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Cross-Linkings Between Spectrin and Band 3 in Human Erythrocyte Membranes

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A specific structural association between spectrin component 1 and band 3 in human erythrocyte membrane has been demonstrated by covalent cross-linkings, specific labeling, and the technique of two-dimensional gel electrophoresis. A complex of 330,000 daltons, representing 1 + 3, was produced in mildly oxidized membranes at physiologic pH and isotonic conditions but not at hypotonic conditions (< 10 mM KCl or NaCl). The yield of this complex decreased dramatically as the monovalent cation concentration decreased from 90 mM to 30 mM. The presence of Mg^{++} or Ca^{++} (2 mM) at low ionic strength promoted 1 + 3 cross-linking in an amount similar to that produced at isotonic conditions. The specific segment of band 3 involved in the cross-linking was also investigated by means of chymotrypsin digestion of band 3 in the intact red cells. The results showed the cross-links between spectrin component 1 and the 55,000-dalton fragment of band 3 at physiologic pH and isotonic conditions. This is consistent with the idea that band 3 is anchored on or contacted with the submembrane meshwork at the cytoplasmic membrane surface.

Key words: spectrin, band 3, covalent cross-linking, two-dimensional gel electrophoresis, ^{125}I-labeling, chymotrypsin digestion

Spectrin, the major "peripheral" protein located at the cytoplasmic surface of erythrocyte membrane [1–3], is thought to form a submembrane meshwork with actin, which influences cell shape, viscoelastic properties, and transmembrane communications [4–15]. This submembrane meshwork may also provide anchoring sites at the inner membrane surface for transmembrane proteins, thereby affecting their surface topography and lateral mobility. However, detailed interactions between spectrin and transmembrane proteins at molecular level are not clear.

Abbreviations: SDS) sodium dodecyl sulfate, PAGE) polyacrylamide gel electrophoresis, ATP) adenosine triphosphate, PAS) periodic acid Schiff (stain), Tris) tris-(hydroxymethyl)-aminomethane, HEPES) N-2-hydroxy-ethyl piperazine-N-2-ethanesulfonic acid, DTT) dithiothreitol, CuP) $CuSO_4$/o-phenanthroline mixture.

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Recently, the techniques of covalent cross-linking and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis have been employed to identify the nearest neighbors among membrane proteins of red cells [16–21]. The data suggest that the proteins in the red cell membrane are organized into stable homooligomers and, less commonly, into heterocomplexes derived from different proteins. The spontaneous cross-linkings among spectrin, actin, and band 3 (the major integral membrane protein) were first observed in ghosts incubated at acid pH (4.0–5.5) [20]. These cross-linkings were disulfides and were related to the isoelectric precipitation of spectrin (and probably band 3) at this pH.

In this investigation, we studied further the protein cross-linkings at physiologic pH and isotonic conditions. The data indicate structural contacts between spectrin component 1 (240,000 daltons) and band 3 (90,000 daltons) at physiologic conditions, the amount of which was affected by the concentration of mono- and divalent cations present. In addition, the segment of band 3 that was cross-linked with spectrin was also investigated by pretreating the intact cells with a proteolytic enzyme, chymotrypsin. The enzyme cleaved band 3 at the outer surface into two fragments of 55,000 daltons and 38,000 daltons [22]. We now demonstrate that the 55,000-dalton fragment of band 3 can be cross-linked to spectrin component 1 at the cytoplasmic surface.

MATERIALS AND METHODS

Lactoperoxidase, glucose oxidase, chymotrypsin, and o-phenanthroline were purchased from Sigma Chemical Co. Sodium iodide I^{125} was obtained from New England Nuclear Co. Erythrocyte membranes were prepared from freshly drawn blood by the hypotonic lysis method of Fairbanks et al [23] except the buffer, which was adjusted to pH 7.4. All membrane preparations were carried out as described at 0–4°C. Catalytic oxidation of ghosts with $CuSO_4$/o-phenanthroline mixture was performed as described by Steck [16] except that some ghosts were suspended in isotonic buffer (5 mM Na phosphate, 150 mM KCl or NaCl, pH 7.4) and the reaction temperature was 0°C. The two-dimensional SDS-PAGE and the designation of the major membrane polypeptides in both first and second dimensions were the same as described previously [20]. The 2.5% polyacrylamide/0.3% agarose composite gels were used in the first dimension. The discontinuous gel system described by Laemmli [42] was employed in the second dimension. Radioactive iodination of surface proteins with lactoperoxidase, glucose oxidase and sodium iodide I^{125} in intact cells was done according to Reichstein and Blostein's procedures [24]. Chymotrypsin treatment of red cells (10% suspension) was carried out in the ATP-maintaining buffer which contains 50 mM glycylglycine, 5 mM KCl, 2 mM $MgCl_2$ 200 mg% glucose, 0.54 mM adenine, 12.7 mM inosine, 2 mM phosphate, and NaCl to obtain 290–300 mOsm, pH 7.4. Chymotrypsin (0.2 mg/ml) was added to initiate the digestion. After 22 h incubation at 37°C under nitrogen, digestion was terminated with diisopropyl phosphofluoridate (final concentration 0.05 mM [22]).

RESULTS

Mild Oxidation and Two-Dimensional SDS PAGE

Disulfide couplings of proteins in isolated erythrocyte membranes were produced by catalytic oxidation with $CuSO_4$/o-phenanthroline mixture (CuP) [16, 17]. The results

depended on the tonicity of the suspending solution (Fig. 1A). Under hypotonic condition (5 mM Na phosphate, pH 7.4), the complexes of 450,000, 260,000, and 175,000 daltons were observed (gel II) as previously described by others [16, 17]. Under isotonic conditions (5 mM Na phosphate, pH 7.4, 150 mM KCl or NaCl) (gels III–V), an additional complex of 330,000 daltons were found. More extensive oxidation did not increase the yield of 330,000-dalton complex (gels III–V). Instead, it produced additional large aggregates retained at the origin of the gel, (gel V). The disulfide couplings were reversible by reduction (gel VI), and were utilized subsequently to identify the participating components.

The cross-linked products in the first dimension were reversed by DTT reduction, and reelectrophoresed into the second dimension, in which the released constituents appeared as "off-diagonal" spots (Fig. 1B–E). We have identified the constituents of the complexes from membrane samples oxidized under isotonic conditions. Several off-diagonal spots appear to represent dimers (ie, 1 + 2, 3_2, 4.1_2 and 5_2) and trimers (ie, 3_3) of membrane proteins. Heterocomplexes of 1 + 3 and 2 + 4.9 are also evident on the slabs of higher resolution (Fig. 1C and 1D). The very high-molecular-weight material at the origin of the gel produced by more extensive oxidation contained essentially all of the membrane protein components (Fig. 1E) and is enriched with spectrin. The data are summarized in Table I.

Cross-Linkings of ^{125}I-Labeled Band 3 With Spectrin

To further substantiate that the above 330,000-dalton complex was in fact 1 + 3, we labeled band 3 with ^{125}I and studied the incorporation of the label into the complexes (Fig. 2). With the enzymes lactoperoxidase and glucose oxidase, band 3 and a 150,000-dalton band in intact cells were labeled extracellularly (Fig. 2C) as shown previously by others [24]. The 150,000-dalton band probably represents the major sialoglycoprotein, PAS-1, as judged from the relative mobility of PAS-1 in the gel system of low concentration of polyacrylamide [20, 25]. Catalytic oxidation of isolated ghosts derived from ^{125}I-labeled intact cells under isotonic conditions produced new radioactive peaks corresponding to 330,000, 240,000, and 175,000 daltons (Fig. 2D). The 330,000-dalton peak has the same relative mobility as 1 + 3. The 240,000- and 175,000-dalton peaks probably represent dimers and trimers, respectively, of band 3. The involvement of PAS-1 in the 330,000-dalton peak is unlikely because the size of PAS-1 is too large to be accounted for; the complex of spectrin and PAS-1 would be about 390,000 rather than 330,000 daltons.

Effect of Salt Concentration on Protein Cross-Linkings

It is known that the interaction of spectrin with membrane is influenced by the ionic strength in the medium [26]. We explored the effect of salt on protein cross-linkings and specifically the cross-linkings of spectrin itself and those between spectrin and band 3 (Fig. 3A). The quantitative analysis (Fig. 3B) showed that the decrease of salt gradually diminished the amount of 1 + 3 and 1 + 2. On the other hand, the amount of band 3 dimer did not change significantly. The change of the amount of 1 + 3 was most noticeable in the salt concentration ranging from 30 to 90 mM (Fig. 3B).

A concomitant depletion of monomeric band 6 (glyceraldehyde 3-phosphate dehydrogenase) was also shown in membranes incubated with high concentration of salt. This was due to the release of band 6 from membranes by salt [27–29] rather that its involvement in the cross-linking reactions, because the subsequent DTT reduction did not normalize the amount of band 6 in the gel (Fig. 1A, gel VI). The range of salt concentration

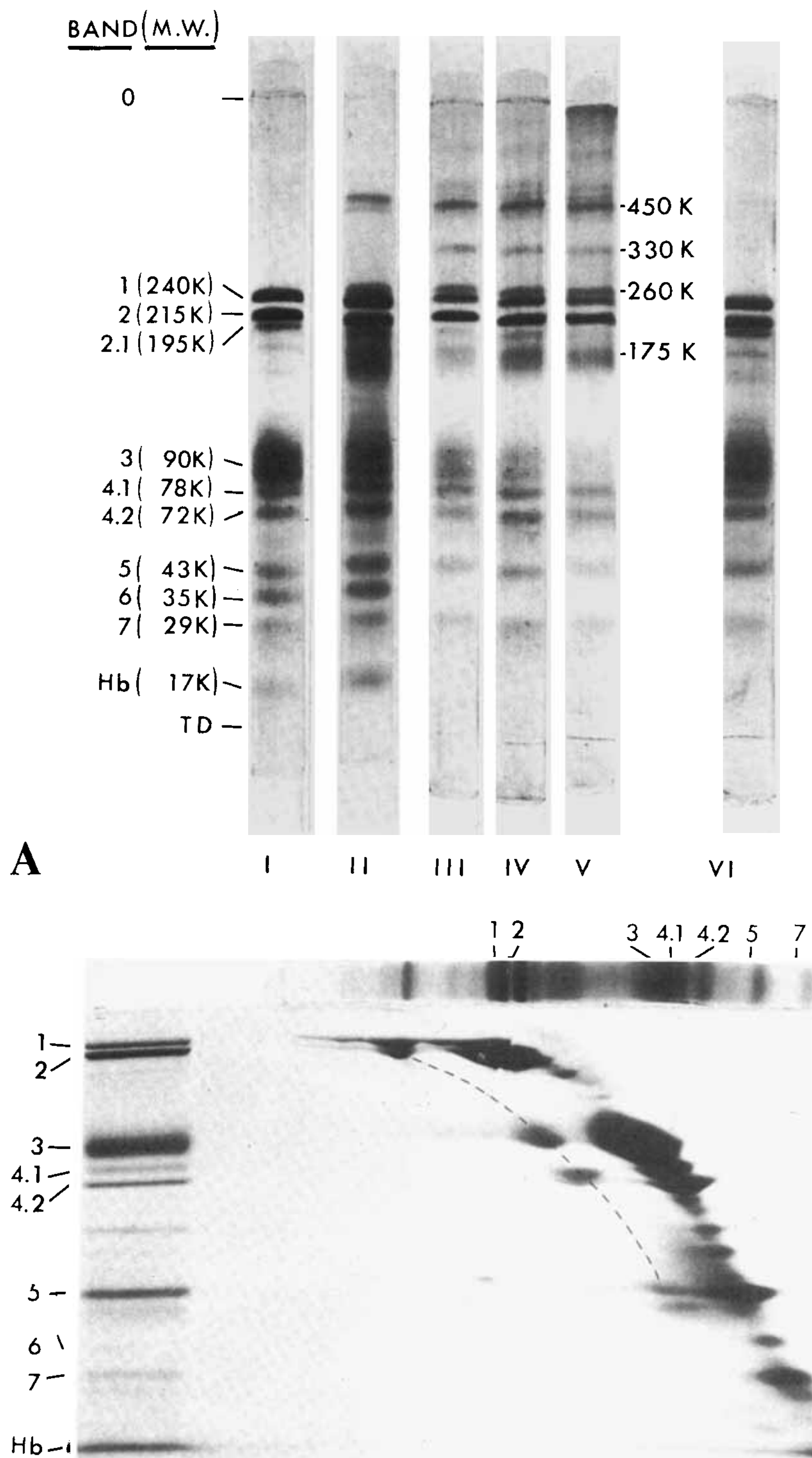
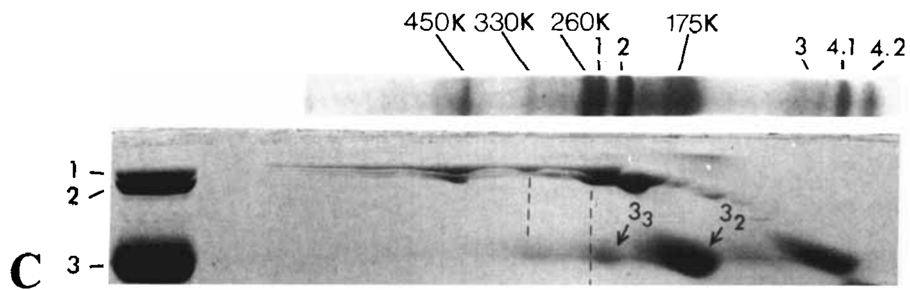
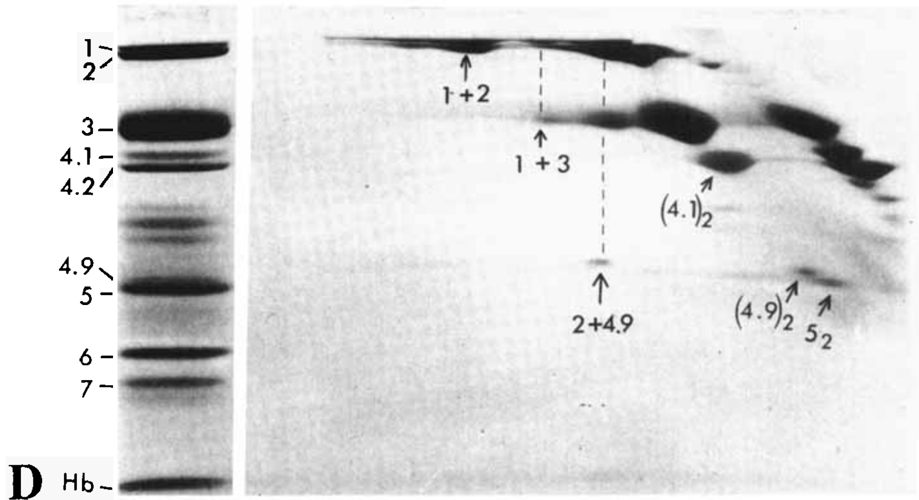
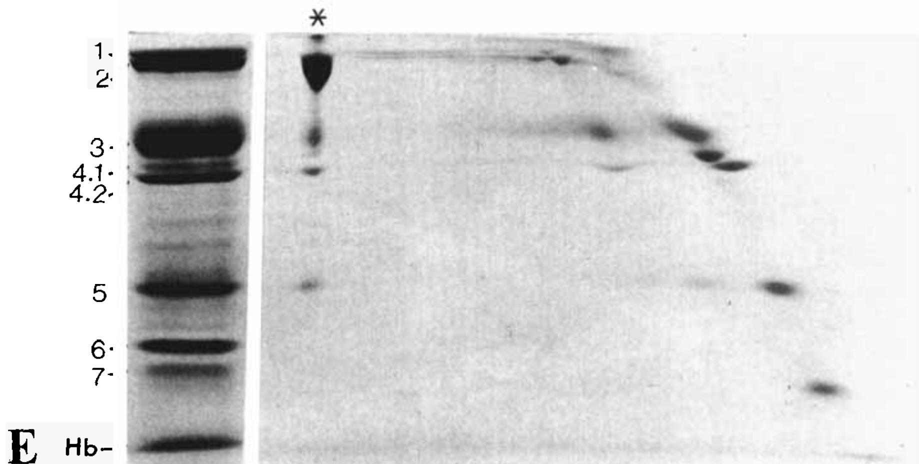


Fig. 1. The cross-linking of proteins in isolated erythrocyte membranes produced by oxidation catalyzed by $CuSO_4$/o-phenanthroline (CuP). A: Cross-linkings under hypotonic and isotonic conditions. Gel I) Untreated ghosts. Packed ghosts (10 μl or 25 μg of protein) dissolved in SDS without reduction were electrophoresed. Gel II) Ghosts in hypotonic buffer (Na phosphate, 5 mM, pH 7.4) were incubated with CuP(5μM/25μM) at 0°C for 20 min. The reaction was quenched by ethyline-diaminetetracetic acid (EDTA) (2 mM). Gels III–V) Ghosts in isotonic buffer (Na phosphate 5 mM, KCl 150 mM, pH 7.4) were incubated at 0°C for 20 min with the following concentrations (μM/μM) of







CuP: 1/5, 5/25, 10/50, respectively. Gel VI) Same as in gel IV, except that the sample was reduced with 25 mM DTT prior to electrophoresis. B–E: Two-dimensional analysis of protein cross-linkings under isotonic conditions. B: Ghosts were oxidized the same as in gel III; 150 μg of membrane protein was applied. A replicate gel of the first dimension is placed at the top of the slab. A membrane sample applied directly on the second-dimension slab is shown at the left for reference. Dashed curve indicates alignments of spots corresponding to polypeptides released after cleavage of the dimers of membrane proteins. C: Ghosts were oxidized the same as in gel IV. The cross-linked sample (100 μg of protein) was subjected to prolonged electrophoresis in the first dimension. The spectrin and band 3 zones of the two-dimensional pattern are shown. D: Same as in C, except that 150 μg of protein was applied. Tentative identification of the cross-linked complexes giving rise to off-diagonal spots is indicated. E: Ghosts were reacted with CuP(10 μM/50 μM) at 0°C for 1 h; 75 μg of protein was applied. The asterisk denotes protein components released from the large aggregates at the origin of the first dimension.

TABLE I. Tentative Identification of the Major Cross-Linked Protein Complexes From Human Erythrocyte Ghosts

Approx MW × 10^{-3a}	Estimated composition
450	1 + 2
330	1 + 3
260	2 + 4.9
175	3_2

[a] A calibration curve for the estimation of aggregate molecular weights was constructed by extrapolating the curve for the major membrane polypeptides through the point representing 1 + 2 complex, which was readily identified before [20].

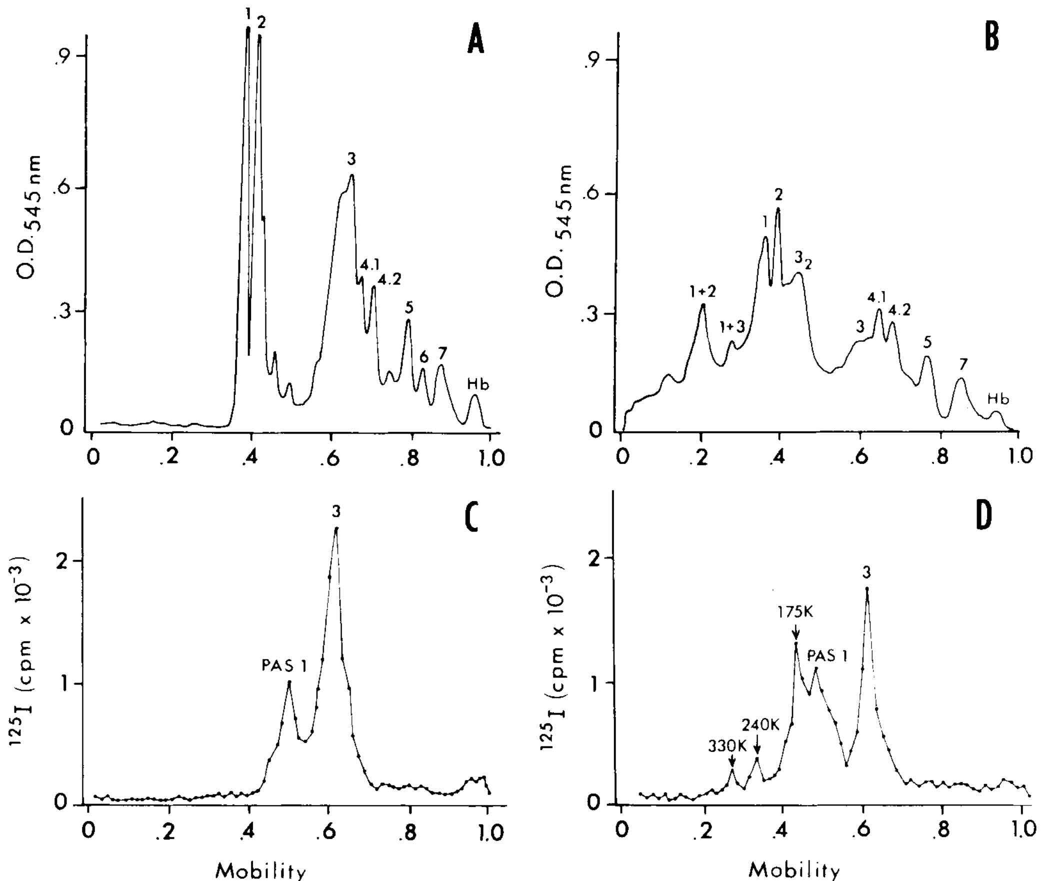


Fig. 2. Cross-linking of ^{125}I-labeled erythrocyte membrane proteins. Intact erythrocytes were labeled with ^{125}I on the outer surface. The isolated ghosts were reacted with CuP(5 μM/25 μM) and electrophoresed as described in the text. Scans of the Coomassie blue-stained gels of the untreated (A) and mildly oxidized (B) ghosts. Radioactive profiles are of the untreated (C) and mildly oxidized (D) ghosts. Radioactivity was determined in 1 mm minced segments of gel.

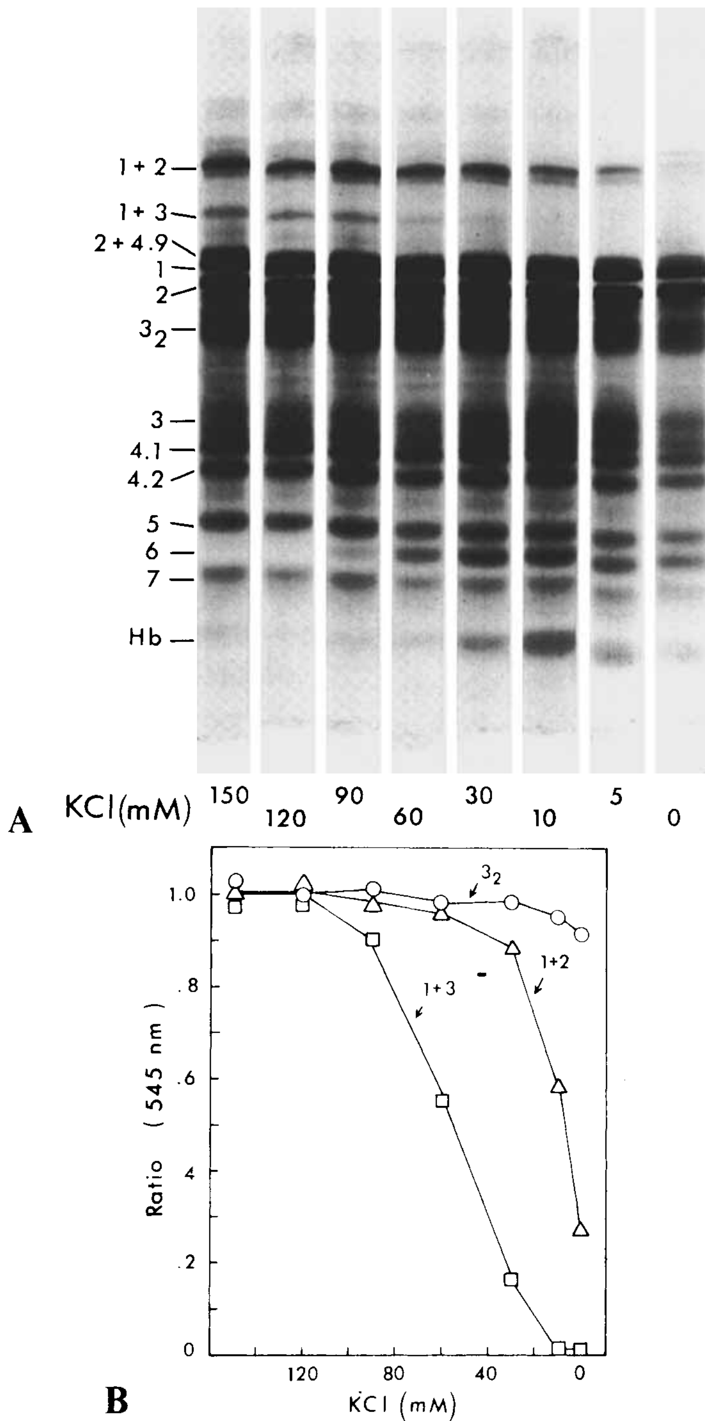


Fig. 3. Influence of decreasing concentrations of KCl on the erythrocyte membrane protein cross-linkings. A: Ghosts were catalytically oxidized with CuP(5 μM/25 μM) in Na phosphate buffer (0.5 mM, pH 7.4) with different concentrations of KCl at 0°C for 20 min. B: Changes in the relative amount of 1 + 3, 1 + 2, and 3_2 as a function of KCl concentration. Depicted are the ratios of the area under each complex (after they were cut out, weighed, and normalized by the total Coomassie stain in the gel) to the area of the same complex produced under isotonic conditions.

(30–90 mM) that influences the retention of band 6 by the membrane has been shown above to affect the amount of 1 + 3. However, a direct correlation between them is not evident (see Discussion).

The effect of Ca^{++} (or Mg^{++}) on membrane protein cross-linking is observed at much lower concentrations than that of KCl (Fig. 4A). Under hypotonic conditions 2 mM Ca^{++} (or Mg^{++}) produced the same effect as that of NaCl (or KCl) at 90 mM or more. In addition, Ca^{++} clearly enhanced the degree of protein cross-linkings when added to the isotonic salt solution (Fig. 4B). A distinct smearing of Coomassie blue stain was shown at the slow-migrating zone of the upper region of the gel.

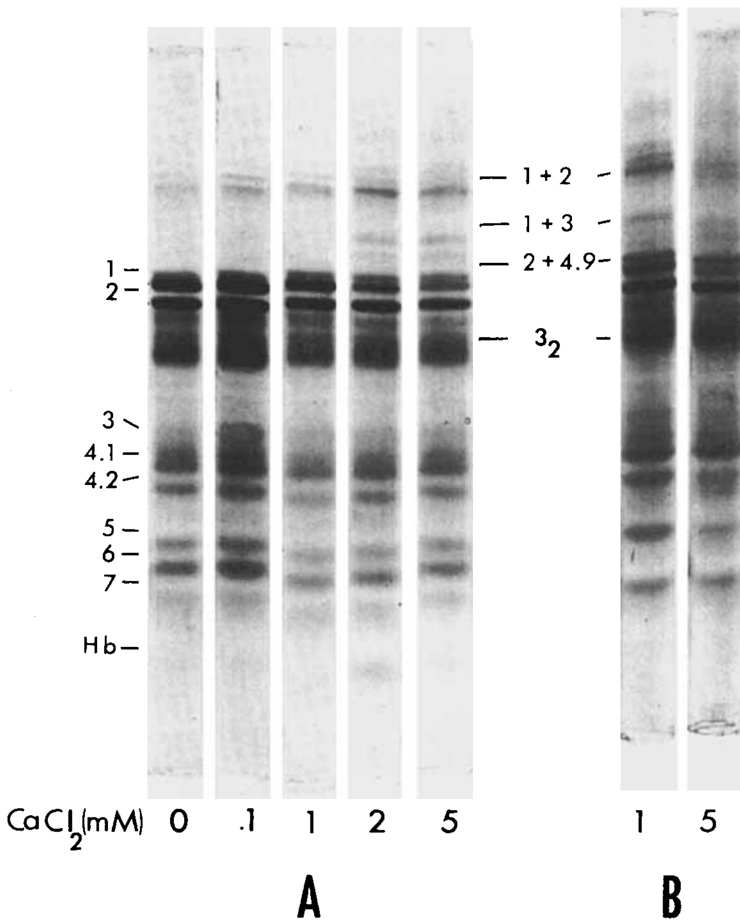


Fig. 4. Influence of $CaCl_2$ on the erythrocyte membrane protein cross-linkings. A: Ghosts were catalytic oxidized with CuP(5 μM/25 μM) in hypotonic buffer (HEPES 2 mM, pH 7.4) with different concentrations of $CaCl_2$ at 0°C for 20 min. B: Ghosts were oxidized in isotonic buffer (HEPES 2 mM, 150 mM KCl, pH 7.4) with 1 or 5 mM $CaCl_2$.

Effect of Chymotrypsin Digestion of Band 3 in Intact Cells on Protein Cross-linking

The mild digestion of intact cells with chymotrypsin cleaved band 3 specifically into two fragments of 55,000 and 38,000 daltons (Fig. 5, gel II; Fig. 6, gel I), which confirmed the previous observation by Steck et al [22]. To examine which fragment was structurally associated with band 1, we oxidized catalytically the ghosts from digested cells with CuP. Three new complexes (110,000, 165,000, and 300,000 daltons) were produced in addition to the 260,000- and 450,000-dalton complexes which were observed previously in undigested cells (Fig. 5, gels III and IV). The two-dimensional analysis revealed that these new complexes were 1 + 55k (300,000 daltons) as well as dimers (110,000 daltons) and trimers (165,000 daltons) of 55k fragments. Two other familiar complexes 2 + 4.9 (260,000) and 1 + 2 (450,000 daltons) were also seen. The 38k fragment of band 3 appeared on the slab as a monomeric spot, involving neither self-polymerization (ie, dimer, trimer) nor coupling with band 1. Compared with the amount of band 3 trimer in undigested sample, the yield of 55k trimer is markedly enhanced (Fig. 1C and Fig. 6C).

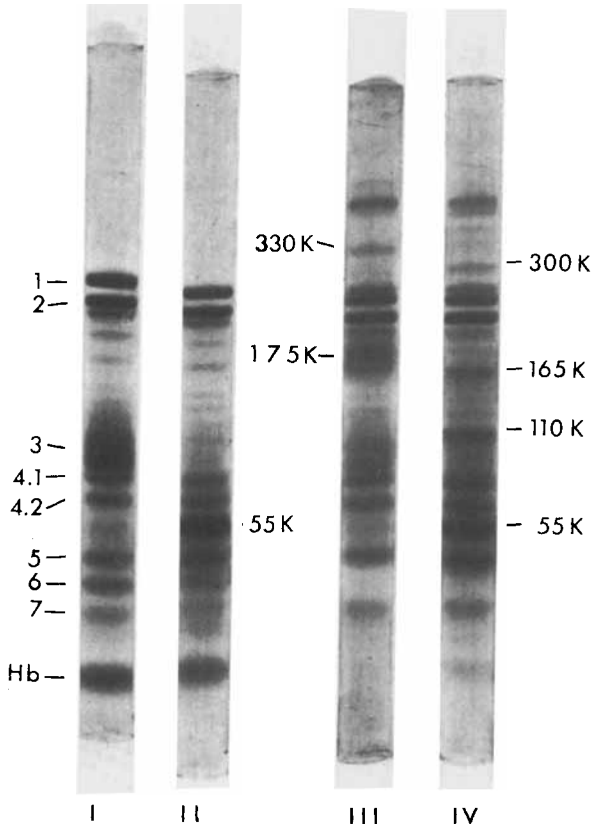


Fig. 5. Protein cross-linkings in isolated ghosts from chymotrypsin-digested erythrocytes. Ghosts were prepared from red cells which had been incubated for 22 h in an ATP maintaining buffer at 37°C lacking (gel I) or containing 0.2 mg/ml of chymotrypsin (gel II). Subsequent oxidation of undigested (gel III) and digested (gel IV) samples were carried out in isotonic buffer (Na phosphate 5 mM, KCl 150 mM) with CuP(5 μM/25 μM) at 0°C for 20 min.

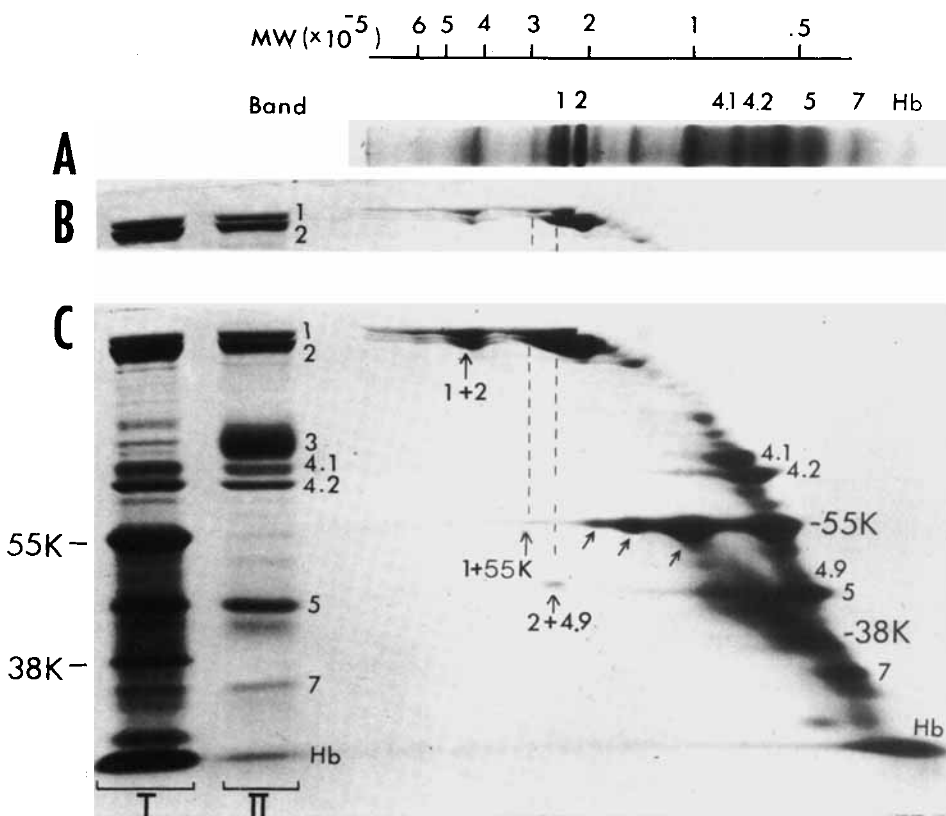


Fig. 6. Two-dimensional analysis of protein cross-linkings in ghosts from chymotrypsin-treated erythrocytes. A. Replicate gel identical to gel IV in Fig. 5. B: Spectrin zone of the two-dimensional pattern with low loading (75 μg of protein). C: Same as (B) with 150 μg of protein applied. Constituents of some complexes are labeled. Arrows indicate the trimers, dimers, and tetramers of 55,000-dalton fragment. Column I) pattern of a membrane sample from chymotrypsin-treated erythrocyte applied directly on the slab, column II) untreated ghost sample in which band 6 has been eluted by salt.

DISCUSSION

The 330,000-dalton complex is one of the major cross-linked products produced by the mild oxidation of ghosts at physiologic pH and isotonic conditions. The complex was identified as 1 + 3 from both the analysis of two-dimensional gel electrophoresis and the specific ^{125}I-labeling of band 3. In addition, prior chymotrypsin digestion of band 3 in intact cells led to the subsequent formation of a smaller complex of 300,000 daltons between spectrin 1 and the 55,000-dalton fragment of band 3.

Previous studies by others [16, 17] using CuP in protein cross-linking of isolated ghosts did not detect the 330,000-dalton complex. This is apparently due to the low tonicity employed in these experiments. The amount of 1 + 3 and 1 + 2 complexes depended on the concentration of mono- and divalent cations in the reaction mixture (Figs. 3 and 4). This is not unexpected, since both the spectrin and band 3 segments exposed at the cytoplasmic surface are known to contain a large amount of glutamic and

aspartic acid residues [22, 30, 31]. In the absence of salt, the charge repulsion arising from these residues at neutral pH is likely to be enormous; and they may limit the physical contacts of protein chains that are necessary for intermolecular disulfide couplings to occur. In fact, at low ionic strengths and alkaline conditions, spectrin is known to detach from the membrane [1, 2, 23] and dissociate primarily into noncovalent dimers or tetramers (ie, $(1 + 2)_{1,2}$) in the solution [2, 32, 33]. In contrast to the 1 + 3 or 1 + 2 complexes, the yield of band 3 dimers in membranes was essentially unchanged at various ionic strengths (Fig. 3B). Whether this is due to the close proximity of hydrophobic segments of these transmembrane polypeptides in the membrane is not known. Recently, the band 3 proteolysis in membrane was also found to be affected by salt [34]; band 3 became more resistant to trypsin digestion under isotonic conditions. This phenomenon is consistent with the interpretation of our cross-linking data; the closer contacts of spectrin to band 3 under isotonic conditions decrease the accessibility of the proteolytic enzyme to band 3. The amount of spectrin-band 3 cross-linkings gradually diminished as the salt concentration (NaCl or KCl) decreased from 90 mM to 30 mM. This range of concentration is higher than that detected by Bennett and Branton in the study of spectrin-membrane interactions [26]. They reported the saturation of spectrin binding by inside-out vesicles at 10 mM KCl. Recent data of Litman et al [35] suggest that band 2, rather than band 1, binds to the inside-out vesicles. The discrepancies probably represent the measurement of two different parameters in these experiments: i) the molecular interactions and ii) the degree of physical contacts. It is recognized that cross-linked products may not necessarily represent the stable protein complexes in the membrane. Random collisional interferences may be introduced from either the lateral mobility or the concentration of protein constituents in the membrane [21, 36]. However, recent studies using fluorescein isothiocyanate labeling show that integral proteins (mainly band 3 and the major sialoglycoprotein) have a diffusion coefficient of the order of 10^{-11} $cm^2 \cdot s^{-1}$ at 37°C, or 10^{-12} $cm^2 \cdot s^{-1}$ at 23°C in the erythrocyte membrane [37, 38]. These diffusion coefficients are about two orders below that of rhodopsin in the photoreceptor membranes [39], or of surface antigens in the membranes of cultured muscle fibers [40]. The mobility of these integral proteins is curtailed dramatically at cold temperatures. Following the fusion of labeled and unlabeled cells, incubation at 0°C for up to 20 h failed to produce any fluorescent label redistribution [37]. Since we have oxidized the ghost proteins at 0°C (Fig. 1), and the complexes of 1 + 3 appeared within a minute of oxidation (Liu and Palek, unpublished observations), the interference from the lateral mobility of band 3 in the membrane is probably not significant. Furthermore, the fact that the yield of 1 + 3 complex is dependent on the tonicity (Fig. 3) or pH [20] of the reaction mixture suggests that the cross-linking is governed by the electrostatic interaction rather than by the concentration or lateral mobility of protein constituents in the membrane. However, the exact relationship between the implicated contacts and the molecular interactions is not clear. Recent studies of direct molecular bindings suggest that another protein component may be involved as an intermediate between spectrin and band 3 [26, 35]. Therefore, the sulfhydryl groups involved in the couplings between spectrin and band 3 may not reside directly in the molecular binding site. On the other hand, our cross-linking data suggest a gradual change of structural proximity, which is not detected in binding studies, between spectrin and band 3 in the salt concentration (KCl or NaCl) ranging from 30 to 90 mM. It should be pointed out here that spectrin-band 3 cross-linkings have not yet been detected in intact red cells. Therefore, the conclusive association between these two proteins in situ requires further elucidation. The possibility of subtle rearrangements of membrane proteins during the ghost preparation cannot be excluded at the present time.

A concomitant increase of band 6 (G3PD) retention by the membrane was observed as the NaCl concentration decreased from 90 mM to 30 mM in the incubation mixture (Fig. 3A). Although it has previously been shown by Yu and Steck [41] that G3PD interacts with band 3 in the membrane, the elution of G3PD from membrane (eg, with NaCl (150 mM) or NADH (2 mM)) prior to cross-linking did not affect the amount of 1 + 3 complex subsequently produced (data not shown). Therefore, the binding of G3PD to band 3 seems to have no direct effect on 1 + 3 formation.

The chymotrypsin-produced 55,000-dalton fragment of band 3 that could cross-link with spectrin (Fig. 5) is probably the same segment as that shown by Steck et al [22] to extend from the extracellular space to the cytoplasmic surface. This segment of band 3 appears to contain the reactive SH groups which could form dimers and trimers as the intact band 3 does in the membrane (Fig. 1). The yield of 55k trimers appears markedly enhanced compared to band 3 trimer in intact ghosts (Fig. 1C and Fig. 6). The reason for this is not yet known. Some subtle rearrangement of 55k fragments may occur as a consequence of chymotrypsin treatment. On the other hand, the 38,000-dalton fragment of band 3 did not polymerized into homopolymers or cross-link with spectrin 1. The data are consistent with the idea that the 38,000-dalton fragment resides mainly on the outer surface and within the lipid bilayer core but does not extend to the cytoplasmic side and hence is not in contact with spectrin. However, a definite conclusion of the lack of physical contacts between spectrin and the 38,000-dalton fragment of band 3 awaits further investigation by some other means, because the absence of their cross-linkings may be due to the lack of adequate sulfhydryl groups of two adjacent polypeptide chains [36].

Our cross-linking data reveal a major difference in the behavior of the two spectrin chains at physiologic pH and isotonic conditions (ie, the preferential cross-linking of chain 1 relative to 2 with band 3). While 1 + 3 is one of the major complexes promoted by the presence of salt, the 2 + 3 apparently is not (Fig. 1). The low yield of 2 + 3 does not appear to be due to the lack of -SH groups of chain 2, since the two spectrin chains have virtually identical amino acid composition [31] and they have previously been shown to produce about the same amount of disulfide couplings with band 3 under acidic conditions (pH 4.0–5.5) [20]. Presumably, these results reflect differences in detailed structure or localization of the two chains in the membrane. Other observations of preferential depletion of monomeric chain 1 relative to chain 2 in ghosts incubated at acid pH (4.0–5.5), and the differential cross-linking in complexes (eg, 2 + 4.9, 1 + 5), are added evidence for this suggestion. However, the physiologic implication of the difference of these two chains is not yet evident. One potentially significant difference is that only spectrin 2 is phosphorylated [43–45] by a cAMP-independent protein kinase. The degree of spectrin 2 phosphorylation has recently been shown to correlate with the shape changes of isolated ghosts [46]. The observation that both 1 + 3 cross-linkings and spectrin 2 phosphorylation are affected significantly by mono- and divalent cations is of particular interest and certainly merits further investigation.

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