Excimer Fluorescence of *N*-(1-Pyrenyl)iodoacetamide-Labeled Spectrin

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The structure of spectrin in aqueous solutions or erythrocyte ghosts was characterized by using a fluorophore, *N*-(1-pyrenyl)iodoacetamide (NPIA). The excimer fluorescence of NPIA was clearly observed in isolated spectrin, but not in other proteins such as actin, bovine serum albumin, and lysozyme. The excimer disappeared upon addition of sodium dodecyl sulfate, but reappeared by dialysis. Further, the excimer appeared in spectrin extracts from ghosts labeled at 37 °C with NPIA. Interestingly, no such excimer in spectrin extracts appeared upon pretreatment of ghosts with diamide, and then *N*-ethylmaleimide. However, when such double-treated ghosts were exposed to dithiothreitol before NPIA labeling, the excimer fluorescence reappeared. Further, excimer fluorescence was observed in ghosts which were pretreated above 30 °C, especially at 50 °C, and then labeled at 0 °C with NPIA. The data of nondenaturing polyacrylamide gel electrophoresis and CD spectra showed marked changes in the structure of spectrin at 50 °C. We thus suggest that the excimer fluorescence of NPIA is useful to monitor the structural changes of spectrin.

Erythrocyte spectrin is a major component of the cytoskeleton, and associates with other proteins, such as actin and protein 4.1.[1,2] Spectrin is a rod-like heterodimer molecule comprising two large subunits, α (240 kDa) and β (220 kDa).[3] In the native membrane, spectrin dimers form tetramers upon head-to-head association.[4—6] However, mutations of the α- and β-subunits affect the self-association of spectrin dimers to tetramers.[7] Erythrocyte membranes containing such defective spectrins are morphologically elliptical and are unstable to thermal and mechanical stresses.[8,9] Thus, spectrin plays an important role in the stability and elasticity of the erythrocyte membrane.

A large number of reports on spectrin dimer–tetramer equilibrium have been published.[4—7] According to these results, its equilibrium is reversible and affected by such factors as the temperature, ionic strength, and basic proteins.[10] The dimer is the more stable form above 30 °C and at low ionic strength.[4] On the other hand, the spectrin tetramer is stabilized by heme-containing proteins or basic ones.[10] The physicochemical properties of spectrin have also been studied by using spectroscopic means, such as CD[7,11,12] and ESR.[13] Conformations of spectrin dissolved in an isotonic buffer are α-helix above 75%.[12] The conformations of spin-labeled spectrin are stabilized by divalent cations (Ca^{2+}, Mg^{2+}) against thermal denaturation.[13] To obtain further information about the structure, spectrin was labeled with *N*-(1-pyrenyl)iodoacetamide (NPIA). This fluorophore was used to study actin polymerization.[14] Upon polymerization of actin, the intensity of the fluorescence increases remarkably.[14] In the present work, we report on the properties of the excimer fluorescence observed in NPIA-treated spectrin.

Experimental

Materials. Diazinedicarboxylic acid bis-(*N*′,*N*′-dimethylamide)(diamide) and *N*-(1-pyrenyl)iodoacetamide (NPIA) were purchased from Sigma and Molecular Probes, respectively. Dithiothreitol (DTT), *N*-ethylmaleimide (NEM), and lysozyme from egg white were obtained from Wako Pure Chemicals. Phenylmethylsulfonyl fluoride (PMSF) and bovine serum albumin (BSA) were from Nacalai Tesque. All other chemicals were of reagent grade.

Spectrin Extraction from Ghost Membranes. Human blood was obtained from the Fukuoka Red Cross Blood Center. The blood was centrifuged at 750 *g* for 10 min at 4 °C. The plasma and buffy coat were carefully removed. The erythrocytes were washed three times with phosphate buffered saline (PBS, 150 mM NaCl, 10 mM sodium phosphate, 1 M = 1 mol dm^{-3}, pH 7.4). A preparation of ghosts from intact erythrocytes was carried out under chilled conditions (0—4 °C). The packed erythrocytes were added into a hypotonic buffer (5 mM sodium phosphate, pH 8.0; 5P8), suspended gently, and stood for 10 min at 0 °C. The erythrocyte suspension was centrifuged at 10000 *g* for 20 min at 4 °C. The pellets, i.e. ghosts, were washed three or four times with 5P8 until the red color of the pellet disappeared. For a heat treatment, ghosts suspended at 10% hematocrit in 5P8 were incubated for 30 min at 0, 20, 30, 40, and 50 °C. For chemical modifications of membrane SH groups, ghosts (10% hematocrit in 5P8) were incubated with 0.5 mM diamide for 30 min at 37 °C, washed twice with 5P8, treated with 10 mM NEM for 30 min at 37 °C, and washed three times with 5P8. Parts of these ghosts (10% hematocrit in 5P8) were exposed to 10 mM DTT for 30 min at 37 °C, and washed three times with 5P8. Chemically modified ghosts (10% hematocrit in 5P8) were incubated with NPIA (10 or 100 μg ml^{-1}) at 37 °C for 30 min or at 0 °C over night. These ghosts were washed once with 5P8 containing 0.5% BSA and rinsed three times with 5P8. To prepare spectrin, chemically modified or unmodified ghosts were added into a 3-fold volume of the extraction buffer (0.1 mM EDTA, 0.1 mM PMSF, pH 8.0),[6] suspended gently, and incubated for 20 h at 0 °C or for 30 min at 37 °C. The suspension was centrifuged for 30 min at 56000 *g* and 4 °C. Parts of the supernatant were used to purify spectrin using a Sepharose CL-4B column, and also exposed to heating for 30 min at 50 °C or to 1% sodium dodecyl sulfate (SDS) at room

temperature.

Preparation of Actin. Actin was prepared from rabbit skeletal muscle by the method of Spudich and Watt.[15] The purity of actin was checked by SDS-polyacrylamide gel electrophoresis (PAGE).

NPIA Treatment of Proteins. Spectrin, BSA, and lysozyme in 0.1 mM EDTA, 0.1 mM PMSF, pH 8.0 or actin in 1 mM $NaHCO_3$, 0.1 mM $CaCl_2$, 0.2 mM ATP, pH 8.0 were incubated with NPIA (1—100 $\mu g\,ml^{-1}$) for 30 min at 37 °C. All protein concentrations were almost the same (0.3 $mg\,ml^{-1}$). The sample solutions were centrifuged at 18000 *g* and 4 °C for 30 min. The supernatants were used for fluorescence measurements. To characterize the excimer fluorescence, SDS (1%) was added to the spectrin solution and then removed by dialysis against the extraction buffer at 4 °C.

Nondenaturing PAGE and CD Spectra of Spectrin Extracts. Spectrin extracts prepared at 0 °C as described above were made isotonic by adding salts (0.14 M KCl, 10 mM NaCl, and 1 mM $MgCl_2$). Oligomeric states of spectrin which had been incubated for 30 min at 25, 40, and 50 °C were analyzed by nondenaturing PAGE.[16] For CD measurements, spectrin extracts (0.3 $mg\,ml^{-1}$) in an isotonic buffer were incubated for 30 min at 20, 30, 40, and 50 °C. The CD spectra were recorded at room temperature (ca. 20 °C) on a model J-600 spectropolarimeter (JASCO, Tokyo, Japan). The CD spectra were acquired with 20-nm min^{-1} scan speed, 1.0-nm band width, 20-mdeg sensitivity and 20-s time constant. The molar ellipticities were obtained using a mean residue molecular mass of 115.2.[11]

Fluorescence Measurement. Fluorescence spectra of NPIA were measured using a model FP-750 spectrometer (JASCO, Tokyo, Japan) with excitation at 345 nm (slit width 5 nm).

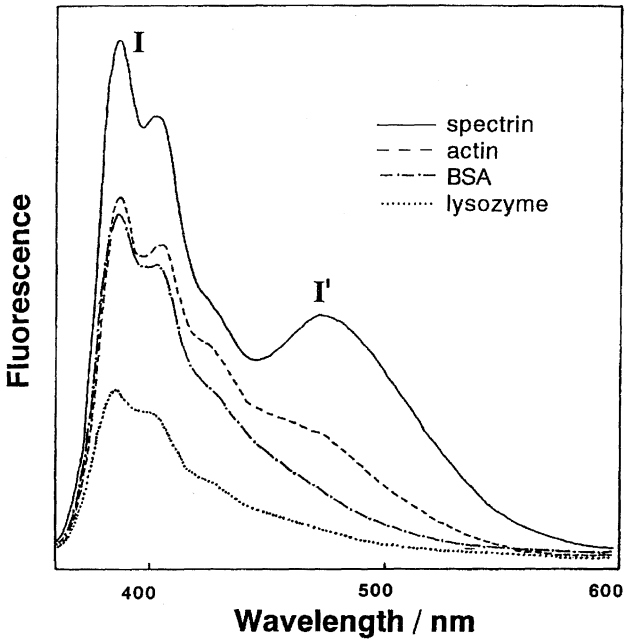

Fig. 1. Fluorescence spectra of NPIA probe molecules added into protein solutions. NPIA probe molecules (10 $\mu g\,ml^{-1}$) were added into each protein solution, and incubated for 30 min at 37 °C. Spectrin was purified from extracts obtained by incubating ghosts in extraction buffer for 20 h at 0 °C. *I* and *I'* indicate the fluorescence intensities at 387 and 465 nm, respectively.

Results

Fluorescence Spectra of NPIA Probe Molecules in Spectrin Solutions. When the NPIA probe molecules were added into a spectrin solution, one broad signal at around 465 nm was observed in addition to the two signals at 387 and 405 nm (Fig. 1). Such a broad signal is reported in a high concentration of pyrene dissolved in organic solvents and referred to as excimer fluorescence.[17] The excimer fluorescence was dependent on the NPIA concentration. Namely, the NPIA excimer to the monomer fluorescence intensity ratio,[18] I'/I, changed from 0.4 to 1.2, corresponding to probe concentrations from 1 to 100 $\mu g\,ml^{-1}$, respectively. Such excimer fluorescence disappeared upon addition of an anionic detergent, SDS, but reappeared upon conducting dialysis (data not shown). Further, NPIA excimer did not appear clearly in 10 mM NEM-pretreated spectrin (data not shown). To examine whether the excimer fluorescence appears in other proteins such as actin, BSA, and lysozyme, NPIA probe molecules were similarly added to each protein solution. However, no such excimer signal was clearly observed in these proteins (Fig. 1).

Properties of Excimer Fluorescence in Spectrin Extracts from NPIA-Labeled Ghosts. To examine whether the excimer fluorescence is due to NPIA molecules which are covalently bound with SH groups of spectrin, ghosts were treated with NPIA and washed with a buffer containing BSA. The cytoskeleton was extracted from NPIA-labeled ghosts. Such extracted solutions, which contain spectrin as a major component,[4] also showed excimer fluorescence (Fig. 2). The fluorescence intensity of this excimer decreased upon heating at 50 °C and the excimer signal disappeared upon addition of SDS, as mentioned above (data not shown). On the other hand, when the ghosts were double pretreated with diamide and then NEM before NPIA labeling, the fluorescence spectra of the extracted solution showed no excimer, irrespective of the same concentration of extracted spectrin. However, when such double pretreated ghosts were exposed to DTT to reduce the cross-linking of membrane proteins before NPIA labeling, the extracted solutions from such ghosts clearly showed the excimer (Fig. 2).

Temperature Effects on Appearance of Excimer Fluorescence in NPIA-Labeled Ghosts. The ghosts, which were prepared from red blood cells at 0—4 °C, were preincubated at various temperatures (0—50 °C) and labeled at 0 °C with NPIA. The excimer fluorescence appeared clearly in 50 °C-treated ghosts (Fig. 3, inset). The temperature dependence of I'/I in NPIA-labeled ghosts is shown in Fig. 3. The values of I'/I remained small up to 30 °C, but increased at higher temperatures, especially at 50 °C. To characterize such excimer fluorescence, the nondenaturing PAGE of spectrin was carried out (Fig. 4A). When the spectrin was prepared at low temperature (0—4 °C) from intact erythrocytes, spectrin tetramers were predominant. The spectrin tetramers were considerably stable below 30 °C, but shifted to dimer forms at higher temperatures. Upon heating at 50 °C of such a spectrin solution, the spectrin dimer and tetramer bands disappeared and diffused bands were observed. The CD spectra of spectrin solutions heated at 20, 30, 40, and 50

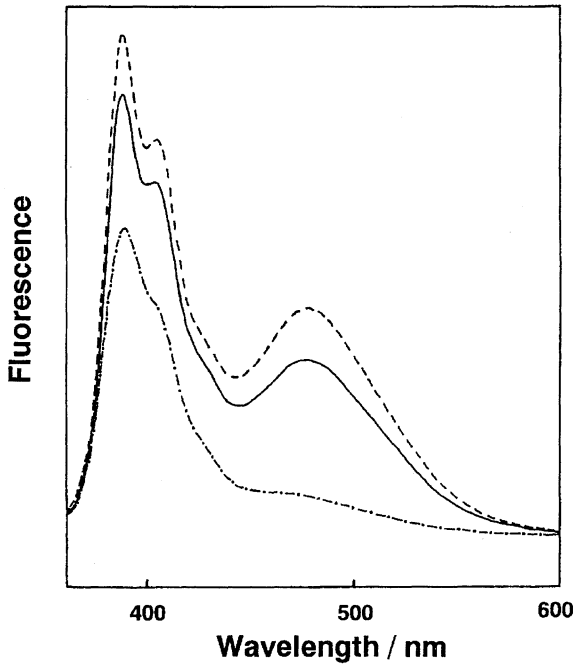

Fig. 2. Effects of SH-reactive agents on excimer fluorescence. Ghosts were treated with 0.5 mM diamide and then 10 mM NEM (–•–). Parts of these double treated ghosts were further exposed to 10 mM DTT (----). Thus, chemically modified and unmodified (—) ghosts were incubated with NPIA (10 μg ml^{-1}) for 30 min at 37 °C and washed with 5P8. NPIA-labeled spectrin was extracted at 37 °C for 30 min from these ghosts.

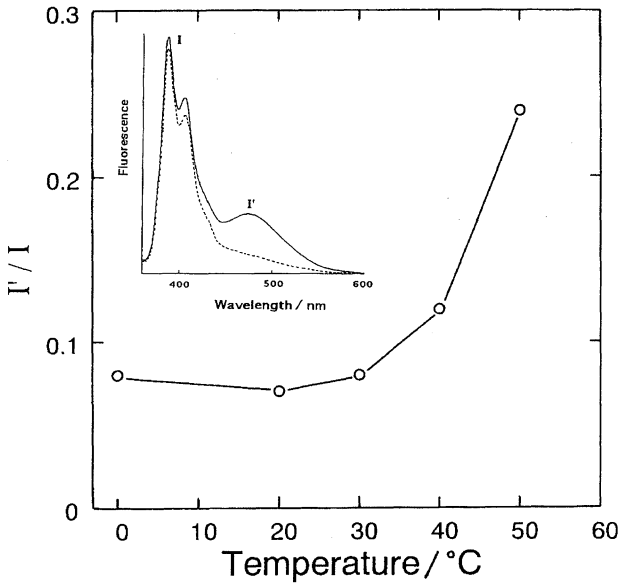

Fig. 3. Effects of preincubation temperature on excimer fluorescence (I'/I) in NPIA-labeled ghosts. Ghosts were preincubated for 30 min at 0, 20, 30, 40, and 50 °C, treated with NPIA (10 μg ml^{-1}) for 20 h at 0 °C, and washed with 5P8. (Inset) Fluorescence spectra of ghosts pretreated at 20 (···) and 50 °C (—) before labeling NPIA.

°C were also measured. The CD spectra of spectrin did not change up to 40 °C, but upon heating at 50 °C the *a*-helix content of spectrin was decreased (Fig. 4B). These results indicate that the denaturation of spectrin occurs at 50 °C.

Discussion

The excimer fluorescence appears when NPIA molecules are added into the spectrin solution. The fluorescence intensity of the excimer increases at higher concentrations of NPIA. Here, it is of importance to remember that a NPIA molecule must be in contact with other NPIA for excimer fluorescence. The NPIA excimer in spectrin indicates that NPIA bound with SH groups of this protein can interact with

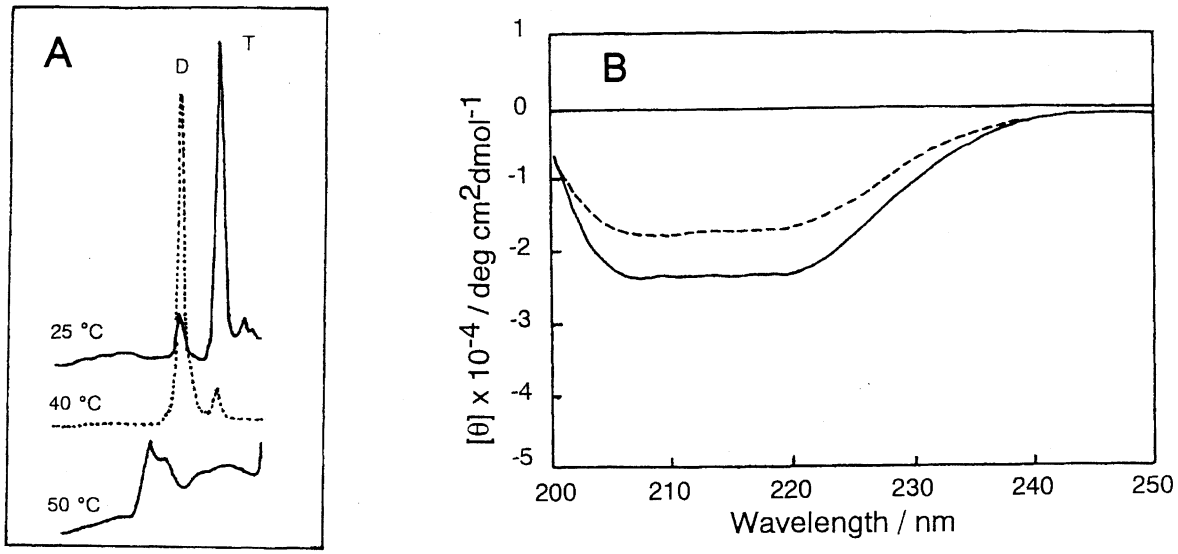

Fig. 4. Structural changes of spectrin by heating. A) Spectrin extracts were incubated for 30 min at 25, 40, and 50 °C. Oligomeric states of spectrin were analyzed by nondenaturing PAGE. T and D indicate spectrin tetramer and dimer, respectively. B) CD spectra of spectrin extracts exposed to 40 (—) and 50 °C (···). The CD spectra of spectrin extracts exposed to 20 and 30 °C were almost the same as that exposed to 40 °C.

other NPIA molecules within spectrin molecules. However, such interactions between NPIA molecules may be affected by the structural changes of spectrin. SDS is widely used to induce the structural changes of proteins. Thus, excimer fluorescence in spectrin seems to be affected by SDS. Indeed, such excimer fluorescence disappears upon addition of SDS into the spectrin solution. Here, it is useful to consider the structure of spectrin. Spectrin is a long, fibrous protein which consists of α, β-subunits.[1—3] Each subunit is characterized by a triple helical motiff of 106 amino acid repeated units.[19—21] Spectrin α, β-subunits comprise 19 and 13 cysteines, respectively.[22,23] The NPIA excimer fluorescence of spectrin diminishes upon pretreatment with NEM. The CD spectra show no change in the secondary structure of spectrin by NEM (T. Yamaguchi et al. unpublished data). Therefore, it seems unlikely that NPIA molecules are unable to access to NEM-treated spectrin. Thus, the role of these cysteins for the appearance of the NPIA excimer has been examined using another proteins. BSA and lysozyme are globular proteins and contain 35 and 8 cysteines, respectively. However, the number of free cysteins is one for BSA and zero for lysozyme. The structure of these proteins is stabilized by many disulfide bonds. No NPIA excimer appears in BSA and lysozyme, suggesting that NPIA probe molecules can not contact each other within these proteins. On the other hand, actin consists of 375 amino acid residues and contains five free cysteines. A globular monomer of actin is called G-actin, whereas a filamentous polymer called F-actin. The NPIA excimer fluorescence of G-actin is not so apparent as seen in spectrin. These results suggest that the binding of NPIA to free cysteines may be necessary for the appearance of NPIA excimer fluorescence.

Does the excimer fluorescence result from the NPIA molecules bound covalently to SH groups of spectrin? To test this possibility, spectrin is extracted from NPIA-labeled ghosts in which unbound NPIA molecules are removed by washing. Spectrin prepared thus shows the excimer fluorescence. However, no such excimer is observed in spectrin which is extracted from the ghosts treated with diamide and NEM before NPIA labeling. Spectrin α, β-subunits are readily cross-linked by oxidants, such as diamide.[24] Such cross-linking of spectrins is reduced by β-mercaptoethanol or DTT.[24] Indeed, when the ghosts treated double with diamide and NEM are exposed to DTT before NPIA labeling, the excimer fluorescence reappears. These data suggest that SH-groups within spectrin α-subunit are in contact with those within the spectrin β-subunits. Thus, the excimer in spectrin may be ascribed to NPIA molecules bound to such SH groups. On top of that, the SH groups which are accessible to each other within each subunit may also contribute the NPIA excimer. Interestingly, the excimer fluorescence is also observed in NPIA-labeled ghosts. In this case, is the excimer due to spectrin? The denaturation of spectrin occurs upon exposure to 49—52 °C of ghosts or intact cells.[25,26] Such heat-induced denaturations of spectrin are detected by CD spectra and nondenaturing PAGE, as demonstrated in this work. Particularly, it is of importance that the excimer fluorescence of NPIA appears more clearly in 50 °C-heated ghosts. Spectrin, which is extracted from NPIA-labeled ghosts, shows the excimer fluorescence. Upon treating the ghosts with *N*-ethylmaleimide spin labels, about 80% of the label is bound to the spectrin–actin complex.[27] In erythrocyte membranes, actin exists as an oligomer of about 13 monomers[28] and is associated with spectrin.[1,2] When NPIA-labeled monomer actins, where unbound NPIA is removed, polymerize to form F-actin filaments, the fluorescence intensity increases but no excimer appears.[4]

Other major proteins in erythrocyte ghosts may also be considered as a candidate for the excimer fluorescence. Band 3 as well as spectrin is a major protein in the erythrocyte membrane. The contents of spectrin and band 3 are about 30 and 24% by weight of the total membrane proteins, respectively.[29] Human band 3 is a 911 amino acid glycoprotein and a anion transporter.[30] This protein comprises four free cysteines because Cys-843 is palmitylated.[31] Only two cysteines (Cys-201 and Cys-317) in the cytoplasmic domain of band 3 are cross-linked with each other by oxidants.[32] Thus, the fluorescence intensity of the NPIA excimer seems to be weak in band 3. In fact, the NPIA excimer fluorescence of band 3 is not so apparent, as seen in spectrin (T. Yamaguchi et al. unpublished data). These data suggest that excimer fluorescence in NPIA-labeled ghosts may be largely ascribed to NPIA bound to spectrin. In intact erythrocytes, spectrin exists mainly as the tetramer. This association state of spectrin is stabilized by hemoglobin.[10] Therefore, spectrin tetramers are predominant components at physiological temperature in intact cells. On the other hand, when ghost membranes are incubated at 40 °C, spectrin tetramers shift to dimers. However, there have been few reports concerning the structural changes of spectrin induced by such transitions. In the present work, we have shown that there is no significant difference in the CD spectra for the spectrin tetramer-dimer transitions, as seen at 40 °C. When ghosts were labeled with NPIA at 0 °C, excimer appeared in membranes pretreated above 30 °C. In ghost membranes, spectrin tetramers shift to dimers above 30 °C.[4] The increment of I'/I above 30 °C suggests that the structural changes of spectrin occur during a transition from the spectrin tetramer to the dimer. Upon shift to spectrin dimers, NPIA molecules become more accessible to SH groups which are in contact with each other within and/or between spectrin subunits. Similar phenomena are observed in NEM-treated ghosts.[33] When the NEM-treated ghosts are incubated at 0 or 37 °C, and then labeled with *N*-ethylmaleimide spin label at 0 °C, the ESR signal appears in 37 °C-treated ghosts.[33] This indicates that the conformational changes of membrane proteins in NEM-treated ghosts occur at 37 °C. In other words, the SH groups buried within the membrane proteins seem to appear on their surface. From the ESR spectra, however, it is difficult to obtain information about the distance between SH-groups. Thus, we expect that NPIA excimer fluorescence will provide useful information to examine the role of spectrin in the membrane structure of erythrocytes.

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