPM is a highly specific reagent for thiol group. Because it is not fluorescent in the free form, and turns to be fluorescent when covalently linked with the thiol groups of the protein, the number of free thiol groups of the protein can be deduced on the basis of its fluorescence intensity. Based on the results of NPM labeling studies of Cd²⁺-SPT complexes, Cd²⁺ ions are bound to a very small fraction of thiol groups at low Cd²⁺ concentration. The dramatic reduction in the intensity at higher Cd²⁺ concentration indicates that Cd²⁺ ions mainly bind to thiol groups and left only a small number of thiol groups to link with NPM. It is reasonable to say that the conformation change at low R value clears the way for more Cd²⁺ ions binding to thiol groups. Yang et al have

Figure 1 Effect of cadmium on intrinsic fluorescence of SPT $C_{\text{sp}}=0.26\mu\text{M}$, buffer: 0.1M NaCl, 0.05M Tris-HCl pH 7.4.

Figure 2 SPf was titrated with Cd^{2+} and then saturated with NPM
 $C_{\text{sp}}=0.43\mu\text{M}$, buffer :0.1M NaCl, 0.05M Tris-HCl
 pH 7.4

observed similar result in case of the interactions between SPT and SeO_3^{2-} [11-13], but it has not been interpreted.

(3) NPM labelled SPT titrated by Cd^{2+} NPM is frequently employed as a probe for conformation changes related to the microenvironment of thiol groups in proteins. As shown in Fig. 3, the fluorescence of NPM-SPT was quenched slightly in the course of titration in lower Cd^{2+} concentration, but as the R value increases over 60, the fluorescence was quenched abruptly. It reveals that Cd^{2+} binding makes the NPM labeled on the thiol groups turns from hydrophobic to hydrophilic environment.

Figure 3 Effect of Cd^{2+} on NPM fluorescence of NPM-SPT

SPT was first labeled with NPM and the unlabelled NPM was removed completely by dialyzing against the same buffer exhaustively and then titrated with Cd^{2+} . $C_{\text{sp}}=0.23\mu\text{M}$, buffer: 0.1M NaCl, 0.05M Tris-HCl, pH 7.4

(4) CD spectra The CD spectra of SPT, characterized by two negative peaks at 208nm and 220nm, are typical α helix-rich protein. The molar ellipticity θ_{208} is dependent on the

Cd^{2+} concentration. From the titration curve (Fig.4), it can be seen that following the increasing of the Cd^{2+} concentration, θ_{208} values move towards slightly negative value. When R value is

Figure 4 CD spectra of SP1 titrated with Cd^{2+}
 $C_{\text{sp}} = 0.125 \mu\text{M}$, buffer: 0.1M NaCl, 0.05M Tris-HCl,
 pH 7.4

higher than 60, the changes become more significant. This indicates that the α -helix contents are decreased when Cd^{2+} increases. These results indicate that a low Cd^{2+} concentration has only a small effect on the secondary structure of spectrin. However, high Cd^{2+} concentration destroys it.

Discussion

The interaction between Cd^{2+} and SPT is featured by a biphasic stage as a function of R and $R=60$ is the turning point. When R is below 60, only a slight conformation change was observed as shown by the low quenching of intrinsic fluorescence and NPM labeled fluorescence. When the R is raised to above 60, the conformation changes abruptly. This biphasic feature might be explained as follows: Cd^{2+} ions bind to a small fraction of the thiol groups and the carboxylic groups on the surface of SPT molecules at first, and the thiol groups which are of high affinity to Cd^{2+} , are buried in SPT molecules. The Cd^{2+} binding to the surface carboxylic groups resulted in only little conformation change, Nevertheless, the binding

facilitates the buried thiol groups to turn out to more open sites, exposing them to attacking Cd^{2+} ions. The conformation changes significantly beyond the turning point, which makes a large number of Cd^{2+} ions to bind to the thiol groups of SPT. These results demonstrate that the thiol groups are very important in maintaining the secondary structure of SPT. In this case, Cd^{2+} binding in the first stage results in a conformation to facilitate Cd^{2+} binding to more thiol groups when R reaches a critical value. This might be considered as a special case of cooperative effect among different binding groups. The present works show that the fluorescence labeling method is helpful to get an insight to the metal ion binding to proteins.

Our previous study on the interaction between Tb^{3+} and SPT shows that Tb^{3+} binds to SPT in a monophasic mode. This is reasonable because the hard acid Tb^{3+} binds to carboxylic groups solely. The difference in hardness of these ions causes binding to different binding groups of the protein and thus induces different conformation change of SPT.

References

- [1] S.R. Goodman, and S.Kathleenr, *Am. J. Physiol.*, 244, c121-c141(1983)
- [2] D.M. Shotton, B. Burke and D. Braton, *J. Mol. Biol.*, 131, 303-329(1979)
- [3] E. Brauer, K.D. Kupka and V. Rudloff, *Bioelectrochem. Bioenerg.*, 3, 509-518(1976)
- [4] B. Lamel and G. Maier, *Biochim. et Biophys. Acta*, 622,245-258(1980)
- [5] C.J. Wallis, E.F. Wenegieme and J.A. Bahitch, *J. Biol. Chem.*, 267(7), 4333-7(1992)
- [6] S.Lundberg, V.-P. Lehto and L. Backman, *Biochemistry*, 31, 5665 (1992)
- [7] C.J. Wallis, E.F. Wenegieme and J.A. Babitch, *Biochemistry*, 32, 5045(1992)
- [8] H.-Y. Sun,H.-K. Lin, Y. Cao, R.-C. Li, K. Wang, R.-T. Chen, *J. Inorg. Biochem.* Submitted
- [9] G.H. Beaven, and W.B. Gratzer, *Biochim. et. Biophys. Acta.*, 600,140-149 (1980)
- [10] W.B. Gratzer, G.B. Beaven, *Eur. J. Biochem.*, 558, 403(1975)
- [11] G.H. Beaven, J. Parmar, G.B. Nash, P.M. Bennett and W.B. Gratzer, *J. Membrane Biol*, 118, 251- 57(1990)
- [12] R.-C. Li, H.-Q. Cai, X.-G. Yang, B.-W. Chen, K. Wang, *Journal of Beijing Medical University*, 24(6), 481(1992)
- [13] J. Yang and F.-Y. Yang, *Acta Biophysica Sinica*, 4(4), 372 (1988)
- [14] M.-Z. Yang and F.-Y. Yang, *Acta Biophysica Sinica*, 4(4), 379(1988)
- [15] F.-Y. Yang, J. Yang, Z.-M. Liu, *BioFactors* , 3(1), 49(1991)
- [16] I.T. Dodge, C. Mitchell and D.J. Hanahan , *Arch. Biochim. Biophys*, 100, 119(1963)
- [17] W.B. Gratzer, *Methods In Enzymology*, 85, 475(1982)

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