Cross-linking of Hemoglobin to the Cytoplasmic Surface of Human Erythrocyte Membranes

IDENTIFICATION OF BAND 3 AS A SITE FOR HEMOGLOBIN BINDING IN Cu²⁺-o-PHENANTHROLINE CATALYZED CROSS-LINKING*

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Mitchel Sayare and Marina Fikiet
From the Biochemistry and Biophysics Section, Biological Sciences Group, The University of Connecticut, Storrs, Connecticut 06268

The addition of hemoglobin to isolated membrane ghosts of human erythrocytes followed by catalytic oxidation with Cu²⁺-o-phenanthroline results in the covalent attachment of hemoglobin to the membrane. A decrease in the mobility of Band 3 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis corresponding to an increase in molecular weight of approximately 16,000 suggests that Band 3 is a site for the covalent attachment of hemoglobin to the membrane under these conditions. This conclusion was confirmed using two-dimensional gels in which the covalent linkage was cleaved with 2-mercaptoethanol in the second dimension. ¹⁴C-labeled hemoglobin was used to show that the new band was composed of one globin monomer per Band 3 monomer. Additionally, it was demonstrated that only the β chain of hemoglobin is covalently bound to the Band 3 protein.

Band 3 is one of the best studied of the human erythrocyte transmembrane proteins. Present at a concentration of 1.2 × 10⁶ copies/cell (1), Band 3 accounts for about 25% of the total Coomassie blue-stainable protein of the erythrocyte membrane (2). It is strongly implicated in mediating anion exchange (3-7), and has been shown to be distributed as a dimer in the membrane (8-15). During SDS-polyacrylamide gel electrophoresis, it migrates as a diffuse band with an apparent molecular weight ranging from 90,000 to 96,000 (16). Band 3 is known to bind to its cytoplasmic arm Bands 2.1 (17) and 4.2 (18), glyceraldehyde-3-P dehydrogenase (10), as well as aldolase (18) and phosphofructokinase (19).

There have been numerous reports that hemoglobin binds to the cytoplasmic surface of unsealed erythrocyte membrane ghosts (20-26), and recent quantitative data suggest that at pH 6.0 and low ionic strength, each ghost has 1.2-1.3 × 10⁶ sites for the binding of hemoglobin A (27-29). It has also been shown that glyceraldehyde-3-P dehydrogenase competes with hemoglobin for binding to the membrane as measured by fluorescence energy transfer (28, 29) and by kinetic measurements of CO binding to membrane-bound hemoglobin (30). Moreover, the binding of 4,4'-dis(iodothiocyanato)-2,2'-stilbenedisulfonate to whole erythrocytes, known to bind to Band 3 anddisable anion transport, results in a reduction in hemoglobin binding to the membrane in isolated ghosts, as measured by light scattering (29). From these observations it was inferred that Band 3 may be a binding site for hemoglobin (27, 29).

A recent report (31) that hemoglobin can be cross-linked into a very high molecular weight complex containing Band 3 has also been used to suggest that hemoglobin binds to Band 3. However, since the complex is composed of numerous membrane proteins cross-linked together, it is not clear to which specific protein hemoglobin is cross-linked. A preliminary report (32) from our laboratory using similar techniques suggested that a change in the electrophoretic mobility of Band 3 after catalytic oxidation of membranes in the presence of hemoglobin was due to the covalent attachment of the latter to Band 3.

In the experiments reported here, SH-group oxidation of unsealed ghosts in the presence of hemoglobin was used to establish whether hemoglobin is localized on the membrane such that cross-linking can occur between it and specific membrane proteins, presumably those to which it is bound. The results permit the conclusion that hemoglobin binds to Band 3 with a stoichiometry of one Band 3 monomer/hemoglobin β chain.

EXPERIMENTAL PROCEDURES

Materials

O-Phenanthroline, PhCH₃SO₂F, and EDTA were purchased from Sigma; acrylamide from Eastman; SDS and 2-mercaptoethanol from Bio-Rad; and K¹⁴CN0 from New England Nuclear.

Methods

Membrane Ghost Preparation—Venous blood was drawn from healthy volunteers into heparinized vacuum tubes and used within 2 h. Erythrocytes were isolated by centrifugation at 2000 × g for 10 min at 4 °C. The supernatant fraction and buffy coat were removed and the cells were washed three times by suspension in buffer (0.15 M NaCl, 0.02 M sodium phosphate, pH 8.0, with 10 μg/ml of PhCH₃SO₂F) followed by centrifugation, as above, at 4 °C. Unsealed ghosts were prepared by hypotonic lysis as outlined by Dodge et al. (20) and modified by Fairbanks et al. (2). The membranes were washed five times by suspension in 5 mM sodium phosphate buffer (pH 8.0), followed by centrifugation at 20,000 × g for 20 min at 4 °C. Care was taken that the cream colored "button" was completely removed from the membrane pellet at the end of the first wash. Resealed ghosts...
were prepared in an identical manner except that the lysis and wash buffers contained 1 mM MgSO$_4$ (33). The numbers and size heterogeneity of ghosts were monitored with a Coulter electronic particle counter and multichannel analyzer.

**Hemoglobin Preparation**—Human oxyhemoglobin A was prepared by the method of Williams and Tsay (34). Concentrations were determined by absorbance measurements using E$_{280}^{	ext{mm}} = 14.6$ (35). For some experiments, this hemoglobin was carboxamylated using K$^+$CN0 according to the method developed by Williams et al. (36).

The term hemoglobin ref to the Erythrocyte Membrane

such that stoichiometric binding of hemoglobin prevails (see Ref. 27), followed by catalytic oxidation with Cu$^{2+}$-o-phenanthroline, results in the disappearance of Band 3 upon gel electrophoresis and the appearance of a new band at a position corresponding to a molecular weight of 106,000 (Fig. 1, Tracks III and IV). If no hemoglobin is added to these ghosts (Fig. 1, Track II), or if hemoglobin is added to whole cells or resealed right-side-out ghosts (data not shown), the 106,000-dalton band does not appear upon oxidation. As Steck has noted (8), when membranes are cross-linked by this method in the absence of hemoglobin, the disappearance of Band 3 is accompanied by the increased appearance of a band corresponding to Band 3 dimer, as well as by larger cross-linked aggregates at the top of the gel. Inspection of Tracks II, III, and IV of Fig. 1 suggest that the amount of 106,000-dalton species formed increases as cross-linking is carried out in the presence of increasing amounts of added hemoglobin. It is interesting to note that the increase in the amount of the 106,000-dalton species formed is accompanied by a decrease in the amount of Band 3 dimer formed as a result of cross-linking. The possible significance of these observations is discussed in the last section.

Cross-linking of membrane-free solutions containing hemoglobin at concentrations used in the experiments above yield no bands other than the 16,000-dalton material at the bottom of the gel. At concentrations of hemoglobin approximately 100-fold higher however, some globin dimer (molecular weight, approximately 32,000) is seen to occur (data not shown). No higher order globin polymer is detectable. In all cases of hemoglobin and/or membrane cross-linking, the nor-
Cross-linking of Hemoglobin to the Erythrocyte Membrane

TABLE I

| Sample              | Protein pmol | Radioactivity dpm | Specific activity mol of 14C/mol of subunit |
|---------------------|--------------|-------------------|------------------------------------------|
| Hemoglobin tetramer | 186          | 21,500            | 0.99                                     |
| Isolated α-subunit  | 23.0         | 2,730             | 1.02                                     |
| Isolated β-subunit  | 23.1         | 2,660             | 0.99                                     |

14C-Hemoglobin was prepared by carbamylation with K14CNO as described under "Experimental Procedures." Protein in tetramer was determined spectrophotometrically on hemoglobin solutions, based on E280nm = 14.6 (35). 10 μg of protein was separated into subunits on SDS-PAGE using 15% acrylamide gels. The gel was scanned densitometrically, and the resulting signals integrated to yield absolute protein content. All protein concentrations are based on a subunit molecular weight of 16,000. Dpm of solution aliquots (tetramer) or solubilized gel slices (subunits) were converted to moles of subunit using the K14CNO specific activity of 53 mCi/mmol.

Concentration was constant (and sufficient to saturate the putative binding sites), while the proportion of 14C-hemoglobin to cold hemoglobin varied. As seen in Fig. 2A, and verified by densitometry (not shown), no significant difference in the amounts of protein in the 106,000-dalton species could be detected. The corresponding autoradiogram (Fig. 2B), however, shows that the amounts of radioactivity seen in this band decrease as the ratio of 14C-hemoglobin to cold hemoglobin in the incubation mixture is decreased.

In order to quantitate this relationship, the radioactive bands were carefully cut out of the stained gel, the slices were solubilized and the eluted material counted. The total label recovered from the four regions sliced out of the gel was consistently better than 90% of the total applied. The results, shown in Table II, indicate that the amount of label recovered in the 106,000-dalton band (and other bands) is closely related to the extent of dilution of the 14C-hemoglobin by unlabeled hemoglobin.

The recoveries of 14C from the top of the gel do not seem to be consistent with a simple dilution in this type of experiment. However, as can be seen by the magnitude of the standard deviation, the amount of label recovered from this region is highly variable. Repeated attempts to control the amount of cross-linked material recovered at the top of the gel by varying the incubation time and temperature met with little success. Since this problem is most likely not related to the use of labeled hemoglobin, and since the remaining bands as well as the total of all four showed recoveries of label close to the proportion of 14C used in the experiment, it is reasonable to assume that the behavior of the cold and 14C-hemoglobin is essentially identical as far as cross-linking is concerned.

The Identity of the 106,000-dalton Peptide—In order to assign an identity to the 106,000-dalton band as well as the other bands revealed on autoradiography, the reversibility of formation of the disulfide bond produced by cross-linking was exploited. A sample of ghosts cross-linked with 14C-hemoglobin as described above was run on a tube gel. The gel was placed horizontally on a slab gel, atop a layer of agarose containing 2.5% 2-mercaptoethanol (Fig. 3A). In the second dimension, the 106,000-dalton band, which is reduced by the 2-mercaptoethanol, runs with a mobility equivalent to Band 3. This position is significantly below the diagonal formed by peptides that were not affected by the catalytic oxidation. An identical two-dimensional gel was prepared for fluorography.

Cross-linking Using 14C-Hemoglobin—The molecular weight of Band 3 is generally reported to be between 90,000 and 96,000 (16). Since the globin subunit molecular weight is between 15,000 and 16,000, the molecular weight of the new 106,000-dalton peptide suggests that Band 3 is covalently bound to a single subunit of hemoglobin after cross-linking. To test this possibility, 14C-labeled hemoglobin was prepared by reacting human hemoglobin with fresh K14CNO as described under "Experimental Procedures." The distribution of label in the α and β chains of this hemoglobin was determined by quantifying the concentration of hemoglobin in solution spectrophotometrically, then solubilizing the protein in SDS and running the sample on a 15% acrylamide gel (SDS-PAGE) in the presence of 2.5% 2-mercaptoethanol. Under these conditions, the globin subunits are readily separated. The resulting Coomassie blue staining patterns were scanned densitometrically to determine the distribution of protein corresponding to the α and β subunits. The bands were then sliced out and counted as described under "Experimental Procedures." As shown in Table I, both the protein and label distributed nearly equally between the two subunits of globin, and they, as well as the original hemoglobin tetramer, contained essentially one derivative/subunit.

14C-hemoglobin was used in experiments analogous to those described in Fig. 1. As shown in Fig. 2A, Track IB, cross-linking in the presence of 14C-hemoglobin results in patterns of stained protein very similar to those with unlabeled hemoglobin (Fig. 1, Track IV). Fig. 2B, Track III shows the autoradiography patterns on x-ray film exposed to a replicate gel prepared for fluorography. Note that a large portion of the label is distributed in the region of globin monomer, while most of the remainder is superimposable with the 106,000-dalton cross-linked peptide. Small amounts of 14C are also present at the top of the gel and in the region of Band 3 dimer.

Before a description of the stoichiometry of label and protein in these regions can be made, it is essential to show that the 14C-carbamoylated hemoglobin behaves identically to unlabeled hemoglobin. The following experiment was performed to test this. Mixtures of unlabeled and 14C-hemoglobin were added to aliquots of ghosts such that the total hemoglobin concentration was constant (and sufficient to saturate the putative binding sites), while the proportion of 14C-hemoglobin to cold hemoglobin varied. As seen in Fig. 2A, and verified by densitometry (not shown), no significant difference in the amounts of protein in the 106,000-dalton species could be detected. The corresponding autoradiogram (Fig. 2B), however, shows that the amounts of radioactivity seen in this band decrease as the ratio of 14C-hemoglobin to cold hemoglobin in the incubation mixture is decreased.

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Cross-linking of Hemoglobin to the Erythrocyte Membrane

FIG. 2. Cross-linking of membrane ghosts in the presence of varying proportions of $^{14}$C-hemoglobin and cold hemoglobin. Hemoglobin was added to solutions of fresh ghosts (3.5 x $10^{10}$ ghosts/ml) to yield a final concentration of 4.6 x $10^{12}$ molecules of hemoglobin tetramer/ml. The hemoglobin was composed of various proportions of $^{14}$C- and unlabeled-protein. The ghost-hemoglobin mixtures were incubated, and cross-linking was carried out as detailed under “Experimental Procedures” and as indicated below. Membrane pellets (15 µg protein) were applied to 4.5% acrylamide gels for Coomassie blue staining and fluorography. Band nomenclature is as in Fig. 1. A, Track IA, 100% $^{14}$C-hemoglobin, no cross-linking; Track IB, 100% $^{14}$C-hemoglobin, cross-linked; Track IIA, 60% $^{14}$C-hemoglobin, no cross-linking; Track III, 60% $^{14}$C-hemoglobin, cross-linked; Track IIIB, 40% $^{14}$C-hemoglobin, no cross-linking; Track IIIC, 40% $^{14}$C-hemoglobin, cross-linked; Track IVA, 20% $^{14}$C-hemoglobin, no cross-linking; Track IVB, 20% $^{14}$C-hemoglobin, cross-linked. B, autoradiogram of an identical gel prepared for fluorography.

and the resulting autoradiogram is shown in Fig. 3B, which compares it with an autoradiogram of the same sample run only in one dimension (without 2-mercaptoethanol). Note that there are four radioactive bands at the bottom of the two-dimensional gel corresponding to the four positions seen in autoradiography of the first dimension. Globin at the top of the gel is associated with a high molecular weight complex which, upon reduction and electrophoresis in the second dimension (as shown in Fig. 3A), yields a number of peptides as well as globin. One of these peptides is Band 3, whose presence in this complex is consistent with results from other laboratories (12, 13). Its presence could very well account for the association of globin with the complex as has been reported earlier (31, 32).

It is noteworthy that nearly all of the labeled globin can be accounted for in association with Band 3 (either as Band 3 monomer, dimer or at the top of the gel), or as free globin running at a molecular weight of 16,000. The complete absence of label from regions of the gel where one would expect to recover cross-linked products of globin and Bands 1, 2, 4.1, 4.2, 5, or 6 suggests that hemoglobin cross-links rather specifically to Band 3.

Stoichiometry—In an effort to determine the stoichiometry of the association of Band 3 and hemoglobin, gels similar to those shown in Fig. 2, Track IB were scanned densitometrically to determine the protein content of each of the bands. The four bands which were shown to contain over 90% of the $^{14}$C-globin were then sliced out of replicate gels, solubilized and counted. The results of eight such experiments are shown in Table III.

The values indicate that the stoichiometry between Band 3 and globin found in the 106,000-dalton species is essentially 1:1 (globin monomer:Band 3 monomer). No such stoichiometric relationship between globin and the Band 3 dimer is evident. However, it should be noted that autoradiograms of this region (see Fig. 2B, Track IB) suggest that in addition to a major band, two minor bands of cross-linked $^{14}$C-globin migrate in the region of the gel designated and collected as Band 3 dimer. Although other peptides may be present, two-dimensional gels (see Fig. 3) reveal that only Band 3 and globin arise after reduction of the protein in this region. In contrast to autoradiography, Coomassie blue staining yields only one relatively broad band (Fig. 2A, Track IB), with no definition of discrete bands in the region of Band 3 dimer. Thus, it is difficult to assign accurate protein concentrations to the specific radioactive bands in this region of the gel. The stoichiometry reported in Table III for the Band 3 dimer simply indicates the number of moles of Band 3 dimer and the number of moles of globin associated with this entire region of the gel.

In the description of the experiments shown in Fig. 1, we pointed out the existence of an inverse relationship between the formation of the 106,000-dalton species and the formation of Band 3 dimer. This suggests that hemoglobin might compete for the same sulfhydryl group on Band 3 as is used by Band 3 in dimer formation. However, this explanation is in conflict with Figs. 2 and 3 which clearly show the presence of $^{14}$C-globin in the region of Band 3 dimer—an unexpected result if simple competition between hemoglobin cross-linking and Band 3 dimerization were the case. An alternative explanation is provided by the data of Reithmeier and Rao (39) which suggests that any one of three sulfhydryl groups on a Band 3 monomer is capable of mediating the disulfide bond resulting in covalent dimer formation. It seems likely that hemoglobin cross-links with one of the free sulfhydryl groups of the Band 3 monomer and that its presence, presumably because of steric hindrance, reduces but does not eliminate the formation of Band 3 dimer. This would account for the presence of globin cross-linked to the dimer. Of course, it is possible that hemoglobin cross-links directly to preformed dimer, but then we are left with no explanation of how the

4 Membranes cross-linked in the presence of $^{14}$C-hemoglobin that are not washed with PBS, pH 8.0, and thus retain Band 6 (glyceraldehyde 3-P-dehydrogenase), yield the same four bands on fluorography (data not shown) as obtained after PBS washing. The only difference is seen in the 16,000-dalton band, containing approximately five times more label.
Cross-linking of Hemoglobin to the Erythrocyte Membrane

Membranes were cross-linked in the presence of hemoglobin at 100% saturation of putative binding sites (see “Experimental Procedures”), and then washed with PBS, pH 8.0, to remove uncross-linked hemoglobin. The proportion of this hemoglobin that was 14C-labeled is indicated in the left column below. The resulting membrane pellet was applied to 4.5% SDS-PAGE, the gels stained, and radioactive bands sliced out and counted. The percentages represent averages of four separate experiments ± standard deviations. In the top row, numbers represent actual dpm recovered in a typical experiment in which 20 µg of protein was applied to the gel.

| % Recovery of 14C-globin relative to undiluted 14C-globin | Dpm recovered from undiluted 14C-hemoglobin |
|----------------------------------------------------------|---------------------------------------------|
| 100                                                      | 610  494  1491  3218  5813                  |
| 60                                                       | 46 ± 7  59 ± 8  59 ± 3  60 ± 3  56 ± 3       |
| 40                                                       | 44 ± 8  41 ± 4  37 ± 3  39 ± 2  37 ± 4       |
| 20                                                       | 25 ± 8  14 ± 3  11 ± 1  17 ± 1  16 ± 1       |

The experiments reported in this paper were designed to ascertain if specific proteins exposed on the cytoplasmic sur-

### Table II

| % Hemoglobin/total hemoglobin | Top Dpm  | Band 3 dimer | 106,000-dalton band | 16,000-dalton band | 16,000-dalton band | Total recovered |
|------------------------------|----------|--------------|---------------------|--------------------|--------------------|----------------|
| 100                          | 610      | 494          | 1491                | 3218               | 5813               |                |
| 60                           | 46 ± 7   | 59 ± 8       | 59 ± 3              | 60 ± 3             | 56 ± 3             |                |
| 40                           | 44 ± 8   | 41 ± 4       | 37 ± 3              | 39 ± 2             | 37 ± 4             |                |
| 20                           | 25 ± 8   | 14 ± 3       | 11 ± 1              | 17 ± 1             | 16 ± 1             |                |

presence of hemoglobin in the incubation mixture reduces the extent of dimer formation. Firmer and more detailed conclusions must await the results of experiments being carried out to determine whether or not hemoglobin cross-links to a specific sulfhydryl group of Band 3.

We noted that the sum of the radioactivity in the three major bands running above the 16,000-dalton globin monomer (top of gel, Band 3 dimer, and 106,000-dalton peptide) was consistently close to half (45.6% ± 1.7%, n = 9) of the total label recovered from the gel (see top of Table II for typical data). Consistent with this observation, 57.0% ± 1.8% (n = 9) of the label was localized in the region of the 16,000-dalton subunit of globin. This raises the possibility that only one of the two different hemoglobin subunits actually covalently binds to the membrane upon cross-linking. This possibility is also suggested by the following considerations. The concentrations of hemoglobin used in the experiments described here are well below the $K_d$ for tetramer-dimer dissociation (40), and thus it is reasonable to assume that hemoglobin is binding to the membranes as an $\alpha$-$\beta$ dimer (30). Furthermore, since both the $\alpha$ and $\beta$ chains have one relatively unreactive cysteine (41) buried in the quaternary structure of the dimer (42), it is unlikely that either of these sulfhydryl groups participates in the cross-linking seen here. The $\beta$ chain, however, has an additional cysteine ($\beta$ 93) whose side chain is accessible on the surface of the dimer (41, 42), suggesting that it may be the $\beta$ chain which is responsible for disulfide bridging to the membrane in these studies. Further support comes from the work of Marley (43) who showed that upon derivatization of the $\beta$ 93 sulfhydryl group with a bivalent reagent possessing an amino-group reacting moiety, hemoglobin was capable of being cross-linked to the membrane.

If this hypothesis is correct, it would be expected that upon SDS solubilization of the $\alpha$-$\beta$ dimer covalently bound to the membrane, only the $\beta$ chain would be found associated with the cross-linked bands on gels. The $\alpha$ chain, which is not cross-linked, would run to the 16,000-dalton subunit position. To test this possibility, experiments were carried out using 15% acrylamide gels (SDS-PAGE) which are capable of resolving the two different globin subunits. Fig. 4A, Track II, shows a 15% gel which has had a sample applied similar to that in Fig. 2. As compared to pure globin shown in the accompanying track, globin from cross-linked membranes that runs in the 16,000-dalton position on 4.5% PAGE, runs in the 15% gels as $\alpha$ chain only. No $\beta$ chain is seen in this region. If a similar sample is run in two dimensions, where the first dimension is a standard 4.5% gel and the second a 15% gel, the results shown in Fig. 4B are obtained. The globin running to the bottom of the first dimension runs in the second dimension as a chain only. On the other hand, the globin subunit that is directly cross-linked to the membranes and therefore appears at higher molecular weights in the first dimension, runs after reduction and electrophoresis in the second dimension, with a mobility indicative of $\beta$ chain. This observation provides strong evidence that the $\beta$ chain is responsible for the cross-linking seen here.

### CONCLUSIONS

The experiments reported in this paper were designed to ascertain if specific proteins exposed on the cytoplasmic sur-
Cross-linking of Hemoglobin to the Erythrocyte Membrane

Fig. 4. One and two-dimensional SDS-PAGE on 15% acrylamide gels of membrane ghosts cross-linked with ¹⁴C-hemoglobin. Conditions of cross-linking as in Fig. 2, Track IB. A. Coomassie blue staining pattern. Track I, hemoglobin alone; Track II, membranes cross-linked with hemoglobin. B. Coomassie blue staining pattern of a sample identical with that shown in Track II separated in two dimensions. Preparation of the two dimensional gel as in Fig. 3 except that the second dimension was 15% acrylamide. The orientation of the first dimension is right to left, and the second dimension, top to bottom.

TABLE III

Stoichiometry of Band 3 and hemoglobin upon cross-linking

Membranes (3.5 x 10⁷/ml) were cross-linked with ¹⁴C-hemoglobin at a concentration (4.6 x 10⁴ mol) molecules tetramer/ml sufficient to saturate the putative binding sites. Membrane pellets (20 µg of membrane protein) were applied to 4.5% SDS-PAGE, and the gels were stained and scanned densitometrically to determine protein concentrations in selected bands. The indicated concentrations are based on a Band 3 monomer molecular weight of 90,000. The radioactive bands, as determined by fluorography, were sliced out of the gels, solubilized, and counted. Dpm were converted to picomoles of 16,000-dalton subunit based on the specific activity of ¹⁴C-hemoglobin (see Table I). The calculated protein values in each band were reduced by the amount contributed by globin, based on radioactivity recovered from the gel slice. The numbers given are averages of eight different experiments ± standard deviation.

| Gel Slice       | Protein    | ¹⁴C-globin |
|-----------------|------------|------------|
| 106,000-dalton Peptide | 13.0 ± 0.3 | 12.9 ± 0.7 |
| Band 3 dimer     | 11.3 ± 0.2 | 4.4 ± 0.8  |

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Cross-linking of Hemoglobin to the Erythrocyte Membrane

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