Biochimica et Biophysica Acta 812 (1985) 786–792
Elsevier

BBA 72442

Interaction between the subunits of human erythrocyte spectrin using a fluorescence probe

Hidenori Yoshino [a,b] and Vincent T. Marchesi [b]

[a] *Department of Chemistry, Sapporo Medical College, S-1, W-17, Chuo-ku, Sapporo, 060 (Japan) and* [b] *Department of Pathology, Yale University School of Medicine, New Haven, CT 06510 (U.S.A.)*

(Received April 19th, 1984)
(Revised manuscript received October 25th, 1984)

Key words: Spectrin; Subunit interaction; Erythrocyte membrane; Cytoskeleton; Fluorescence probe

Fluorescence labeling of spectrin subunits was performed with *N*-(1-anilinonaphthyl-4)maleimide (ANM) to study the interaction between α and β subunits. The fluorescence anisotropy of both ANMα and ANMβ increased linearly with the addition of nonfluorescent β or α subunit, and saturated at a protein ratio about 1, indicating that 1 mol α subunit binds to 1 mol β subunit with high affinity in vitro. Furthermore, this binding seemed to be reversible, because the anisotropy value decreased when an excess of nonfluorescent α was added to the ANMα/β mixture. The anisotropy of ANMα attained a maximum level within 1 min after addition of the same quantity of nonfluorescent β at 12°C, and the anisotropy of this mixture decreased rapidly when an excess of nonfluorescent α was added. These findings suggested that both the binding process of β to ANMα and the dissociation step of ANMα from the ANMα-β complex were quite rapid. The results obtained here imply that dynamic interaction between α and β subunits of spectrin should be taken into account in understanding the role of the spectrin molecule in the cytoskeletal mesh.

Introduction

It has been well-established that spectrin is one of the major structural proteins in the cytoskeleton of the human red blood cell. The isolated spectrin molecule consists of two unique subunits referred to as the α ($M_r = 240\,000$) and β ($M_r = 220\,000$) subunit, and the basic functional unit of the spectrin molecule is an $\alpha\beta$ dimer [1,2]. In isotonic salt solution, however, spectrin forms a tetramer [3,4] or higher oligomers [5,6]. We recently described that each spectrin subunit could be isolated in high yield by ion-exchange column chromatography with 3 M urea [7,8]. Both α and β subunits were completely soluble in low- and high-salt solution, and spectrin-like high-molecular weight complexes could be reconstituted from isolated subunits [8].

Besides the human erythrocyte cell, spectrin-like proteins were isolated from various types of cell [9]. An important concept in those studies is that the subunit compositions of those spectrins were very similar to those of erythrocyte spectrins [10,11]. Consequently, it is rational to contemplate that the $\alpha\beta$-type hetero dimer should be the essential unit of various spectrin-like molecules.

Up until recently, however, there has been little information available about the mechanism of the interaction between α and β subunits. In this paper, we describe the stoichiometry and rate of binding of one subunit to the other in vitro using fluorescent-labeled subunits. ANM reacts selec-

Abbreviation: ANM, *N*-(1-anilinonaphthyl-4)maleimide.

tively with thiols to give addition products which are strongly fluorescent, though ANM in the free form is less fluorescent [12]. The cysteine content of spectrin is 9.7 residues per 100 kDa [13]. It seems reasonable to consider that α and β subunits have some cysteine residues to be able to react with ANM, although the content of cysteine residues in α and β subunits is still uncertain.

Materials and Methods

Protein preparation. Spectrin was prepared from fresh human blood by the methods previously described [8]. Spectrin subunits were isolated from purified spectrin fraction using DEAE-cellulose column chromatography with 3 M urea, as reported previously [8].

Fluorescence labeling. Isolated spectrin subunits were adjusted to 20 mM Tris-HCl (pH 8.0)/1 mM EDTA by dialyis. ANM dissolved in acetone (approx. 30 mM) was diluted with ice-cold absolute ethanol just before use (usually, 10 μl ANM stock solution was added to 90 μl absolute ethanol). The fluorescence-labeling reaction was started by addition of aliquots of the diluted ANM to the spectrin subunit solution on ice. The concentration of ANM in the reaction mixture was less than 15 μM, because the solubility of ANM in aqueous solution was about 15 μM, and the final concentration of ethanol was less than 1% (v/v). The concentration of the subunits was 0.5–0.8 mg/ml. Labeling reaction was terminated with the addition of 140 mM β-mercaptoethanol at final concentration 7 mM, and then the mixture was dialyzed against 20 mM Tris-HCl (pH 8.0)/1 mM EDTA/2 mM β-mercaptoethanol in a cold room for 24 h with several renewals of external buffer solution. The binding numbers of ANM to α and β subunits were monitored by absorbance at 350 nm, and increased almost linearly with increasing additions of ANM up to 15 μM, and 5 min was enough time for the reaction, even on ice for both subunits. When ANM was added in a 6-fold molar excess to the α or β subunit solution, the binding molar ratio of ANM to the subunits was about 3, in both subunits, which was estimated using 13 200 as the molar absorption coefficient of ANM at 350 nm [12]. These subunits, which had about 3 mol ANM/mol, were used as ANMα and ANMβ. The protein concentration was determined by the method of Lowry et al. [14]. Polyacrylamide gel electrophoresis of fluorescent-labeled subunits with sodium dodecyl sulfate (SDS) showed that only the α and β subunits were fluorescent and there were no other fluorescent bands under ultraviolet light.

Binding of the subunits to inside-out vesicles. The inside-out vesicles were prepared as follows. Washed erythrocyte ghosts were incubated in 10 vol. excess of 0.1 mM EDTA/20 μM phenylmethane-sulfonyl fluoride/125 μM diisopropylfluorophosphate/1 mM β-mercaptoethanol, titrated to pH 9.3 (normal spectrin extraction solution) at 37°C for 10 min. Vesicles were then pelleted by centrifugation. The pellets obtained were rewashed using the method described above to remove the spectrin completely. The resulting vesicles were suspended in the binding assay buffer of 20 mM Tris-HCl (pH 8.0)/ 1 mM $CaCl_2$/1mM $MgCl_2$/120 mM KCl/10 mM NaCl, and used as spectrin-depleted inside-out vesicles. In the binding assay, 0.1 ml of these vesicles (protein content was 0.8 mg) was mixed with 0.4 mg spectrin, 0.2 mg each ANMα or ANMβ, or 0.2 mg of both ANMα and ANMβ on ice under the assay buffer conditions described above. Exactly the same protein quantities were used for nonmodified subunits. 5 min after the mixing, the mixture was diluted with 5 ml of the same buffer and then pelleted by centrifugation at 4°C. The pellet was washed twice with 10 ml of the same buffer by centrifugation, and the resulting pellet was dissolved in 1% SDS solution for SDS-polyacrylamide gel electrophoresis analysis.

Gel electrophoresis. SDS-polyacrylamide gel electrophoresis was performed using 2-mm thick slab gels with 7% acrylamide, according to the procedure of Laemmli [15]. Nondenaturing polyacrylamide gel electrophoresis was also performed in slab gels, 3 mm thick, with an acrylamide gradient of 2–4% as reported previously [8].

Fluorescence measurements. Fluorescence spectra and anisotropies were obtained with a SLM 4800 spectrofluorimeter. In anisotropy measurements the exciting light was polarized and the fluorescence from samples was automatically analyzed through two photomultipliers for parallel and perpendicular emissions. Anisotropy, r, is de-

fined as:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities observed parallel and perpendicular, respectively. The exciting wavelength for ANM was 350 nm, and all fluorescence above 418 nm was collected through a cutoff filter (Scott KV 418).

ANM was purchased from Wako Chemicals (Japan) and was used without further purification.

Results

Fig. 1 shows the fluorescence spectra of ANMα, ANMβ, ANMα-β, ANMβ-α and ANM with β-mercaptoethanol, respectively. The maximum wavelengths of the fluorescence spectra were 441 nm for ANMα and ANMα-β, 431 for ANMβ and ANMβ-α, and 490 nm for ANM in 2.8 mM β-mercaptoethanol. The concentrations of ANM were about 0.6 μM for ANMα and ANMβ, and 40 μM for ANM in β-mercaptoethanol. In these measurements, all fluorescence spectra were obtained at the same fluorometer sensitivity. Fluorescence intensities of both ANMα and ANMβ decreased slightly with the addition of equal amounts of nonfluorescent β or α.

High-molecular weight complexes formed when ANMα or ANMβ was mixed with the α or β subunit, and even in the mixture of ANMα with ANMβ, as shown in Fig. 2. In this assay, ANM-modified subunits in 20 mM Tris-HCl (pH 8.0)/120 mM NaCl/1 mM EDTA/2 mM β-mercaptoethanol were mixed with their counterparts on ice, and 5 or 10 min later, the mixture was

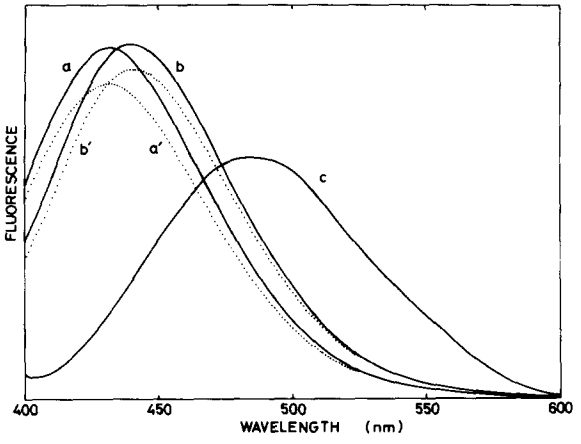

Fig. 1. Fluorescence spectra of ANM-modified spectrin subunits. (a) 0.049 mg/ml of ANMβ, (a') nonfluorescent α added to (a) as 0.05 mg/ml. (b) 0.054 mg/ml of ANMα (b') nonfluorescent β added to (b) as 0.056 mg/ml. (c) 40 μM of ANM with 2.8 mM β-mercaptoethanol. The binding molar ratio of ANM to the subunits was about 3. All spectra were measured in 20 mM Tris-HCl (pH 8), 1 mM EDTA, 120 mM NaCl and 2.8 mM β-mercaptoethanol at 25°C.

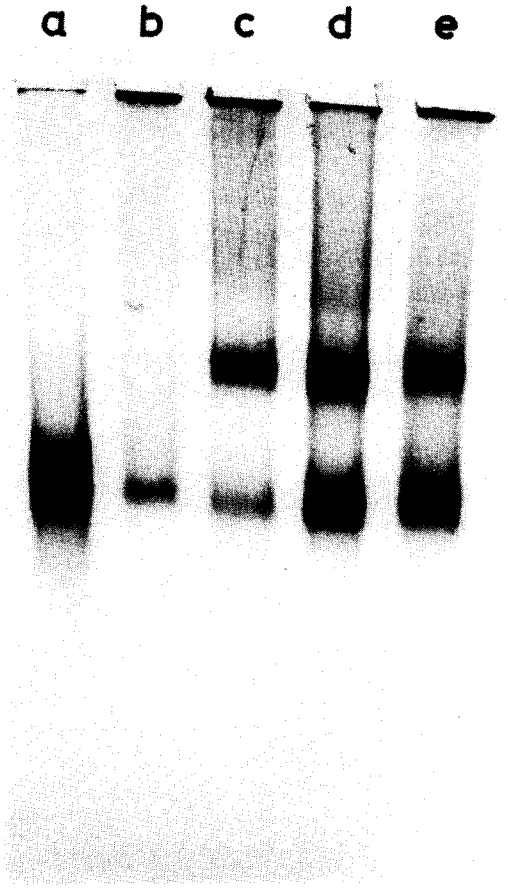

Fig. 2. Recombinations of ANM-modified spectrin subunits analyzed by nondenaturing polyacrylamide gel electrophoresis. In lanes a (ANMα) and b (ANMβ), 40 μg of the subunits were applied to 2–4% acrylamide gradient gel. In lanes c (ANMα + β), d (ANMβ + α) and e (ANMα + ANMβ), ANM-modified subunits were mixed with an equivalent amount of their counterparts in the presence of 20 mM Tris-HCl (pH 8.0)/120 mM NaCl/1 mM EDTA on ice, and 40 μg of the proteins were applied to the gel.

applied to nondenaturing polyacrylamide gel electrophoresis in a cold room. Both the dimer and tetramer bands at lanes c, d and e [5,8] were strongly fluorescent during electrophoresis. It is noted (Fig. 2) that the lower bands in c, d and e should be $\alpha\beta$-dimers as previously described, though their mobilities were not so different from these of α and β (a and b) [8]. These results indicated that spectrin-like molecules could be reconstituted from ANM-labeled subunits as well as from nonlabeled subunits. In lanes a and b, almost the same quantities of ANMα and ANMβ were applied to the gel, but ANMβ showed a weak band and many of the proteins remained on top of the gel. In comparison with the result of unlabeled β [8], ANM labeling might be the cause for aggregation of the β subunit.

Fig. 3 shows clearly that ANMβ bound to spectrin-depleted inside-out vesicles as well as β (compare lane 6 with 3) [16], and ANMα also bound to inside-out vesicles with ANMβ (lane 5). Panel B shows fluorescence bands of ANM-conjugated proteins illuminated with 302 nm light. The faint bands additional to the ANM may be due to intrinsic protein fluorescence. Lanes 4 and 7 in panel A and lane 3 in panel B show the direct binding of the α subunit to inside-out vesicles without added β subunits, though the binding affinity of α seems to be less than that of β. The effect of trace contamination which appeared at the β position (see lane 9) would be negligible, since the densities of protein bands in both lanes 4 and 7 are much higher than that of the contaminant band in lane 9. Furthermore, the anisotropy value of ANMα increased remarkably upon the addition of purified band 4.1, indicating the direct interaction between them (data not given).

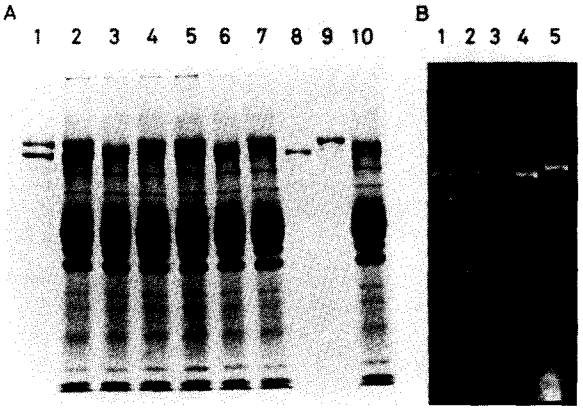

Fig. 3. Binding of ANM-subunits to spectrin-depleted inside-out vesicles analyzed by SDS-polyacrylamide gel electrophoresis. Panel A depicts the Coomassie blue-stained gels and panel B shows the fluorescence bands, from the ANM-subunits, which were obtained upon illumination with 302 nm light. In panel A, lanes 2–7 were inside-out vesicles with pectrin (2), β (3), α (4), ANMα + ANMβ (5), ANMβ (6) and ANMα (7), and lanes 1 and 8–10 were spectrin (1), ANMβ (8), ANMα (9) and inside-out vesicles (10), respectively. In panel B, lanes 1–3 were inside-out vesicles with ANMα + ANMβ (1), ANMβ (2) and ANMα (3), and lanes 4 and 5 were ANMβ (4) and ANMα (5), respectively. The details of the binding assay are described in Materials and Methods.

Fluorescence anisotropy values (r) of both ANMα and ANMβ increased remarkably with the addition of nonfluorescent α or β subunit, and in both cases, r values were saturated at the protein ratio of 1, as shown in Fig. 4. These changes in r were reversible, since r decreased significantly when excess of nonfluorescent α was added to the ANMα/β mixture, and increased again when nonfluorescent β was added to that mixture, as shown in Fig. 5. Consequently, the increase of anisotropy of ANMα or ANMβ induced by the addition of the β or α subunit should reflect the association step of the subunits, and also the decrease in r should reflect the dissociation step of ANMα from

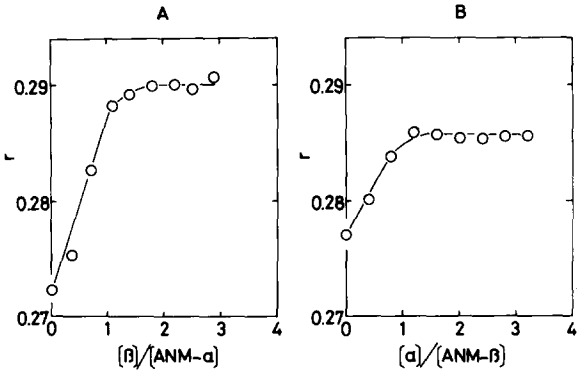

Fig. 4. Anisotropy changes of ANMα and ANMβ induced by β or α. (A) 0.13 mg/ml of ANMα was titrated by 4.25 mg/ml of β. (B) 0.11 mg/ml of ANMβ was titrated by 2.5 mg/ml of α. The conditions were 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 120 mM NaCl and 2 mM β-mercaptoethanol at 25°C. The ratios of the subunits were calculated on a weight-to-weight basis.

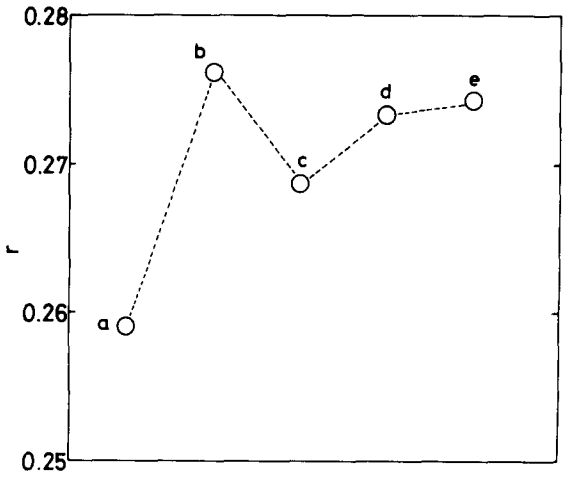

Fig. 5. A sequential change of anisotropy of ANMα induced by nonfluorescent subunits. (a) 0.022 mg/ml of ANMα. (b) β was added to (a) as 0.02 mg/ml. (c) α was added to (b) as 0.19 mg/ml. (d) β was added to (c) as 0.20 mg/ml. (e) more β was added to (d) as 0.29 mg/ml. The fluorescence anisotropy was measured in 20 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1 mM EDTA and 2.8 mM β-mercaptoethanol at 20°C.

the ANMα-β complex upon addition of nonfluorescent α. It was noted that the anisotropy values of ANMα depended on the protein concentration; for instance, above 0.02 mg/ml, r was constant and independent of the concentration, but at concentrations below 0.015 mg/ml, it decreased drastically.

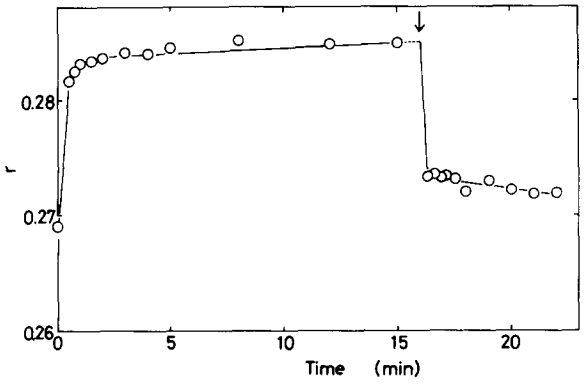

Fig. 6. Time-course of anisotropy changes of ANMα induced by β and α. The reaction was started by addition of nonfluorescent β (20 μl of 2.6 mg/ml β) to a final concentration of 0.029 mg/ml to ANMα (0.026 mg/ml), after 16 min (shown in the figure by the arrow) nonfluorescent α was added to the mixture to a final concentration of 0.33 mg/ml. The reaction was performed in 20 mM Tris-HCl (pH 8.0), 120 mM NaCl, 1 mM EDTA and 2 mM β-mercaptoethanol at 12°C.

The time-courses of association of ANMα to β and of dissociation of ANMα from the ANMα-β complex are shown in Fig. 6. The reaction rates of both steps seem to be quite high in our experimental conditions. Almost the same time-courses as in Fig. 6 were obtained in the presence of NaCl (0–120 mM), divalent metal ions as Mg^{2+} or Ca^{2+} (approx. 2 mM), ATP (0.5 mM), and Mg- or Ca-ATP (0.5 mM).

Discussion

In this paper, we present evidence for the binding stoichiometry and the binding rate of ANM-modified spectrin subunits in vitro. Both α and β subunits of spectrin still were able to form dimers and tetramers after chemical modification with ANM. In our previous studies, using fluorescence polarization and low-angle rotary shadowing and electron microscopy, it was clear that spectrin-like molecules could be reconstituted, and that there were not significant amounts of free subunits when nonmodified α and β subunits were mixed. The nondenaturing gel electrophoresis patterns of those mixtures were very similar to those of intact spectrin [8]. Since the electrophoretic patterns of c, d and e of Fig. 2 were almost the same as those of nonmodified subunits, it is reasonably to conclude that the faster-running bands should be the $\alpha\beta$ dimers, and slower ones should be the $\alpha_2\beta_2$ tetramers. It is noted that tetramers are always formed when α or ANMα is mixed with β or ANMβ, even on ice, because intact spectrin dimers were kinetically trapped and did not convert to tetramers at 0°C [17]. Therefore, $\alpha\beta$ dimers which were structurally different from intact spectrin dimers might be formed at first when the subunits were mixed on ice, then converted into tetramers or stable dimers, and/or $\alpha_2\beta_2$ tetramers might be formed directly from 2α and 2β. Furthermore, ANMβ could bind to spectrin-depleted inside-out vesicles; also ANMα bound to inside-out vesicles with ANMβ. These observations permitted fluorescence-labeled α and β to be used to study the interaction between these subunits. The nonspecific binding of spectrin to inside-out vesicles was estimated about 15% under the condition of

3-fold excess of spectrin to membrane protein on a weight-to-weight basis [18]. In our binding assay, on the other hand, such nonspecific binding was not a serious problem, because the ratios of spectrin or ANM subunits to membrane protein were less than one-half and the mixture of inside-out vesicles with the proteins was washed twice using a large excess of the buffer solution as described in Materials and Methods.

It is very interesting that the anisotropy of ANMα and ANMβ increased linearly with the addition of nonfluorescent β or α and saturated at a protein ratio about 1, indicating 1- to -1 mol binding with considerably high affinity. Refering to the observationos of electron micrographs in which the α subunit bound to the β subunit side-to-side [4], it seems reasonable that the anisotropies of ANMα or ANMβ increase when ANMα-β or ANMβ-α complexes are formed, because mobility of ANM on the subunits should be restricted directly by the side-to-side associaton. Formation of tetramers or oligomers from dimers, however, may not contribute to anisotropy changes, since these associations are of the head-to-head type [5]. In addition, account should be taken of local conformational change in the vicinity of ANM-binding sites on the subunits induced by binding of the counterpart. Fluorescence properties of ANM might be sensitive to its microenvironment [12]. For instance, both fluorescence intensities of ANMα and ANMβ dropped-off about 10% with the addition of nonfluorescent β or α without any changes of maximum wavelength of both spectra, as shown in Fig. 1. It was difficult to explain exactly the main reason for these phenomena. But it was emphasized that those fluorescence anisotropy and intensity changes saturated at the protein ratio of 1, indicating that they were induced by binding of ANM-subunits to their counterparts.

It is clearly shown that both the binding rate of β to ANMα and the exchange rate between ANMα and nonfluorescent α on ANMα-β complex were considerably rapid (Fig. 6). Initial phase of the time-course (within 1 min) conformed to simple second-order reaction kinetics, and rough estimation of the rate constant obtained from the slope was about $1 \cdot 10^6/M^{-1} \cdot s^{-1}$. But even at 2°C, the increase in anisotropy was too fast to follow exactly by our method. The anisotropy value of ANMα, on the other hand, hardly increased when the dimer or tetramer fraction of intact spectrin was added up to a 10-fold mol excess (data not given). Presumably, the spectrin dimer or tetramer was more stable than the ANMα-β complex in equilibrium state, so free ANMα was dominant in an equimolar mixture of ANMα and spectrin. And also in the mixture of a 10-fold molar excess of spectrin, almost all ANMα was in the free form, since the ratio of ANMα to α was one-tenth.

Taken together, these results seem to imply that side-to-side interaction between α and β subunits should be important for understanding the molecular role of the spectrin mesh on the red cell membrane. Of course, this idea should provide a clue to substantiate the mechanism, because some problems still remain, as will be described below.

The anisotropy value of ANM in the system of ANMβ with spectrin-depleted inside-out vesicles was lower than that of ANMβ in solution, and the anisotorpy of ANMβ bound to the vesicle showed a nonsaturable and small increase upon the addition of nonfluorescent α (data not given), though even ANMα could bind to ANMβ with inside-out vesicles (Fig. 3). These findings suggested that the interaction mode of α with β subunit on the membrane might be something different from that in solution. Presumably, the interaction between spectrin subunits on the membrane is more complicated, since not only the direct interaction between them but also the interactions with some spectrin-binding proteins such as ankyrin [19] and band 4.1 protein [20] on the red cell membrane has to be taken into consideration. Further experiments, including those on spectrin-binding proteins, need to be done to understand the interaction between subunits on the erythrocyte membrane.

The binding of ANM to α and β subunits was not so specific under our coupling conditions, since more than five strong fluorescent bands could be seen on the SDS-polyacrylamide gels after mild trypsin digestion of ANMα and ANMβ, which had about 3 mol ANM/mol subunit. The similar heterogeneous binding of ANM described above was observed under different coupling conditions such as pH 6–8 or in phosphate buffer at pH 6–8. These observations indicated that both α and β subunits had several thiols which were in very

similar chemical circumstances. These results would be quite natural, because spectrin subunits are long, mammoth-sized proteins; furthermore, each subunit had some structurally similar domains [21]. This heterogeneous binding of ANM to the subunits, however, did not cause many difficulties for the experiments on the interaction between spectrin subunits described here. The anisotropy values of ANM subunits were somewhat different from preparation to preparation. This difference might be caused by heterogeneous binding of ANM described above. But it was noted that the degree of anisotropy changes of the ANM subunit induced by binding of the subunit counterpart was almost constant and independent of initial anisotropy values.

References

1 Marchesi, V.T. (1979) J. Membrane Biol. 51, 101–131
2 Speicher, D.W., Morrow, J.S., Knowles, W.J. and Marchesi, V.T. (1980) Proc. Natl. Acad. Sci. USA 77, 5673–5677
3 Kam, Z., Josephs, R., Eisenberg, H. and Gratzer, W.B. (1977) Biochemistry 16, 5568–5572
4 Shotton, D., Bark, B. and Branton, D. (1979) J. Mol. Biol. 131, 303–329
5 Morrow, J.S. and Marchesi, V.T. (1981) J. Cell Biol. 88, 463–468
6 Morrow, J.S., Haigh, W.B. and Marchesi, V.T. (1981) J. Supramol. Struct. Cell. Biochem. 17, 275–287
7 Yoshino, H. and Marchesi, V.T. (1982) Fed. Proc. 41, 512 (Abstr.)
8 Yoshino, H. and Marchesi, V.T. (1984) J. Biol. Chem. 259, 4496–4500
9 Lazarides, E. and Nelson, W.J. (1982) Cell 31, 505–508
10 Davis, J. and Bennett, V. (1983) J. Biol. Chem. 258, 7757–7766
11 Nelson, W.J., Granger, B.L. and Lazarides, E.J. (1983) J. Cell Biol. 97, 1271–1276
12 Kanaoka, Y., Machida, M., Machida, M. and Sekine, T. (1973) Biochim. Biophys. Acta 317, 563–568
13 Marchesi, S.L., Steers, E., Marchesi, V.T. and Tillack, T.W. (1970) Biochemistry 9, 50–56
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 256–275
15 Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685
16 Morrow, J.S., Speicher, D.W., Knowles, W.J., Hsu, C.J. and Marchesi, V.T. (1980) Proc. Natl. Acad. Sci. USA 77, 6592–6596
17 Ungewickell, E. and Gratzer, W. (1978) Eur. J. Biochem. 88, 379–385
18 Bennett, V. and Branton, D. (1977) J. Biol. Chem. 252, 2753–2763
19 Bennett, V. and Stenbuck, P.J. (1979) J. Biol. Chem. 254, 2533–2541
20 Tyler, J.M., Hargreaves, W.R. and Branton, D. (1979) Proc. Natl. Acad. Sci. USA 76, 5192–5196
21 Speicher, D.W., Morrow, J.S., Knowles, W.J. and Marchesi, V.T. (1982) J. Biol. Chem. 257, 9093–9101