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# Proteolytic Degradation of Human Erythrocyte Band 3 by Membrane-Associated Protease Activity

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*Summary.* Antisera directed against the cytoplasmic portion of human erythrocyte Band 3 were used to follow the degradation of the band 3 molecule. Small amounts of Band 3 were degraded when well-washed red cell membrane ghosts were incubated in the cold; this process was greatly accelerated by incubating ghosts at 37 °C. Band 3 labeled with pyridoxal-phosphate was digested at comparable rates. Band 3 digestion also took place when alkali-extracted ghost membranes were incubated at 37° for prolonged periods. These results suggest that human erythrocytes contain tightly bound, membrane-associated proteolytic activity.

Band 3 is the predominant integral protein of the human erythrocyte membrane [15]. This protein has been studied in some detail over the past few years, largely because there is good evidence that it is associated in some way with the movement of anions across the red cell membrane [14]. It has also been established with reasonable certainty that Band 3 is a transmembrane glycoprotein [4] with parts of its polypeptide chain situated on both sides of lipid bilayer, connected by an intramembranous domain. This arrangement is similar in overall design to the transmembrane conformation proposed for glycophorin A [21], but with some significant differences. The membrane-spanning segment of glycophorin A seems to be a relatively short stretch of polypeptide composed of approximately 25 amino acids [12]; in contrast, the membrane spanning or intramembranous segment of Band 3 appears to be considerably larger. Present estimates based on sequential proteolytic digestion experiments indicate that the intramembranous segment of Band 3 may be as large

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as 17,000 daltons [17]. Band 3 also differs from glycophorin A in that its cytoplasmic segment is the largest of the three domains; its size is estimated to be close to 40,000 daltons [16]. Another distinctive feature of Band 3 is that its cytoplasmic segment contains the N-terminus of the molecule [9], in contrast to glycophorin A, whose N-terminus is located on the external segment of the polypeptide chain.

In the course of a study of the structure and functional properties of the cytoplasmic portion of Band 3 we have developed methods for the isolation of specific peptides from this end of the molecule and have generated antisera specific for certain subregions of the polypeptide chain [11]. These antisera have been characterized by appropriate absorption studies and immunoelectrophoresis and have been shown to be unreactive with other components of human red cell membranes and only weakly cross-reactive with Band 3 components of red cells from different animal species.

In the course of applying these antisera to different membrane preparations using a newly developed method to localize antigenic activity on SDS gels (as described below), we discovered that our anti-Band 3 antisera reacted with a number of smaller molecular weight components in addition to the major Band 3 fraction. The results described below indicate that the smaller molecular weight fragments represent proteolytic degradation products of Band 3 rather than contaminating cross-reacting antigens. We have also explored the factors which promote the production of these small molecular weight fragments. Our findings suggest that human erythrocyte membranes have tightly bound proteolytic activity capable of degrading the cytoplasmic segments of Band 3 molecules into discrete peptide fragments.

## Materials and Methods

### *Preparation of Erythrocyte Membranes*

Membranes were prepared from fresh human blood drawn in citrate-dextrose anticoagulant (U.S.P. formula A) according to the method of Fairbanks *et al.* [10]. The pellets of white ghosts were resuspended at 5 mg protein per ml in 5 mM phosphate buffer, pH 8.0, containing 0.02% of sodium azide and incubated at 37 °C in a shaking waterbath. At various times aliquots were taken and immediately prepared for electrophoresis.

### *Preparation of the Antibodies to Band 3*

Antiserum to purified Band 3 was raised in sheep as described previously [11]. Antibodies were purified by affinity chromatography on an immunoadsorbent prepared by

coupling two partially overlapping peptides (24,000 and 36,000 daltons, respectively) derived from the cytoplasmic segment of Band 3 to sepharose 4 B. Specific antibodies were eluted in 4 M guanidine HCl at pH 7.4 and immediately dialyzed against phosphate-buffered saline. About 4 mg of specific antibodies were recovered from 1 ml antiserum.

### *Labeling with Pyridoxal Phosphate*

Saline-washed red cells were suspended in 20 mM Hepes buffer, 135 mM NaCl, pH 7.2 (HBS) at a hematocrit of ~30%, and pyridoxal phosphate was added to final concentration of 1 mM. The cells were incubated at 37 °C for 10 min. The suspension was then chilled on ice, centrifuged at 2,500 rpm 10 min, and the red cells were washed with 30 volumes of HBS (pH 8.0), collected by centrifugation again and then resuspended in 0.67 vol HBS, pH 8.0 (hematocrit ~50%). $^{3}$H-$NaBH_4$ (79 mM, 316 mCi/mmol) was added to a final concentration of 3.9 μM, and the cells were incubated in an ice bath for 30 min. The labeled cells were washed with 30 vol HBS (pH 8.0) four times and ghost membranes prepared as described above.

### *Staph Aureus A Protein and Antibody Labeling of SDS Gels*

Slab gels prepared by the method of Fairbanks *et al.* [10] were electrophoresed and fixed overnight in 46% methanol, 8% acetic acid (Burridge Fixative). All incubation and washing procedures were performed at room temperature and with gentle shaking on a Junior Orbit Shaker (Lab-Line). The fixative was removed and the gels were washed several times over a 12–24 hr period with 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 0.05% sodium azide (Burridge Buffer) until the pH was raised to 7–7.4. The gels were then immersed in sheep antisera, diluted 1:100 in Burridge Buffer, and incubated overnight. The antiserum had been absorbed previously with type AB washed red blood cells. After overnight incubation the antibody solution was removed and stored at 4 °C for re-use. The gels were again washed with several changes of Burridge Buffer over 12–24 hr to remove all unbound antibody. $^{125}$I-Protein A from *Staphylococcus aureus* (*SPA) was incubated with the slabs overnight to allow binding of the *SPA to the *Fc* region of the sheep antibodies. Protein A (Pharmacia Fine Chemicals) was iodinated by the chloramine T method. The SPA, $Na^{125}I$ (New England Nuclear) and ~25 mM chloramine T were reacted for 1 min at room temperature with vigorous agitation, and the reaction was stopped by the addition of an excess of tyrosine. The iodinated SPA was separated from free $^{125}$I on a Sephadex G-25 column. The iodinated SPA was diluted in Burridge Buffer with Ovalbumin at 0.5 mg/ml as carrier to yield a concentration of ~25 μg/ml, $4 \times 10^6$ cpm/μg. The $^{125}$I-SPA was decanted from the gels and stored at 4 °C for re-use. The gels were washed with several changes of Burridge Buffer over 12–24 hr until the wash contained less than 100 cpm above background. The washed gels were dehydrated by immersion in dimethylsulfoxide (DMSO) for 30 min at room temperature and then suspended in fresh DMSO for an additional 30 min. The dehydrated gels were then incubated for 3 hr in PPP/DMSO (20% wt/wt), after which the PPO/DMSO was removed and the PPO within the gels precipitated by washing the gels several times over a 1-hr period with double distilled water. The gels were dried under vacuum from 2 hr to overnight and exposed for 12 hr–10 days with Kodak Royal X-Omat XR-2 film. Development of the film was done at the YNHH radiology department.

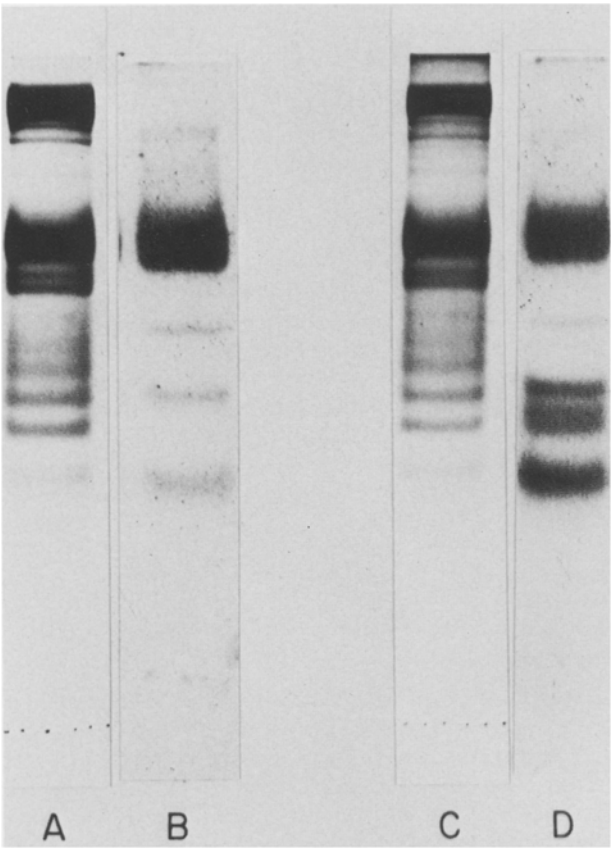


Fig. 1. SDS gels of human ghost membranes. Membranes were analyzed directly after preparation by hypotonic lysis (gels *A, B*) or after storage at 4 °C for several days (gels *C, D*). *A* and *C* were stained by Coomassie Blue and *B* and *D* were "stained" by anti-Band 3 antisera followed by $^{125}I$ staph A protein and then analyzed by autoradiography. Anti-Band 3 sera react with Band 3, as expected, but other lower mol wt components are also seen (*B*); these are present in greater amounts in ghosts after storage at 4 °C (D)

## Results

When purified anti-Band 3 antibodies are applied to appropriately prepared SDS gels of intact human erythrocyte ghost membrane, the antibody-Staph A protein complex "stains" the Band 3 region of the gel most intensely [Fig. 1], but it also invariably stains three other lower mol wt bands [Fig. 1] which have approximate mol wt 60,000, 40,000, and 24,000. No staining was observed when nonimmune sera or the radioactive protein A alone were applied to the erythrocyte membrane gel electrophoresis. The staining of these minor bands has been seen

in erythrocyte samples from at least two dozen normal donors. Furthermore, the same pattern was generated when the isotonic lysis method [2] was used to prepare ghosts; in fact, we have never encountered an erythrocyte ghost sample which did not have these lower mol wt forms. Such low mol wt bands are not found when normal serum samples are run without membranes or when membrane fractions of human neutrophils or human platelets are run under the same conditions. Thus the most obvious sources of possible contamination, cross-reactions with other blood elements or blood proteins, are not responsible for the presence of these minor bands.

The "staining" of the lower mol wt bands is increased when erythrocyte ghosts are stored in the cold for prolonged periods before the SDS gel electrophoresis step (Fig. 1*b*). This finding prompted us to study the conditions under which the small mol wt forms could be enhanced. We found that the smaller mol wt forms were invariably enhanced by simply incubating "white" human ghosts in a variety of different buffer conditions and in a wide range of pHs and salt concentrations (as described in *Methods*). Figure 2 shows the results of incubating red cell ghosts at 37° for varying periods at pH 8.0. Examination of red cell ghosts by SDS gels after 19 hr incubation at 37° suggests that there is a small amount of the Band 3 remaining, when assayed by Coomassie Blue staining [Fig. 2]; however, the antibody staining of a comparable gel [Fig. 2], indicates that there is little or no intact Band 3 remaining in these preparations. Instead, the entire Band-3 fraction has been converted to lower mol wt forms. It is also evident that most of the major polypeptide chains of the red cell ghosts have also been digested under these conditions. However, by analyzing gels of ghost membranes incubated for shorter periods of time, the antibody staining procedure shows that the Band-3 polypeptide is almost completely degraded before the bulk of the spectrin polypeptides have been attacked.

It was this initial observation which suggested to us that the proteolytic activity in these membranes may have greater specificity for the Band 3 components than the other more loosely bound membrane proteins. Thus we considered it more likely that the proteolytic activity responsible for the breakdown of the membrane proteins was derived from the membrane itself rather than from contaminating leukocytes, since proteases from the latter would be expected to attack all available proteins more or less indiscriminately. Further evidence for the specificity of the protease towards Band 3 was provided by incubating ghost membranes at 37° in the presence of EDTA. Under these conditions Band 3

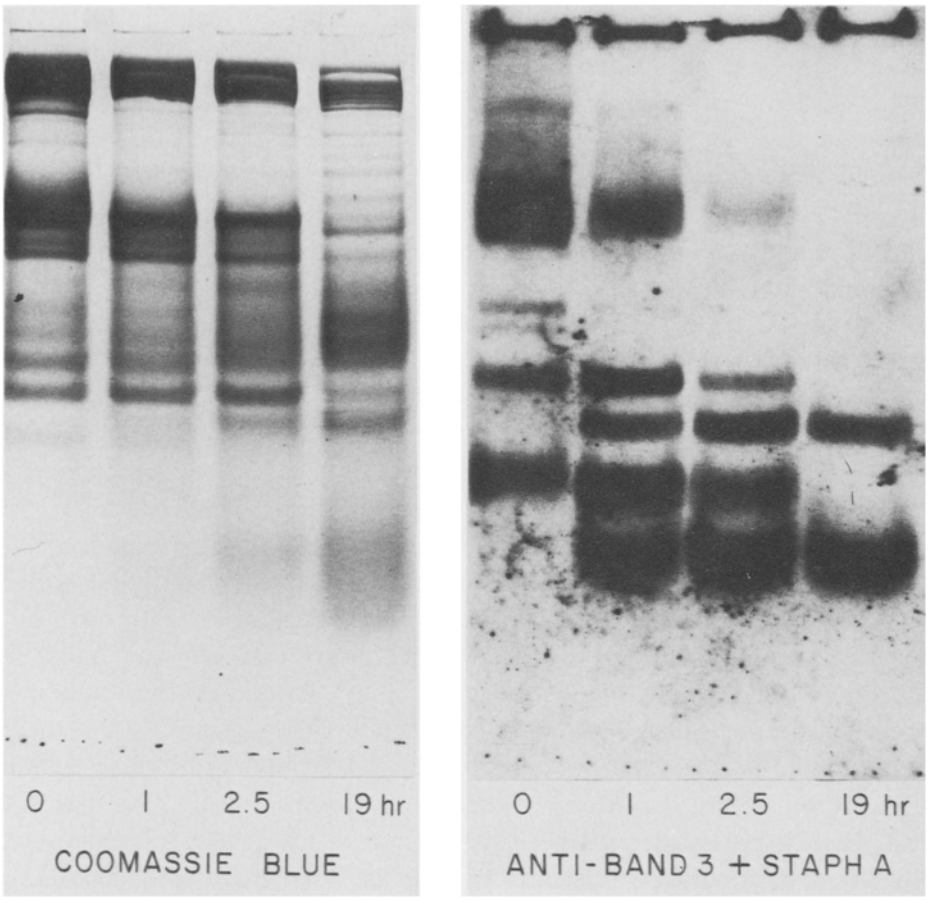


Fig. 2. Freshly prepared RBC ghosts were incubated at 37° in pH 8 buffer for the times indicated. Samples were run on SDS slab gels and stained with either Coomassie Blue or anti-Band 3 antisera followed by $^{125}$I staph A protein and autoradiography. Prolonged incubation results in the disappearance of Band 3 and the accumulation of lower mol wt peptides

was almost totally degraded after an 18-hr incubation while the spectrin polypeptides appeared to be unaffected.

The degradation of Band 3 seemed to proceed step-wise with the production first of 40,000- and 24,000-dalton peptides, then following prolonged incubation these peptides seemed to break down into smaller forms with approximate sizes of 30,000 and 12,000. The generation of such discrete peptides suggests that the proteolytic enzymes acting on Band 3 act at specific sites and are not nonspecific exopeptidases. It is also interesting that the digestion of Band 3 seems to be confined entirely to the cytoplasmic segment of this molecule. Following prolonged digestion at 37°, the proteolytic fragments of Band 3 and any other

remaining membrane proteins can be stripped from the membrane vesicles by alkali treatment [18] and the only component left in significant amounts is the polypeptide portion of Band 3 which seems, on the basis of its mobility, to represent the combined intramembranous and external pieces. It is also interesting that the external portion of glycophorin A also appears undegraded during this process since the PAS-stainable bands are not diminished or shifted in position after such incubations. These findings suggest that the proteolytic activity responsible for Band 3 degradation is confined to and probably bound to the cytoplasmic surface of the erythrocyte ghost membrane.

In order to confirm that degradation of Band 3 as analyzed by these techniques is the "anion channel protein," labeling of Band 3 was carried out with pyridoxal phosphate [6] and labeled membranes were subjected to the same digestion procedures described above. Figure 3 shows that radio-labeled Band 3 is entirely degraded under these conditions, generating a tritiated fragment of the size expected on the basis of cleavage of the Band 3 polypeptide at its cytoplasmic interface.

We have made some preliminary attempts to identify the source of the proteolytic activity responsible for Band 3 degradation. Although our bias was in favor of a membrane-associated protease, we were well aware of the many observations suggesting that serum enzymes, leukocytic and platelet enzymes, or red cell cytoplasmic proteases could bind to membranes and digest their proteins. The following observations suggest that the proteolytic activity responsible for Band 3 digestion is a tightly bound membrane-associated enzyme. To assess whether leukocytes and/or platelets contribute to this proteolytic activity, samples of red cells were taken for leukocyte and platelet counts and subjected to three different isolation procedures. Ghost membranes were prepared from: (i) crude unfractionated whole blood; (ii) red cells washed extensively with phosphate buffered saline to remove all gross contamination by leukocytes and platelets, and (iii) red cells fractionated on Ficoll gradients. Platelet and leukocyte counts were taken of each sample and showed that there was significant decrease in contaminating elements as the red cells were processed. Membranes prepared from these three samples were incubated under the same conditions as described above and SDS gels prepared. Antibody and Coomassie Blue staining showed that the Band 3 polypeptides were degraded to the same extent, regardless of the degree of platelet of leukocyte contamination. Since no evident change occurred when red cells were purified free of gross cellular elements on Ficoll gradients, attempts were made to differentially extract

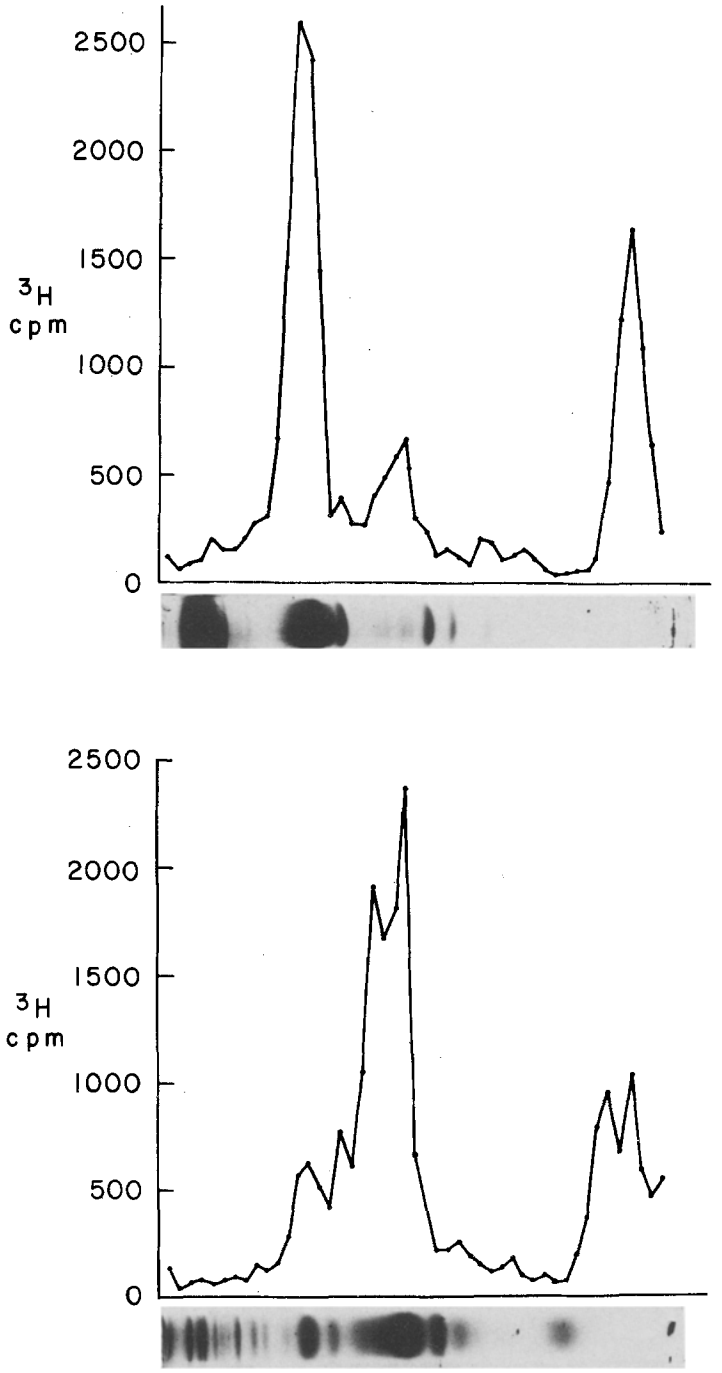


Fig. 3. SDS gels of peridoxal-phosphate labeled RBC ghost membranes before [top] and after [bottom] incubation at 37° for 18 hr. The bulk of the label is associated with Band 3 in the fresh ghosts; this shifts to lower mol wt forms as a result of prolonged incubation at 37°

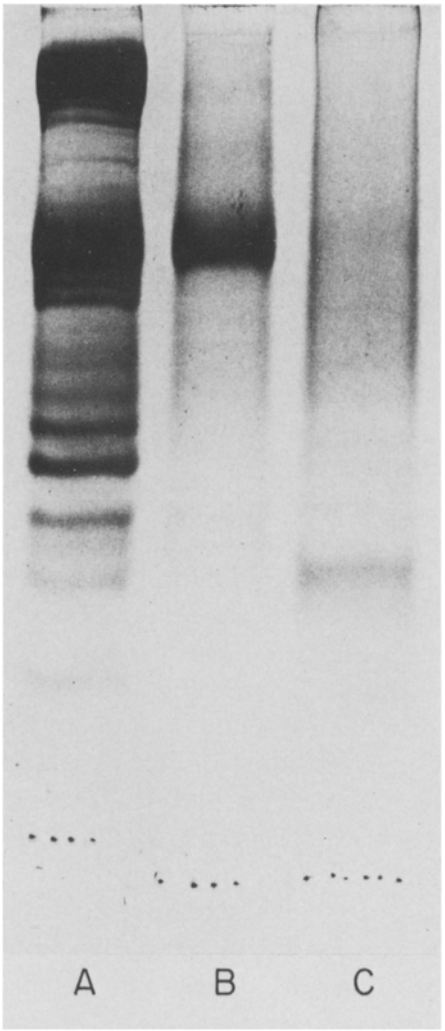


Fig. 4. SDS gels of normal red cell ghosts (*A*) and ghosts stripped of loosely bound proteins by 0.1 N sodium hydroxide treatment (*B, C*). The alkali-extracted ghosts (*B*) also convert Band 3 to lower mol wt forms after prolonged incubation at 37° (*C*)

red cell membranes with salt solutions, nonionic detergents, and strong alkali treatment. The latter has been shown by Steck and coworkers to strip red cell membranes of all their nonintegral membrane proteins [18]. An example of red cell membranes treated with 0.1 N sodium hydroxide is shown in Fig. 4. When such membrane fragments, which contain only the integral membrane proteins [Bands 3, 7, and the sialoglycopeptides] are incubated under the conditions described above, there is essentially complete degradation of the Band 3 polypeptides. These re-

sults show that the enzyme or enzymes responsible for Band 3 degradation are indeed intimately associated either with the lipids or the integral protein elements of the red cell membrane.

## Discussion

These results are most consistent with the idea that human red blood cells have a proteolytic activity which is tightly bound to their membranes. Since this activity resists solubilization by salt extractions, nonionic detergents, and even 0.1 N sodium hydroxide, this enzyme or enzymes must be bound to the membrane at least as tightly as the integral membrane proteins. Since this activity is so tightly associated with the ghost membranes, we consider it likely that this enzyme represents an endogenous red blood cell protein rather than being derived from other blood cells or the serum. Its capacity to digest Band 3 rather than spectrin in the presence of EDTA also favors this interpretation.

This membrane-associated protease appears to have relatively low specific activity since it requires hours of incubation at 37° to digest all of the Band 3 substrate. This could be due to a low intrinsic activity of the enzyme itself or to the fact that it may be present in only relatively small numbers on the membrane. The slow rate of Band 3 degradation might also be due to the fact that both the enzyme and the substrate are in relatively fixed orientations due to their tight association with the lipids of the membrane.

The proteolytic activity of the alkali-treated ghost membranes does not appear to attack glycophorin A, even after prolonged incubation. This is somewhat surprising since there is convincing evidence that glycophorin A has a polypeptide segment inside the cytoplasm of the red cell [7], and this segment is also sensitive to proteolytic cleavage when the isolated molecule is exposed to appropriate enzymes [20].

Proteolytic activity has been found by previous investigators in different red cell membrane preparations [1, 13, 19], and others have found that red cells are capable of binding exogenously added proteases [5]. Tokes and Chambers [19] have identified two apparently different proteolytic activities associated with human ghost membranes, one of which is active at alkaline pH, the same conditions used in this present study. They found this activity to be DFP-inhibitable and concluded that the enzyme is located on the external surface of the membrane. On the basis of the findings described above we feel that the proteases we have observed are tightly associated with the inner surface of the membrane.

The activity described here is not affected by incubating the membranes in EDTA or phenanthroline, but it can be inhibited by heating ghost membranes at 80 °C or by incubating them in the presence of red cell hemolysate. The latter suggests that intact red cells contain inhibiting factors which keep this proteolytic activity under control. More detailed studies of the characteristics of this protease and the properties of the cytoplasmic inhibitor activity are now in progress.

Why does the mature human erythrocyte contain proteolytic activity capable of digesting one of its most valuable membrane proteins? Such an activity could be a useful way for a cell to rid itself of defective or obsolete membrane proteins. However it seems unlikely that this activity could play much of a role in the normal workings of the mature red cell since the latter is unable to synthesize replacement parts. Such an activity could be part of a self-destruct mechanism, possibly part of a more general mechanism for removing old or damaged cells from the circulation.

The proteolytic activity demonstrated here could also be residual activity, left over from an earlier more active stage of the red cell's life. Perhaps it is part of the proteolytic activity thought to be involved in secreting newly synthesized polypeptide chains [3], some of which seem to be tightly associated with membranes.

Membrane-bound proteases may also function to convert other membrane-associated proteins to soluble forms. Recent evidence suggests that erythrocyte cytochrome $b_5$ is converted from a membrane-bound form to a soluble protein by proteolytic activity which cleaves off a 95 amino acid peptide that apparently seems to anchor the hemoprotein to the membrane [8].

It is clear that we need to know much more about the functional properties of this proteolytic activity before we can begin to explore the implications of these findings.

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