Restriction of the Lateral Motion of Band 3 in the Erythrocyte Membrane by the Cytoskeletal Network: Dependence on Spectrin Association State†

Akihiko Tsuji and Shun-ichi Ohnishi*

Department of Biophysics, Faculty of Science, Kyoto University, Sakyo-ku, Kyoto 606, Japan

Received December 19, 1985; Revised Manuscript Received June 13, 1986

ABSTRACT: The effects of incubation of erythrocyte ghosts under various conditions (ionic strength or addition of ankyrin, diamines, or ATP) on the lateral motion of band 3 in the membranes were studied by using the fluorescence photobleaching recovery technique. Incubation of ghosts with exogenous ankyrin increased the immobile fraction of band 3, from 0.6 in intact ghosts to 0.8–0.9 when an average of 0.2 mol of extra ankyrin was bound per mole of band 3. Ankyrin-free band 3 proteins were mobile, but their mobility was governed by the spectrin association state in the cytoskeletal network. The diffusion constant was 5.3×10^{-11} cm^2 s^{-1} at a spectrin tetramer mole fraction of 0.3–0.4 in 10 mM NaCl/5 mM sodium phosphate, pH 7.8, and decreased 1 order of magnitude when the tetramer fraction increased to 0.5 in higher NaCl concentration (150 mM NaCl). A similar decrease was observed when the spectrin tetramer fraction was increased by 0.2 mM spermine in 10 mM NaCl/10 mM tris(hydroxymethyl)aminomethane hydrochloride, pH 7.6. On the other hand, the rotational motion of band 3 in the membranes was not affected by the spectrin association state. Trypsin treatment of ghosts cleaved off the cytoplasmic domain of band 3 and caused a marked (8-fold) increase in the lateral mobility, $D = 4.0 \times 10^{-10}$ cm^2 s^{-1}. These results indicate that the lateral mobility of ankyrin-free band 3 protein is restricted by interactions of their cytoplasmic domain with the cytoskeletal network. A model is presented that band 3 can pass the network when spectrins are in dissociated dimers and cannot pass when they are tetramers. The lateral diffusion constant is thus determined by the spectrin dimer population in the network.

Proteins can diffuse rapidly in lipid bilayer membranes in the liquid-crystalline phase with the lateral diffusion constant in a range of $(1–3) \times 10^{-8}$ cm^2 s^{-1}. The lateral diffusion can be well described as the motion of a cylinder in a two-dimensional matrix with a viscosity of a few poise (Saffman & Delbruck, 1975). However, the protein mobility in cell plasma membranes is largely restricted. For example, while the diffusion constant of band 3 in dimyristoylphosphatidylcholine-reconstituted membranes at a lipid/protein ratio of 250 was 1.6×10^{-8} cm^2 s^{-1} at 30 °C (Chan et al., 1981), that of band 3 in erythrocyte membranes was a few orders of magnitude smaller, $(4–6) \times 10^{-11}$ cm^2 s^{-1}. This large restriction has been ascribed to interactions of the integral protein with cytoskeletal proteins. Sheetz et al. (1980) showed that the diffusion constant of band 3 in a spectrin-deficient mutant of mouse erythrocytes was 2.5×10^{-9} cm^2 s^{-1} (24 °C), in contrast to 4.5×10^{-11} cm^2 s^{-1} for normal mouse erythrocytes. Golan and Veatch (1980) observed a large increase in the diffusion constant of band 3 in human erythrocyte membranes on decreasing ionic strength, $D = 2 \times 10^{-9}$ cm^2 s^{-1} in 13 mM sodium phosphate (NaP_i).[1] A diffusion constant value of the order of 10^{-9} cm^2 s^{-1} can be explained by the simple two-dimensional diffusion model, taking into account the large concentration of proteins in cell membranes (Peters & Cherry, 1982).

In order to obtain more insight into the mechanism of restriction, we studied the lateral motion of band 3 in erythrocyte membranes with a modified cytoskeletal network or with modified band 3 by FPR measurements. The peripheral proteins spectrin, actin, and band 4.1 form a flexible two-dimensional network underneath the erythrocyte membrane, and another peripheral protein, ankyrin, anchors band 3 to the network by binding to band 3 on one hand and to spectrin on the other hand (Lux, 1979). However, the amount of ankyrin in erythrocytes is much smaller as compared with that of band 3, the molar ratio of ankyrin to band 3 being 0.10–0.15 (Bennett & Stenbuck, 1979). This indicates that only a fraction of band 3 can be linked to ankyrin, 20–30% if band 3 existed as a dimer or 40–60% if it existed as a tetramer. We therefore added extra ankyrin to a ghost suspension to investigate the effect of ankyrin binding on the lateral motion of band 3. Spectrin exists predominantly as a tetramer in the erythrocyte membrane under physiological conditions but can be dissociated into dimers on decreasing ionic strength (Ralston et al., 1977; Ungewickell & Gratzer, 1978; Lie & Palek, 1980). We then investigated the effect of this transformation on the lateral mobility of band 3 by changing the ionic strength and also by adding some polyamines and polyphosphates. Finally, we treated ghosts with trypsin to cleave off the cytoplasmic domain of band 3 and investigated its effect on the lateral motion of band 3. The results showed that band 3 proteins anchored to the cytoskeletal network via ankyrin were immobile in the time scale of the recovery measurement. Ankyrin-free band 3 proteins were mobile, but the motion was restricted by the cytoskeletal network and was dependent on the spectrin association state. Band 3 proteins lacking the

† This work was supported in part by Grant-in-Aid for Special Project Research on Molecular Mechanism of Bioelectrical Response 60123002, Ministry of Education, Japanese Government.

[1] Abbreviations: FPR, fluorescence photobleaching recovery; FITC, fluorescein isothiocyanate; PMSF, phenylmethanesulfonyl fluoride; NBD-PE, *N*-(4-nitro-2,1,3-benzoxadiazolyl)phosphatidylethanolamine; NaP_i, sodium phosphate; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hyrochloride; EDTA, ethylenediaminetetraacetic acid; kDa, kilodalton(s).

0006-2960/86/0425-6133$01.50/0

cytoplasmic domain received much smaller resistance.

Materials and Methods

Erythrocytes. Human red blood cells were obtained from Kyoto Red Cross Blood Center by courtesy of Dr. H. Saji. Labeling of erythrocytes with FITC was done according to Fowler and Branton (1977). Ghosts were prepared by lysis of FITC-labeled erythrocytes in an ice-cold 5 mM NaP_i buffer at pH 8.0 containing 50 μg/mL PMSF, followed by centrifugation in a hypotonic phosphate buffer (10 mM NaCl/5 mM NaP_i at pH 7.8) or a hypotonic Tris buffer (10 mM NaCl/10 mM Tris-HCl at pH 7.6). A densitometric analysis of an SDS–polyacrylamide gel electrophoretogram of labeled ghosts showed a strong fluorescence at the position of band 3 but no detectable fluorescence at the position of glycophorin. Trypsin treatment of ghosts was carried out by incubating FITC-labeled ghosts (2 mg of protein/mL) with various concentrations of trypsin (bovine, Seravac) for 20 min at 0 °C in hypotonic phosphate buffer. The reaction was stopped by adding PMSF and ethanol at final concentrations of 0.7 mM and 0.5%, respectively, and centrifugation. Ghosts were washed once by centrifugation in hypotonic phosphate buffer containing PMSF and then twice with the same buffer without PMSF.

A fluorescent lipid probe, NBD-PE, prepared from egg yolk PE, was incorporated into erythrocyte membranes by incubation of ghosts (2 mg of protein/mL) with sonicated vesicles of NBD-PE (0.4 mM) in isotonic phosphate buffer (150 mM NaCl/5 mM NaP_i at pH 8.0) for 20 min at room temperature, followed by two washings with hypotonic phosphate buffer. Protein was determined according to Lowry et al. (1951) or by using fluorescamine (Bohlen et al., 1973). SDS–polyacrylamide gel electrophoresis was performed according to Laemmli (1970).

Isolation of Ankyrin and Its Binding to Ghosts. Ankyrin was extracted from ghosts and purified by DEAE-cellulose column chromatography (Whatman DE 32) as described by Tyler et al. (1979). Purified ankyrin was stored at 4 °C in hypotonic phosphate buffer containing 1 mM EDTA, 0.5 mM dithiothreitol, and 0.004% diisopropyl fluorophosphate.

Isolated ankyrin at various concentrations was added to the ghost suspension (120 μg of protein/mL) and incubated for 2 h at room temperature or at 0 °C in hypotonic phosphate buffer. Ankyrin binding was assayed according to Bennett and Stenbuck (1980). ^{125}I-Ankyrin was prepared by using Bolton–Hunter reagent (New England Nuclear). The specific activity of the product was 43 000–96 000 cpm/μg. ^{125}I-Labeled ankyrin was added to FITC-labeled ghosts in hypotonic phosphate buffer and incubated. The sample (0.25 mL) was then layered on 0.2 mL of 20% (w/w) sucrose in hypotonic phosphate buffer and centrifuged for 25 min at 15000g. The bottom fraction (ca. 0.1 mL) containing membranes was frozen, and the unfrozen top fraction (0.35 mL) was removed. The radioactivity of both fractions was counted. As controls, binding of heat-treated ankyrin (60 °C for 10 min) to intact ghosts and also binding of intact ankyrin to resealed ghosts prepared by incubation in 130 mM KCl/10 mM NaCl/5 mM NaP_i, pH 7.8, were assayed.

Analysis of the Dimer and Tetramer of Spectrin. Spectrin tetramer and dimer were assayed after separation by a sucrose density gradient centrifugation at low temperature. Sample (0.3 mL) was layered over 4 mL of a 5–30% (w/w) sucrose density gradient on 0.5 mL of a 40% (w/w) sucrose cushion and centrifuged at 150000g for 15 h at 4 °C. Fractions (0.2 mL each) were collected, and tetramer and dimer were quantitated by using fluorescamine or the OD at 280 nm. The spectrin association state in the erythrocyte membrane was estimated indirectly after extraction from the ghost at low temperature (4 °C) with a very low ionic strength medium (0.3 mM NaP_i/0.2 mM EDTA, pH 7.6) for 20 h as done by Liu and Palek (1980). It was shown that the dimer–tetramer intercoversion was extremely slowed down at low temperature (Ungewickell & Gratzer, 1978).

The spectrin association state in erythrocyte membranes was modified by incubating ghosts for 20 min at 37 °C in various concentrations of NaCl buffered with 5 mM NaP_i at pH 7.8 or in hypotonic Tris buffer containing various concentrations of diamines, spermine, spermidine, or putrescine (Nakarai Chemicals). For comparison, ghosts were incubated in isotonic phosphate buffer containing ATP (Sigma). Polyphosphates such as ATP were reported to disrupt the Triton-insoluble erythrocyte cytoskeleton (Sheetz & Casaly, 1980). The effects of these reagents or of lowering the ionic strength on isolated spectrin in solution were also examined. Spectrin tetramer was collected by elution of the very low ionic strength extract through a Sepharose CL-4B column. After dialysis against hypotonic Tris buffer at low temperature, spectrin was concentrated by using ultrafiltration membrane cones CF-25 (Amicon) to give an OD value of 0.55 at 280 nm. The final sample contained 74 mol % of tetramer.

FPR Measurements. FPR measurements were made on ghosts at 26 °C by using a 100× objective lens as described previously (Chan et al., 1981). The bleaching beam radius was ca. 0.8 μm. The sample was deoxygenated by nitrogen gas bubbling for 1 h at room temperature and placed on a slide glass under a nitrogen gas stream to avoid possible cross-linking of proteins during photobleaching (Lepock et al., 1978; Sheetz & Koppel, 1979). Most recovery curves were satisfactorily analyzed on the basis of diffusion of a single mobile component and immobile component (Axelrod et al., 1976). When there were two mobile components, the recovery curve was analyzed by

$$F(t)/F(-) = x_1\sum_n^\infty[(-K)^n/n!][1 + n(1 + 2t/\tau_1)]^{-1} + x_2\sum_n^\infty[(-K)^n/n!][1 + n(1 + 2t/\tau_2)]^{-1} + (1 - x_1 - x_2)K^{-1}(1 - e^{-K})$$

where $F(t)$ and $F(-)$ are the fluorescence intensity at time t after bleaching and before bleaching, respectively, x_1 and x_2 are the fraction of the two mobile components, τ_1 and τ_2 are the characteristic time of diffusion for the two components, and K is a constant determined by the extent of bleaching. The least-squares fitting was made by changing x_1 and x_2 as adjustable parameters and using fixed values for τ_1 and τ_2.

Results

Effect of Ankyrin Binding on Lateral Motion of Band 3. FPR curves for band 3 in erythrocyte membranes can be analyzed on the basis of a single mobile component with a lateral diffusion constant of $(5.3 \pm 1.37) \times 10^{-11}$ cm^2 s^{-1} in a hypotonic medium (10 mM NaCl/5 mM NaP_i, pH 7.8). The mobile fraction was 0.40 ± 0.069. The immobile fraction (0.60 ± 0.069) diffused much more slowly, $D < 10^{-13}$ cm^2 s^{-1}. The diffusion constant of the lipid probe NBD-PE in ghost membranes was obtained as $(2.5 \pm 0.34) \times 10^{-9}$ cm^2 s^{-1} with the mobile fraction of 0.79 ± 0.068.

In native erythrocytes, only a fraction of band 3 is linked to ankyrin because of shortage in the latter. We therefore added isolated ankyrin to the ghost suspension followed by incubation. FPR curves of these ghosts (Figure 1A) can be

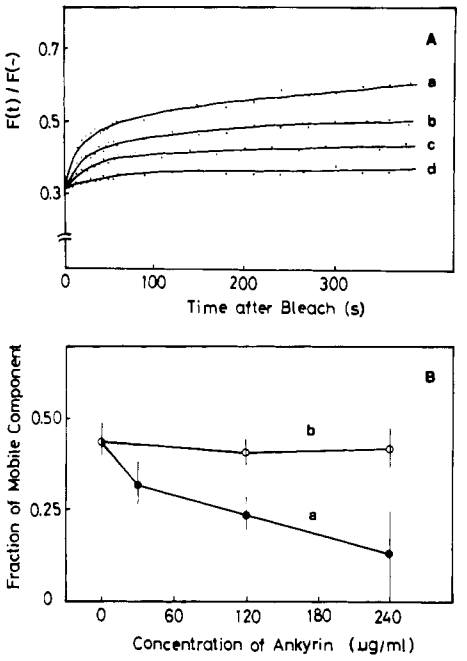

FIGURE 1: Effect of ankyrin binding on the lateral motion of band 3 in erythrocyte membranes. (A) Typical FPR curves for FITC-labeled ghosts incubated in the absence (a) or the presence of various concentrations of ankyrin [30 (b), 120 (c), and 240 μg/mL (d)] in hypotonic phosphate buffer for 2 h at room temperature. The molar ratio of exogenous ankyrin to band 3 in ghosts in the incubation mixture was 0 (a), 0.5 (b), 2.0 (c), and 4.0 (d). (B) Fraction of the mobile component ($D = 5.3 \times 10^{-11}$ cm^2 s^{-1}) against the concentration of ankyrin used for incubation with ghosts: (a) intact and (b) heat treated.

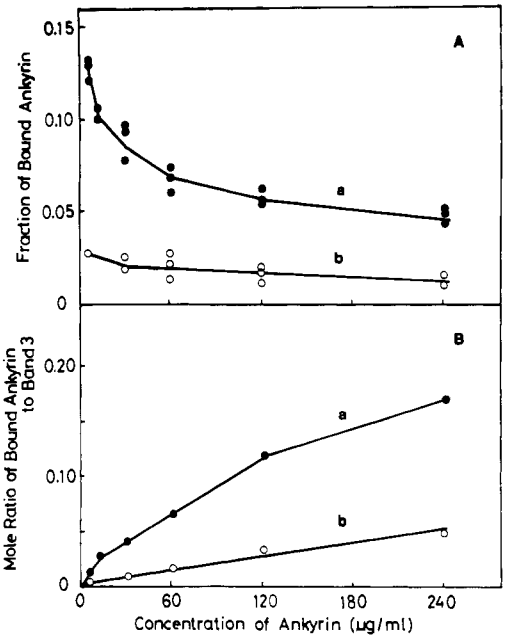

FIGURE 2: Assay of bound exogenous ankyrin to ghost membranes. (A) Fraction of bound ankyrin of total added ankyrin as a function of the concentration of exogenously added ankyrin used for the incubation with ghosts. (B) Mole ratio of bound exogenous ankyrin to band 3 in ghosts calculated by using the data in (A): (a) intact and (b) heat-treated (10 min at 60 °C) ankyrin. ^{125}I-Labeled ankyrin at the indicated concentration was added to FITC-labeled ghosts (120 μg of protein/mL) in hypotonic phosphate buffer and incubated for 2 h at room temperature. Bound ankyrin and free ankyrin were separated by centrifugation on 20% sucrose, and the radioactivity was counted.

analyzed also by a single mobile component. The analysis showed that the incubation with exogenous ankyrin caused a decrease in the mobile fraction of band 3 without affecting the diffusion constant (Figure 1B). The decrease was larger with the increase in the amount of ankyrin added to ghosts. On the other hand, heat-treated ankyrin did not affect the mobile fraction as well as the diffusion constant (Figure 1B).

The amount of exogenous ankyrin bound to ghosts was assayed. It increased from 0 to 0.18 mol/mol of band 3 on incubation of ghosts with increasing concentrations of exogenous ankyrin from 0 to 240 μg/mL (Figure 2). The total amount of bound ankyrin, intact and exogenous, can therefore be made as large as ca. 0.3 mol/mol of band 3 by incubation with exogenous ankyrin at a concentration of 240 μg/mL. The mobile fraction of band 3 in such ghosts decreased to a very small value (0.1–0.2). Binding of heat-treated ankyrin to ghosts was slight (Figure 2).

These results suggest that the mobile band 3 proteins are those free from ankyrin binding and the immobile ones are those linked to ankyrin, which in turn connects to the cytoskeletal network. About 80–90% of band 3 was immobile when 1 mol of band 3 was bound by an average of 0.3 mol of ankyrin.

Effect of Conversion of Spectrin into Tetramer on Band 3 Mobility. In the above experiments, pretreatment of ghosts and FPR measurements were made in a low ionic strength medium (10 mM NaCl/5 mM NaP_i, pH 7.8). Since increasing ionic strength favors tetrameric spectrin, pretreatment and FPR measurements were made in higher ionic strength media to see the effect of the spectrin conversion on the lateral mobility. FPR curves for ghosts under such conditions can also be analyzed by a single mobile component. The analysis showed a decrease in the diffusion constant for the mobile component without affecting its fraction on increasing the ionic

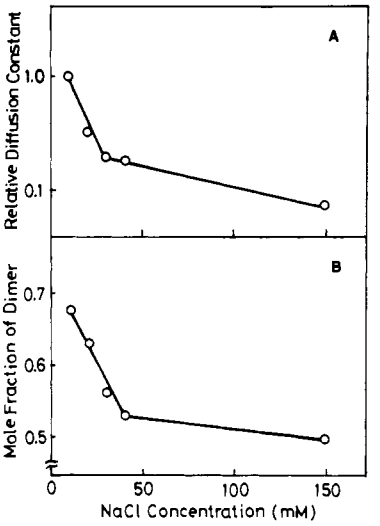

FIGURE 3: Effect of ionic strength on the lateral motion of band 3 in erythrocyte membranes. FITC-labeled ghosts were incubated in various concentrations of NaCl in 5 mM NaP_i at pH 7.8 for 20 min at 37 °C, and FPR curves of these ghosts were measured at 26 °C in the presence of the incubation medium. (A) Diffusion constant relative to that in hypotonic phosphate buffer (10 mM NaCl/5 mM NaP_i) as a function of the concentration of NaCl. (B) Mole fraction of spectrin dimer, f_D = [dimer]/([dimer] + [tetramer]). Spectrin was extracted from ghosts at 4 °C for 20 h and assayed for tetramer and dimer after sucrose density gradient centrifugation.

strength. The diffusion constant became 1 order of magnitude smaller on increasing the NaCl concentration from 10 to 150 mM (Figure 3A).

The association state of spectrin in ghosts was estimated after its extraction from ghosts at low temperature. The tetramer mole fraction (f_T) was obtained as 0.3–0.4 in 10 mM NaCl/5 mM NaP_i, pH 7.8, but sharply increased with the

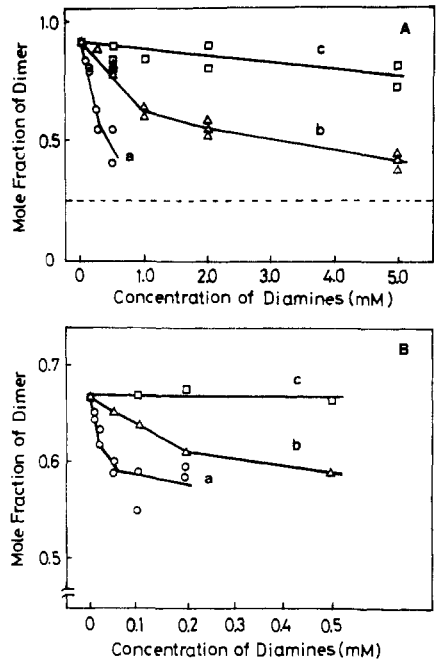

FIGURE 4: Effect of diamines on spectrin association in solution (A) and in ghosts (B). Isolated spectrin tetramer fraction (f_T = 0.74) (A) or erythrocyte ghosts (B) were incubated for 20 min at 37 °C in hypotonic Tris buffer containing the indicated concentrations of spermine (a), spermidine (b), or putrescine (c). Spectrin dimer and tetramer were assayed after sucrose density gradient centrifugation. The association state in ghosts was estimated after extraction from ghosts at 4 °C for 20 h. The dashed straight line in (A) shows the level without incubation.

increase in NaCl concentration: 0.46 in 40 mM NaCl and 0.50 in 150 mM NaCl (Figure 3B). The diffusion constant of the mobile component was about 5×10^{-12} cm^2 s^{-1} when ca. 50 mol % of spectrin was in tetrameric form.

Some diamines affected spectrin association in favor of tetramer. When spermine was added to isolated spectrin tetramer (f_T = 0.74) and incubated in hypotonic Tris buffer, it inhibited dissociation into dimer (Figure 4A, curve a). f_T remained at a high value of 0.60 in the presence of 0.5 mM spermine but decreased to 0.07 in its absence. Spermidine with a shorter inter-amino group spacing had a similar effect but required a higher concentration to give the same strength of effect (Figure 4A, curve b). Putrescine with the shortest inter-amino group spacing of diamines tested gave only a slight effect (Figure 4A, curve c). Spermine and spermidine affected the spectrin association state in ghosts in a qualitatively similar manner (Figure 4B). The f_T value was 0.42 in ghosts treated with 0.2 mM spermine in hypotonic Tris buffer, while it was 0.33 in its absence.

Spermine greatly affected band 3 lateral mobility (Figure 5a). It caused a large decrease in the diffusion constant of the mobile component, about 1 order of magnitude in its presence at 0.2 mM. The fraction of that component was not modified, however. Spermidine had a similar effect but required a higher concentration to give the same strength of effect (Figure 5b). Putrescine showed almost no effect on the mobility (Figure 5c).

The effects of ATP on the spectrin association state and band 3 mobility were studied for comparison. This negatively charged compound affected spectrin in favor of dimer, in contrast to the positively charge diamines. The dimer mole fraction (f_D) in ghosts incubated in the presence or absence of 12.5 mM ATP in isotonic phosphate buffer was 0.58 and 0.46, respectively. ATP caused an increase in band 3 mobility. The diffusion constant was 1.1×10^{-11} cm^2 s^{-1} in the presence

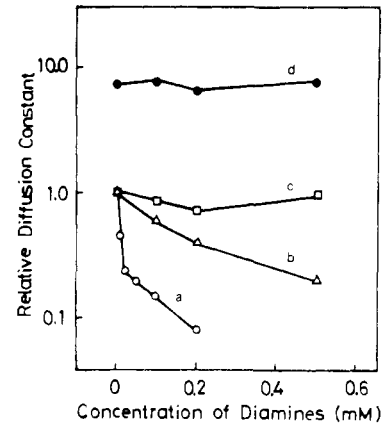

FIGURE 5: Effect of diamines on the lateral motion of band 3 in erythrocyte membranes. FITC-labeled ghost (230 μg/mL) was incubated in hypotonic Tris buffer containing the indicated concentrations of spermine (a), spermidine (b), or putrescine (c) for 20 min at 37 °C, and the FPR curve of these ghosts was measured in the presence of the incubation medium. The lateral diffusion constant of the mobile component relative to that for ghosts incubated without diamines is given in the ordinate. (d) Data for ghosts treated with 0.5 μg/mL trypsin for 20 min at 0 °C and then with various concentrations of spermine.

of 12.5 mM ATP and 5×10^{-12} cm^2 s^{-1} in its absence, respectively. These results are in qualitative agreement with Schindler et al. (1980), who observed immobilization in 0.6 mM spermine and a 2–4-fold increase in the diffusion constant in 12.5 mM ATP or 2,3-diphosphoglycerate.

As a complementary study, the rotational motion of band 3 in erythrocyte membranes was measured by using the phosphorescence depolarization technique (Cherry & Godfrey, 1981). The anisotropy decay curves for eosin-labeled band 3 in ghosts in 10 mM NaCl/5 mM NaP_i, pH 7.8, were satisfactorily analyzed by an equation of two exponentials with two rotational relaxation times, 127 and 1836 μs, with the former fraction of 0.37 at 37 °C. Incubation of ghosts in various concentrations of NaCl up to 150 mM did not affect the rotational characteristics at all. Diamines also did not affect it. At higher concentrations, spermine (>0.2 mM) and spermidine (>0.5 mM) affected the rotational motion slightly, but the effects were much smaller than those on the lateral motion (A. Tsuji et al., submitted for publication).

These results indicate that the lateral motion of band 3 proteins which are not anchored to ankyrin is governed by the spectrin association state in the underlying network, being more restricted with the increase in the tetramer population. On the other hand, the rotational mobility of band 3 was independent of the spectrin association state.

Effect of Cleavage of the Cytoplasmic Domain of Band 3 on Lateral Motion. When ghosts were mildly treated with trypsin, the cytoplasmic domain of band 3 was selectively digested to release a 40-kDa peptide (Steck et al., 1976). The extent of cleavage became larger with the increase in trypsin concentration used. The cleavage was almost complete on treatment with 0.5 μg/mL trypsin at 0 °C for 20 min. However, spectrin, actin, and band 4.1 were not cleaved under this condition (data not shown). Cleavage of ankyrin was not clear on the polyacrylamide gel used (10.5%).

FPR curves for ghosts treated with various concentrations of trypsin are shown in Figure 6A. These curves cannot be analyzed on the basis of a single mobile component and immobile components. At least a faster mobile component with $D = (4.0 \pm 1.63) \times 10^{-10}$ cm^2 s^{-1} was required for better fitting, in addition to the mobile ($D = 5.3 \times 10^{-11}$ cm^2 s^{-1}) and immobile components. The results of such analysis showed that

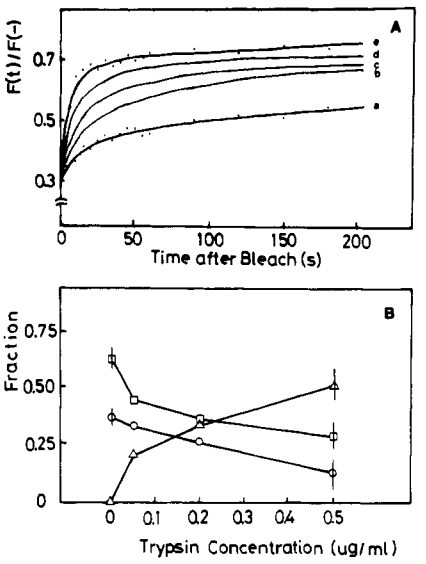

FIGURE 6: Effect of trypsin treatment of ghosts on the lateral motion of band 3. (A) FPR curves for FITC-labeled ghosts treated with various concentrations of tyrpsin: (a) 0, (b) 0.05, (c) 0.1, (d) 0.2, and (e) 0.5 μg/mL. (B) Analysis of FPR curves based on two mobile components with diffusion constants of 4.0×10^{-10} (△) and 5.3×10^{-11} cm^2 s^{-1} (○) and on the immobile component (□). The fraction of the components is plotted against the concentration of trypsin used for the treatment. The trypsin treatment was made at 0 °C for 20 min in hypotonic phosphate buffer using 2 mg/mL ghosts. FPR measurements were carried out in the presence of hypotonic phosphate buffer.

the fast component increased with the extent of band 3 cleavage, accompanied by a decrease in the mobile and immobile components (Figure 6B). The fraction of the fast component increased from 0 to 0.54 when almost all band 3 was cleaved, while the fractions of the mobile and immobile components decreased from 0.37 to 0.15 and 0.63 to 0.31, respectively. These results indicate a marked (8-fold) mobilization of band 3 protein in the erythrocyte membrane by cleaving off the cytoplasmic domain. The mobilization was not due to an increase in the lipid bilayer fluidity since the lateral diffusion of NBD-PE was not affected by trypsin treatment of ghosts.

Conversion of spectrin into tetramer caused a reduced mobility of band 3 as described in the previous section. However, such conversion did not affect the lateral mobility of band 3 lacking the 40-kDa domain. FPR measurements were made on ghosts treated with trypsin at 0.5 μg/mL and then with various concentrations of spermine. The results showed practically no effect of spermine on the fast component of band 3 (Figure 5d). The diffusion constant remained at 4×10^{-10} cm^2 s^{-1}, and the fraction of that component was unaltered in the presence of various concentrations of spermine.

Discussion

The present results can be summarized as follows (Figure 7). (1) Band 3 proteins anchored to the cytoskeletal network via ankyrin were immobile in the time scale of recovery measurements (Figure 7a). (2) Ankyrin-free band 3 proteins were mobile, but the mobility was restricted by the cytoskeletal network (Figure 7b,c). The restriction was greatly dependent on the spectrin association state, becoming smaller with the increase in the dimer population. The diffusion constant was 5×10^{-11} cm^2 s^{-1} for the tetramer mole fraction of 0.3–0.4 but decreased 1 order of magnitude when the fraction was increased to 0.50 by raising the ionic strength or by adding spermine. Consistently, an increase in the diffusion constant

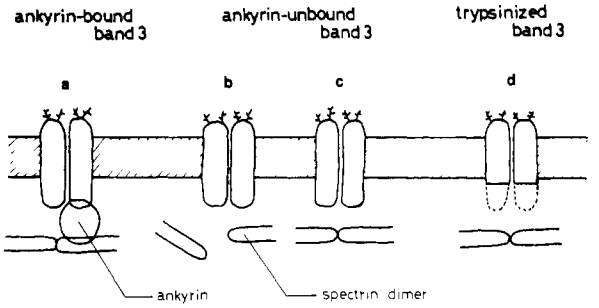

FIGURE 7: Restriction of the lateral motion of band 3 in erythrocyte membranes. (a) Band 3 proteins bound to ankyrin which in turn bound to spectrin in the cytoskeletal network are immobile, $D < 10^{-13}$ cm^2 s^{-1}. (b and c) Band 3 proteins unbound to the cytoskeletal network are mobile. The mobility was dependent on the spectrin association state in the cytoskeletal network, increasing with the dissociated dimer population: $D = 5 \times 10^{-11}$ cm^2 s^{-1} for f_T = ca. 0.33 and $D = 5 \times 10^{-12}$ cm^2 s^{-1} for $f_T = 0.5$. (d) Band 3 proteins lacking their cytoplasmic domain (40 kDa) diffuse much faster, $D = 4 \times 10^{-10}$ cm^2 s^{-1}.

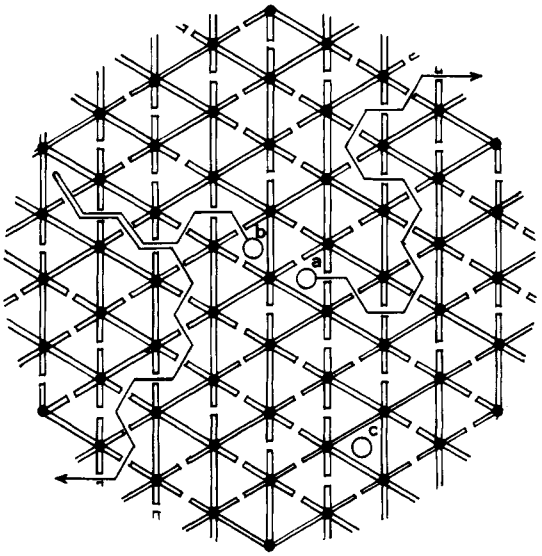

FIGURE 8: Schematic model for the restricted motion of band 3 in the cytoskeletal network (a rear view). Each junction (●) (actin and band 4.1) is drawn to hold six spectrin molecules (stretch length ca. 200 nm) either in the tetramer (closed gate) or in dissociated dimers (open gate). When the cytoplasmic domain of band 3 hits the dissociated dimer edge, it can pass it, while when it hits the tetrameric edge it cannot pass. In the FPR measurement, proteins should travel to reach and pass the bleached area (sphere with $r = 0.8$ μm). Protein b encounters a dead-end triangle, taking more time to reach the sphere than the protein a. Protein c is confined in all tetrameric edges and cannot pass.

was observed when the dimer fraction was increased by adding ATP in isotonic phosphate buffer. (3) The restriction by the cytoskeletal network was greatly reduced when the cytoplasmic 40-kDa domain of band 3 was cleaved off (Figure 7d). The diffusion constant became 1 order of magnitude larger, approaching the value for band 3 in spectrin-deficient erythrocytes. There is a possibility that the cytoskeletal network properties in these trypsin-treated ghosts may be modified because of possible cleavage of ankyrin. These results clearly show that the viscous drag to band 3 in the lipid bilayer is much smaller than the resistive force by the cytoskeletal network, in qualitative agreement with Sheetz et al. (1980), who suggested the band 3 motion to be analogous to diffusion through a polymer network. The lateral motion of band 3 is still confined in a two-dimensional matrix but governed by the motion of its cytoplasmic domain in the cytoskeletal network.

We present here a model for the restriction of band 3 motion that its cytoplasmic domain can pass the cytoskeletal network

when the spectrin molecules are in dissociated dimers but cannot when they are tetramers (Figure 8). The network is drawn as triangles in the figure, taking into account of a recent observation by Byers and Branton (1985) that an average of 5.3 spectrin molecules appear to insert into each junctional complex consisting of actin and band 4.1. Each edge of the triangle consists of a spectrin tetramer or two dissociated dimers, and band 3 can pass the dimeric edge (open gate) but cannot pass the tetrameric edge (closed gate). After random trials of pass and failure, band 3 can reach and pass a sphere of radius r equal to the bleaching beam radius (Figure 8a). The average time for the travel depends on the dimeric spectrin population or the number of open gates. The probability for the open gate in the network with spectrin dimer fractions f_D of 0.5 and 0.67 is 0.33 and 0.5, respectively. The average number of open gates per triangle is therefore 1 and 1.5, respectively. The average travel time greatly (10-fold) decreased with the 1.5-fold increase in the number of open gates. The discrepancy from the proportionality between them may partly arise from the small photobleached area (r = 0.8 μm) as compared with the unit travel distance. The lateral motion of band 3 is an essentially random walk in two dimensions but could deviate from it because of the small area for observation.

Band 3 proteins confined in triangles made only of spectrin tetramers (Figure 8c) should be immobile in the time scale of the recovery measurements and contribute to the immobile fraction. The probability for such triangles would be 0.13 for f_D = 0.67 and 0.30 for F_D = 0.5, assuming the binomial distribution. Triangles with one open and two closed gates would create a dead-end road (Figure 8b). The probability for such triangles would be 0.38 for f_D = 0.67 and 0.45 for f_D = 0.5.

The model is consistent with the observation that FPR curves for ghosts with different populations of spectrin tetramer could be analyzed by a single mobile component and that the diffusion constant of the mobile component was affected by the change in f_T, while its fraction was unaffected. Band 3 proteins should behave as a homogeneous population with an average diffusion constant dependent on the spectrin dimer population.

Strong support for the model was obtained from a different line of experiments. The rotational motion of band 3 was found to be independent of the spectrin association state. Ankyrin-free band 3 proteins undergo rotational motions in the lipid bilayer matrix independent of the cytoskeletal network.

Whether such lateral mobility control exists in intact erythrocytes or such control is significant for the function is not known. Mobilization of band 3 proteins caused by influenza virus may be related to the action of that virus on erythrocytes. The mobilization was observed when erythrocytes were incubated with virus at acidic pH but not at neutral pH (Yoshimura et al., 1985), consistent with the pH dependence of the ability of the virus to fuse with erythrocyte membranes (Maeda & Ohnishi, 1980; Ohnishi, 1985). More recently, we have observed a similar mobilization affected by hemagglutinin glycoproteins isolated from the viral envelope. The lateral diffusion constant of band 3 was 6.1×10^{-12} cm^2 s^{-1} with the mobile fraction of 0.33 when erythrocytes were incubated with hemagglutinin in isotonic phosphate buffer, but increased to a value of 1.6×10^{-10} cm^2 s^{-1} without an effect on the mobile fraction when the incubation was made at pH 5.2 and FPR was measured in isotonic phosphate buffer. The mobilization is again consistent with the pH-dependent hemolytic action of hemagglutinin (Sato et al., 1983). Speculation is that hemagglutinin may affect the spectrin association state, increasing the dimer population, and thus mobilize the band 3 proteins. Such mobilization would produce protein-free lipid areas in the membranes which may be the site of fusion.

Similar lateral mobility control may exist in other eukaryote cells. Physiological signals may lead to modification of the association state of cytoskeletal protein components to change the mobility or the mobile domains of integral proteins. Production or degradation of the ankyrin-like anchor proteins would result in immobilization or mobilization of specific integral proteins.

ACKNOWLEDGMENTS

We thank Dr. Akihiro Kusumi for the rotational measurements in the Microphotonic Center, Medical College of Wisconsin.

Registry No. 5′-ATP, 56-65-5; putrescine, 110-60-1; spermidine, 124-20-9; spermine, 71-44-3.

REFERENCES

Axelrod, D., Koppel, D. E., Schlessinger, J., Elson, E., & Webb, W. W. (1976) *Biophys. J. 16*, 1055–1069.

Bennett, V., & Stenbuck, P. J. (1979) *Nature (London) 280*, 468–473.

Bennett, V., & Stenbuck, P. J. (1980) *J. Biol. Chem. 255*, 6424–6432.

Bohlen, P., Stein, S., Dairman, W., & Udenfriend, S. (1973) *Arch. Biochem. Biophys. 155*, 213–220.

Byers, T. J., & Branton, D. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6153–6157.

Chang, C.-H., Takeuchi, H., Ito, T., Machida, K., & Ohnishi, S. (1981) *J. Biochem. (Tokyo) 90*, 997–1004.

Cherry, R. J., & Godfrey, R. E. (1981) *Biophys. J. 36*, 257–276.

Fowler, V., & Branton, D. (1977) *Nature (London) 268*, 23–26.

Golan, D. E., & Veatch, W. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2537–2541.

Laemmli, U. K. (1970) *Nature (London) 277*, 680–685.

Lepock, J. R., Thompson, J. E., Kruuv, J., & Wallach, D. F. H. (1978) *Biochem. Biophys. Res. Commun. 85*, 344–350.

Liu, S.-C., & Palek, J. (1980) *Nature (London) 285*, 586–588.

Lowry, O. H., Rosebrough, N. Y., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.

Lux, S. E. (1979) *Nature (London) 281*, 426–429.

Maeda, T., & Ohnishi, S. (1980) *FEBS Lett. 122*, 283–287.

Ohnishi, S. (1985) in *Biomolecules, Electronic Aspects* (Nagata, C., Hatano, M., Tanaka, J., & Suzuki, H., Eds.) pp 227–252, Japan Scientific Societies Press, Tokyo, and Elsevier, Amsterdam.

Peters, R., & Cherry, R. J. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4317–4321.

Ralston, G. B., Dunbar, J., & White M. (1977) *Biochim. Biophys. Acta 491*, 345–348.

Saffman, P. G., & Delbrück, M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 3111–3113.

Sato, S. B., Kawasaki, K., & Ohnishi, S. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 3153–3157.

Shindler, M., Koppel, D. E., & Sheetz, M. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 1457–1461.

Sheetz, M. P., & Koppel, D. E. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3314–3317.

Sheetz, M. P., & Casaly, J. (1980) *J. Biol. Chem. 255*, 9955–9960.

Sheetz, M. P., Schindler, M., & Koppel, D. E. (1980) *Nature (London) 285*, 510–512.

Steck, T. L., Ramos, B., & Strapazon, E. (1976) *Biochemistry 15*, 1154–1161.
Tyler, J. M., Hargreaves, W. R., & Branton, D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5192–5196.
Ungewickell, E., & Gratzer, W. (1978) *Eur. J. Biochem. 88*, 379–385.
Yoshimura, A., Yamashina, S., & Ohnishi, S. (1985) *Exp. Cell Res. 160*, 126–137.

Partial Proteolysis as a Probe for Ligand-Induced Conformational Changes in the Isolated β Subunit of the H^+-Translocating $F_0 \cdot F_1$ ATP Synthase†

Daniel Khananshvili and Zippora Gromet-Elhanan*
Department of Biochemistry, The Weizmann Institute of Science, Rehovot 76100, Israel
Received April 11, 1986

ABSTRACT: The isolated β subunit of the *Rhodospirillum rubrum* $F_0 \cdot F_1$ ATP synthase contains two nucleotide binding sites, a Mg-independent and a Mg-dependent site [Gromet-Elhanan, Z., & Khananshvili, D. (1984) *Biochemistry 23*, 1022–1028]. Phosphate (P_i) binds only to the second site [Khananshvili, D., & Gromet-Elhanan, Z. (1985) *Biochemistry 24*, 2482–2487]. Binding of these ligands has been found to induce conformational changes in the β subunit that can be followed by their effect on trypsin sensitivity of the subunit. With a ratio of 1 mol of trypsin/100 mol of β, the subunit is digested in the absence of ligands with a half-time of 10 min. $MgCl_2$ has no effect on the trypsin sensitivity of β, but the other ligands show pronounced effects. Binding of either ADP or ATP to the Mg-independent site results in partial protection of the β subunit against its digestion by trypsin, increasing the $t_{1/2}$ to 20 min. A further decrease in the sensitivity to trypsin occurs on binding of MgADP to the second Mg-dependent site, increasing the $t_{1/2}$ to 30 min. Binding of MgATP or MgP_i to this site causes, however, an opposite effect, resulting in a decrease in the $t_{1/2}$ to 3 and 6 min, respectively. These results indicate that ligand binding induces two distinct changes in the conformation of the isolated β subunit. One conformational state is obtained on occupation of the Mg-independent nucleotide binding site and is further stabilized by MgADP. The second conformational state is obtained on binding of MgATP or MgP_i, suggesting that it is induced by occupation of the γ-phosphoryl subsite in the Mg-dependent catalytic site on the β subunit.

Energy-transducing membranes contain a reversible proton-translocating ATP synthase–ATPase complex $F_0 \cdot F_1$. It is comprised of two portions: an intrinsic membrane portion, F_0, that mediates proton movement and an extrinsic membrane portion, F_1, that is the catalytic sector (Kagawa et al., 1979; Penefsky, 1979; Nelson, 1981; Futai & Kanazawa, 1983; Senior & Wise, 1983). F_1 consists of five types of subunits (α, β, γ, δ, ϵ) and functions also as a soluble ATPase. It has been shown to contain several nucleotide binding sites that reside in the two major subunits, α and β (Harris, 1978; Baird & Hammes, 1979; Cross, 1981; Senior & Wise, 1983), and seem to include both catalytic and noncatalytic sites (Cross & Nalin, 1982; Grubmeyer et al., 1982; O'Neal & Boyer, 1984). However, the subunit location of these sites as well as their exact role in the mechanism of ATP synthesis and hydrolysis is still uncertain. One possible approach to the elucidation of the molecular events controlled by the multisubunit F_1 complex is the study of simpler functional systems such as individual subunits of F_1.

Investigations of individual subunits became possible when reconstitutively active α and β subunits have been purified from several bacterial sources (Yoshida et al., 1977; Futai, 1977; Dunn & Futai, 1980; Philosoph et al., 1977; Khananshvili & Gromet-Elhanan, 1982). Direct ligand-binding studies have been carried out until now on the α and β subunits of *Escherichia coli* F_1 (Dunn & Futai, 1980; Issartel & Vignais, 1984) and on the β subunit of *Rhodospirillum rubrum* F_1 (Gromet-Elhanan & Khananshvili, 1984; Khananshvili & Gromet-Elhanan, 1984, 1985a). These studies have established the presence of one Mg-independent nucleotide binding site on Ecα,[1] with K_d values of 0.1 μM for ATP and 0.9 μM for ADP, and one on Ecβ that binds ADP (K_d = 25 μM) and possibly also ATP (K_d = 50–100 μM). On Rrβ two binding sites have been demonstrated: a high-affinity Mg-independent nucleotide binding site with K_d values of 4 μM for ATP and 7 μM for ADP and a low-affinity Mg-dependent site that binds ATP (K_d = 200 μM), ADP (K_d = 80 μM), and P_i (K_d = 270 μM). Ligand-induced conformational changes have also been investigated, mainly with Tα and Tβ (Ohta et al., 1980a,b) and Ecα (Dunn, 1980; Senda et al., 1983). But only in the case of Ecα have the ATP-induced conformational changes been correlated with its high-affinity binding site that has been characterized by direct binding studies. With Ecβ, in contrast to Ecα, addition of ATP has not been found to induce any change in the trypsin sensitivity (Senda et al., 1983), although it has been found to induce some conformational change that could be detected as a quenching of 8-anilinonaphthalene

† This work was supported in part by the Minerva Foundation, Munich, West Germany.

[1] Abbreviations: Ecα, Tα, Ecβ, Rrβ, and Tβ, isolated reconstitutively active α and β subunits of the $F_0 \cdot F_1$ complex of *E. coli*, *R. rubrum*, and the thermophilic bacterium PS3; Tricine, *N*-[tris(hydroxymethyl)-methyl]glycine; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

0006-2960/86/0425-6139$01.50/0