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Registry No. Chol, 57-88-5; NBD-Cl, 10199-89-0; palmitic acid, 57-10-3.

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Hemichrome Binding to Band 3: Nucleation of Heinz Bodies on the Erythrocyte Membrane†

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ABSTRACT: Hemichromes, the precursors of red cell Heinz bodies, were prepared by treatment of native hemoglobin with phenylhydrazine, and their interaction with the cytoplasmic surface of the human erythrocyte membrane was studied. Binding of hemichromes to leaky red cell ghosts was found to be biphasic, exhibiting both high-affinity and low-affinity sites. The high-affinity sites were shown to be located on the cytoplasmic domain of band 3, since (i) glyceraldehyde-3-phosphate dehydrogenase, a known ligand of band 3, competes with the hemichromes for their binding sites, (ii) removal of the cytoplasmic domain of band 3 by proteolytic cleavage causes loss of the high-affinity sites, and (iii) the isolated cytoplasmic domain of band 3 interacts tightly with hemichromes, rapidly forming a pH-dependent, water-insoluble copolymer upon mixing in aqueous solution. Since the copolymer of hemichromes with the cytoplasmic domain of band 3 was readily isolatable, a partial characterization of its properties was conducted. The copolymer was shown to be of defined stoichiometry, containing ~2.5 hemichrome tetramers (or ~5 hemichrome dimers) per band 3 dimer, regardless of the ratio of hemichrome:band 3 in the initial reaction solution. The copolymer was found to be of macroscopic dimensions, generating particles which could be easily visualized without use of a microscope. The coprecipitation was also highly selective for hemichromes, since, in mixed solutions with native hemoglobin, only hemichrome was observed in the isolated pellet. Furthermore, no precipitate was ever observed upon mixing the cytoplasmic domain of band 3 with oxyhemoglobin, deoxyhemoglobin, (carbonmonoxy)hemoglobin, or methemoglobin. The affinity of the cytoplasmic domain of band 3 was likely much higher for hemichromes than for native hemoglobin, since a 20-fold molar excess of hemoglobin was required to reduce copolymerization by 50%. We suggest that the copolymerization of band 3 and hemichromes in vivo can explain the aggregation of Heinz bodies on the erythrocyte membrane and the resulting hemolysis observed in numerous hemoglobinopathies.

The interaction of native hemoglobin (Hb) with the human erythrocyte membrane has recently received considerable attention. Two classes of binding sites have been identified, and one of these, the higher affinity class, has been located on the cytoplasmic domain of the major erythrocyte protein, band 3 (Shaklai et al., 1977a,b; Salhany et al., 1980; Sayare & Fikiet, 1981). This interaction has been shown to be electrostatic in nature with the affinity of Hb for the membrane increasing as pH and ionic strength decrease (Shaklai et al., 1977a,b; Fung, 1981; Fischer et al., 1975). A similar pH dependence of Hb binding to the membrane has also been reported for intact cells (Eisinger et al., 1982). The isolated cytoplasmic domain of band 3 has also been shown to bind two molecules of hemoglobin, supporting the identification of band 3 as a major membrane attachment site for soluble

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hemoglobin (Cassoly, 1983). Recently, the electron density map of a cocrystal of deoxyhemoglobin and an acidic N-terminal fragment of the cytoplasmic domain of band 3 has been obtained, and the derived three-dimensional structure demonstrates that the band 3 peptide inserts into the 2,3-diphosphoglycerate binding cavity of Hb (Walder et al., 1984).

Denatured hemoglobins also bind tightly to the erythrocyte membrane, often forming dense aggregates on the membrane surface termed Heinz bodies. These patches of denatured hemoglobin are frequently observed in aged cells (Sears et al., 1975), in erythrocytes from individuals with glucose-6-phosphate dehydrogenase deficiency disease and other maladies involving loss of intracellular reducing power (Jandl et al., 1960), and in cells containing unstable hemoglobins (Jacob & Winterhalter, 1972; Winterbourn & Carrell, 1974). The Heinz bodies are believed to be responsible for the hemolysis which commonly accompanies the anemias associated with the latter two diseases. Unfortunately, no studies have been conducted to determine the site or mode of interaction of these modified hemoglobins with the membrane.

The pathway of hemoglobin denaturation is not well characterized but appears to be similar in the situations examined to date (Peisach et al., 1975). Initially, the native Hb is oxidized to methemoglobin. Minor structural rearrangements can then lead to formation of a reversible hemichrome, which under appropriate conditions can revert back to methemoglobin. Otherwise, the reversible hemichromes denature further to form irreversible hemichromes, which eventually aggregate to form the Heinz bodies.

Hemichromes[1] can be studied by artificially inducing hemoglobin denaturation with oxidant-generating drugs such as phenylhydrazine and its derivatives (Itano et al., 1977; French et al., 1978). Good evidence indicates that whether natural or drug induced, Heinz body formation occurs by a similar mechanism (Peisach et al., 1975). Since hemoglobin binds to band 3, we reasoned that band 3 may provide a binding site for hemichromes also. Our results indicate that a tight association between band 3 and phenylhydrazine-treated hemoglobin does indeed exist, both on the membrane and between the isolated components in solution. Furthermore, the interaction tends to propagate, leading to the formation of a copolymer between the two components. We suggest that this unusual tendency of band 3 and hemichromes to copolymerize may be important in the formation of Heinz bodies and in the accompanying distortion of the membrane leading to hemolysis.

Experimental Procedures

Materials

Fresh human blood was obtained from the Central Indiana Regional Blood Bank with citrate/dextrose solution added and was used before it became officially outdated. Myoglobin, ovalbumin, bovine serum albumin, and Triton X-100 were obtained from Sigma Chemical Co. Trypsinogen was from Worthington, glyceraldehyde-3-phosphate dehydrogenase from Calbiochem, and phenylhydrazine from MCB.

Methods

Purification of Hemoglobin and the Cytoplasmic Domain of Band 3. Erythrocyte membranes were prepared by hypotonic lysis of washed erythrocytes in 5 mM sodium phosphate, pH 7.4, as described by (Dodge et al., 1963). The dimeric 90K water-soluble fragment of band 3 was prepared by mild chymotryptic digestion of the erythrocyte membranes as described (Appell & Low, 1981). This procedure yields a major fragment of M_r ~43K and a minor fragment of ~39K which derives from further cleavage of the major fragment (Appell & Low, 1981; Bennett & Stenbuck, 1980). Hemoglobin was obtained and purified essentially as described in Salhany et al. (1972).

Preparation of Hemichromes. Freshly isolated hemoglobin was dialyzed overnight against 10 mM sodium phosphate, pH 7.5, and adjusted to a concentration of 3.5×10^{-4} M tetramer with the same buffer. To this solution (typically 5 mL) was added an equal volume of 10 mM sodium phosphate, pH 7.5, containing 30 mM phenylhydrazine. The resulting brown solution was incubated at 37 °C for 90 min with occasional mixing. The precipitated protein, which began forming soon after the start of the incubation, was removed by a brief centrifugation, and the reddish brown supernatant was immediately passed down a Sephadex G-25 column to remove the phenylhydrazine. The most concentrated fractions were pooled and used for subsequent studies. This phenylhydrazine-treated hemoglobin (hemichromes) was always used within 48 h of preparation.

Preparation of Membranes for Binding Studies. Erythrocyte membranes, prepared by hypotonic lysis of washed erythrocytes, were washed thoroughly with 5 mM sodium phosphate, pH 8.0, until white. Residual hemoglobin and glycolytic enzymes were removed by washing the membranes with 20 volumes of 10 mM sodium phosphate, pH 7.5, containing 0.5 M NaCl for 30 min at 4 °C (McDaniel et al., 1974). The membranes were isolated by centrifugation, washed with 10 mM sodium phosphate, pH 7.5, and then equilibrated with 10 mM sodium phosphate, pH 6.0.

Coprecipitate Formation. The addition of the cytoplasmic fragment of band 3 to a solution of hemichromes under appropriate conditions (see figure legends) resulted in the immediate formation of a turbid solution. The amount of hemichrome–band 3 complex formed was commonly quantitated by measuring the turbidity of the samples at 700 nm in black-side-walled cuvettes. Alternatively, the copolymer was pelleted by centrifugation at 5000*g*, washed once to remove any entrapped protein, and assayed by the method of Lowry et al. (1951) to determine its protein content.

Analytical Procedures. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed according to the method of Fairbanks et al. (1971) using 5.5% slab gels containing 0.2% sodium dodecyl sulfate. The Coomassie blue stained gels were scanned on an E-C Apparatus Corp. densitometer equipped for slab gels. Absorption spectra and turbidity measurements were obtained on a Cary 15 recording spectrophotometer. Protein concentrations were determined according to Lowry et al. (1951).

Results

Characterization of the Hemichromes. Acetylphenylhydrazine and phenylhydrazine are two reagents commonly used to induce Heinz body formation in red cells, and the products obtained upon acetylphenylhydrazine reaction with oxyhemoglobin have been well characterized by Peisach et al. (1975). After extended incubation of oxyhemoglobin with acetylphenylhydrazine, the predominant products are low-spin ferric hemoglobin derivatives termed ferrihemochromes. These compounds form when sufficient structural alteration occurs in the heme pocket to allow occupation of the sixth coordination position of the heme by a ligand endogenous to the protein such as the imidazole of the distal histidine. When

[1] The term hemichrome is used in this paper to connote a group of partially denatured, low-spin ferric hemoglobins termed by (Peisach et al., 1975) ferrihemochromes.

we added phenylhydrazine to a solution of oxyhemoglobin, an immediate color change from red to brown occurred. Further incubation at 37 °C resulted in complete denaturation of part of the hemoglobin, and this was removed by centrifugation and discarded. The resulting clear, reddish brown supernatant, after passage down a Sephadex G-25 column, contained soluble protein which had an absorption spectrum identical with that of ferrihemochrome (Peisach et al., 1975; see below).

In order to determine the oligomeric structure of the isolated hemichromes, the hemichromes were chromatographed on a Sephadex G-100 column which was equilibrated and eluted with 10 mM sodium phosphate, pH 7.5. Two distinct species were observed in the elution profile, and these species occurred in approximately equal amounts. The slower eluting species migrated with an apparent molecular weight of 32 400 on the calibrated column, i.e., the value expected for the $\alpha\beta$ dimer of hemoglobin. The more rapidly eluting species had an apparent molecular weight of 46 700. This component eluted in essentially the same fraction as native oxyhemoglobin when the latter was chromatographed at the same protein concentration. This anomalous behavior of oxyhemoglobin on gel filtration, even at high concentrations, was documented as early as 1964 by Andrews (1964) and was attributed to the reversible equilibrium between $\alpha\beta$ dimers and $\alpha_2\beta_2$ tetramers. The slower eluting species, on the other hand, exhibits the behavior of an irreversible dimer. Since both types of hemichromes interacted similarly with the cytoplasmic domain of band 3, no attempt was made to study them separately.

Hemichrome Binding to Band 3 on the Erythrocyte Membrane. In order to evaluate the affinity of hemichromes for the erythrocyte membrane, leaky ghosts were incubated with the modified hemoglobins, and after centrifugation, the concentration of unbound hemichromes in the supernatant was determined spectrophotometrically (see figure legend). A Scatchard plot of the binding data is shown in Figure 1 (upper curve), and at least two classes of binding sites are evident. Due to difficulty in obtaining highly accurate data at low hemichrome concentrations, a detailed quantitative analysis of the binding data of Figure 1 was not attempted. However, the higher affinity interaction appears to involve band 3, since excess glyceraldehyde-3-phosphate dehydrogenase eliminates this interaction with little effect on the low-affinity sites (lower curve of Figure 1). Since this enzyme is known to bind to band 3 (Yu & Steck, 1975; Tsai et al., 1982), its competition with hemichromes also suggests that the high-affinity hemichrome sites are located on band 3. Other studies have shown that this high-affinity interaction is also removed as the cytoplasmic domain of band 3 is cleaved from the membrane with chymotrypsin (not shown).

Coprecipitate Formation between Hemichromes and the Cytoplasmic Domain of Band 3. In order to study the band 3-hemichrome interaction further, the dimeric 90 000-dalton cytoplasmic domain of band 3 was proteolytically cleaved from the membrane and isolated as described previously (Appell & Low, 1981). Curiously, addition of this water-soluble fragment of band 3 to a solution of hemichromes resulted in the immediate appearance of turbidity. This precipitation reaction was found to be maximal at low pH and low ionic strength and to decrease gradually as either solution variable was raised above its physiological value. The precipitation was also observed to be largely reversible, in that cycling of the pH between pH 6 and pH 8 led to repeated appearance and disappearance of turbidity. However, if the two components were allowed to remain associated for an extended period, their interaction became increasingly irreversible.

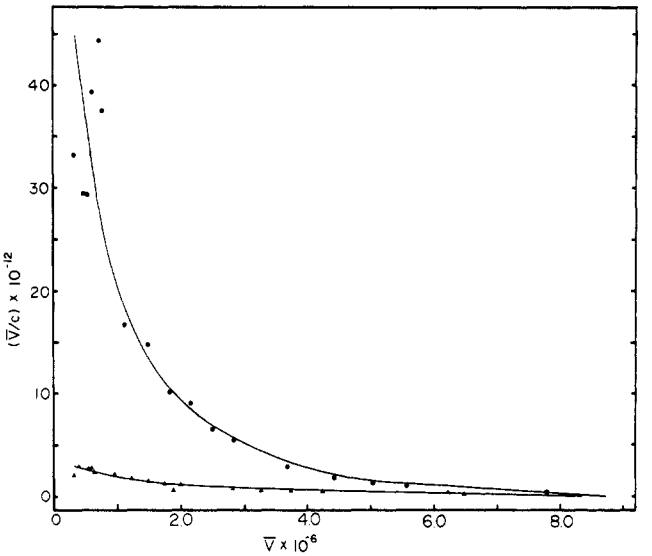

FIGURE 1: Scatchard plot of the binding of hemichromes to the erythrocyte membrane in the presence (▲) and absence (●) of glyceraldehyde-3-phosphate dehydrogenase. Membranes, prepared as under Methods, were mixed with different concentrations of hemichromes, incubated on ice for 60 min, and then pelleted by centrifugation. The concentration of hemichromes in the supernatant was determined by measuring protein absorbance at 410 nm. The amount of bound hemichrome was then calculated from the difference between the total amount added and the amount in the supernatant. The final ghost protein concentration was 0.82 mg/mL in all cases, and glyceraldehyde-3-phosphate dehydrogenase was present, when desired, at a final concentration of 3.9×10^{-6} M. In the competition study this enzyme was incubated with the ghosts 15 min before the addition of the hemichromes. The number of hemichrome molecules bound per ghost, $\bar{\nu}$, was calculated assuming a hemichrome molecular weight of 32 250. The molar concentration of hemichromes not bound to the membrane is denoted by c. The number of ghosts was determined by using a value of 5.13×10^{-10} mg of protein per ghost (Lepke et al., 1976).

The stoichiometry of the hemichrome–band 3 copellet was evaluated by mixing different amounts of the two components in solution and examining the molar ratio of band 3 to hemichrome in the resulting precipitates. This molar ratio was estimated from the staining intensities of the two proteins in polyacrylamide gels after electrophoresis of the pelletable material in the presence of sodium dodecyl sulfate. In the experiment shown in Figure 2, equal amounts of the 90 000-dalton dimeric fragment of band 3 were added to two different concentrations of hemichromes, one sample (lanes D and F) containing a 4-fold higher concentration than the other (lanes E and G). Even though there was noticeably more precipitate formed in the solution containing the higher hemichrome concentration, the relative amounts of the modified hemoglobin and the cytoplasmic fragment of band 3 present in the pellets were the same (lanes D and E of Figure 2). Thus, integration of the peak areas in lanes D and E yielded a ratio of ~5 hemoglobin monomers per band 3 monomer. Importantly, these protein ratios represent, within the accuracy of the measurements, true values and not simply differences in Coomassie blue staining intensity, since equal weights of hemichrome and band 3 fragment (lanes B and C, Figure 2) gave identical integrated peak areas. Thus, it would appear that the copolymer of band 3 and ferrihemochrome forms with a defined stoichiometry, suggesting an association driven by molecular specificity. In other words, the precipitation event is not simply a mass aggregation of denatured protein, where the ratio of protein in the precipitate is dependent upon their ratio in solution, but more likely a copolymerization of tightly interacting, multivalent proteins.

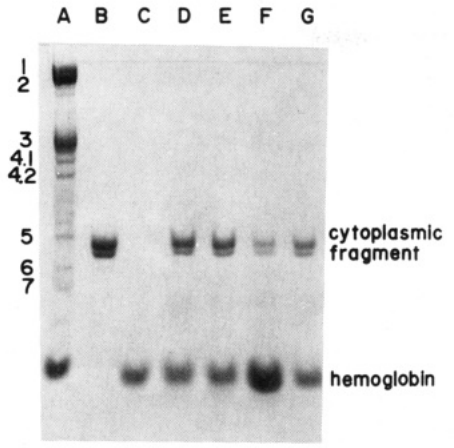

FIGURE 2: Determination of the hemichrome–band 3 stoichiometry in the insoluble coprecipitate. The cytoplasmic fragment of band 3 in 10 mM sodium phosphate, pH 6.0, was added to each of two hemichrome solutions in the same buffer which differed 4-fold in hemichrome concentration. After a 15-min incubation the precipitate in each reaction mixture was isolated by centrifugation, washed with buffer, and solubilized in preparation for electrophoresis. Reaction mixture one contained 4.0 mg/mL hemichrome and 0.53 mg/mL cytoplasmic fragment while mixture two contained 1.0 mg/mL hemichrome and 0.53 mg/mL cytoplasmic fragment. The gel profiles shown contain erythrocyte membranes (A), 15 μg of cytoplasmic fragment of band 3 (B), 15 μg of hemichromes (C), precipitate from reaction mixture one (D), precipitate from reaction mixture two (E), supernatant from mixture one after centrifugation (F), and supernatant from mixture two after centrifugation (G). All samples were treated with 2.5% β-mercaptoethanol prior to electrophoresis. Both bands in lane B derive from the cytoplasmic domain of band 3, the smaller fragment being a proteolysis product of the larger fragment (Bennett & Stenbuck, 1980).

Specificity in Copolymer Formation. In order to investigate the specificity of the copolymerization reaction, ferrihemochrome and band 3 were allowed to react in the presence of excess oxyhemoglobin, and the relative concentrations of hemichrome and native hemoglobin in the pellet were examined. Figure 3 shows the results when oxyhemoglobin and hemichrome were mixed at a concentration ratio of 10:1 followed by addition of the cytoplasmic domain of band 3 to initiate polymerization. After the precipitation was washed in the reaction buffer, the copolymer was dissolved in a solubilizing buffer, and its spectrum was compared with that of pure hemichrome and native hemoglobin. The spectra demonstrate that the hemoglobin component of the dissolved copolymer (Figure 3B) is distinct from oxyhemoglobin (Figure 3C) and closely resembles pure hemichrome (Figure 3A). In fact, the more abundant native hemoglobin appears to be essentially absent from the coprecipitate. Significantly, oxyhemoglobin is not unique in this inability to copolymerize with band 3, since deoxyhemoglobin, (carbomonoxy)hemoglobin, and methemoglobin also formed no precipitate upon addition of the cytoplasmic domain. To demonstrate that the marked specificity of band 3 for hemichromes over native hemoglobin is not an artifact of band 3 proteolysis, a similar competition experiment was conducted with leaky erythrocyte ghosts. Red cell membranes were depleted of both hemoglobin and glycolytic enzymes and were mixed with a solution of hemoglobin and hemichromes at a 20:1 weight ratio of the two proteins. After incubation, the membranes and supernatant were separated, and their absorption spectra were measured. As shown in Figure 4, the absorption spectrum of the supernatant shows no evidence of residual hemichromes, and the absorption spectrum of the pellet appears largely uncontaminated by native hemoglobin. Thus, studies on both the isolated band 3 fragment and on intact band 3 in situ demonstrate a strong

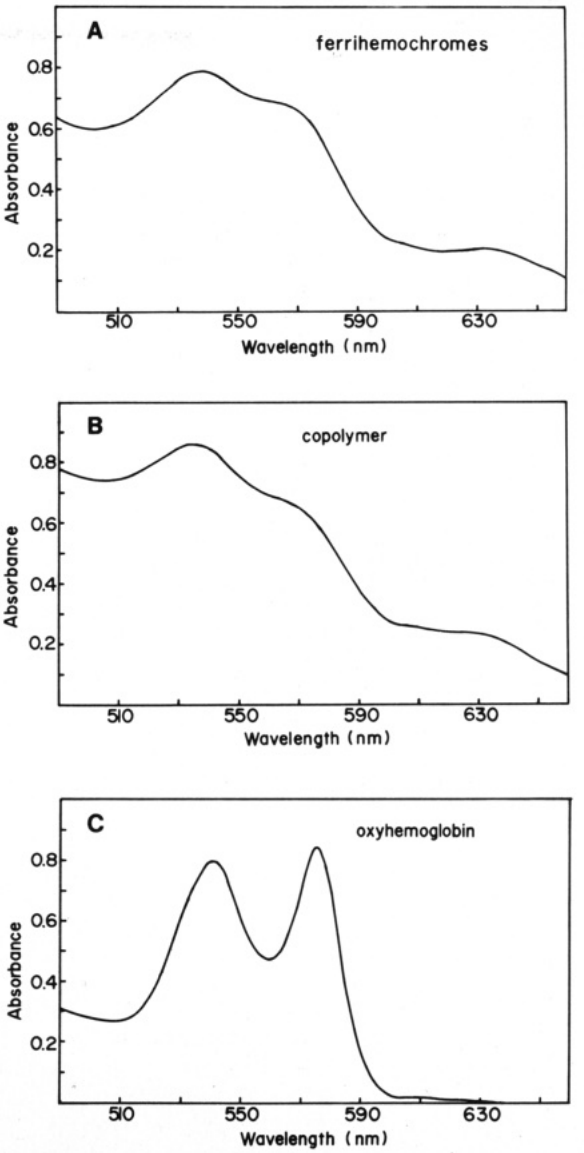

FIGURE 3: Specificity of the reaction between soluble hemichromes and the cytoplasmic fragment of band 3. (A) The absorption spectrum of hemichromes (1.38 mg/mL) in 10 mM sodium phosphate buffer and 0.5% Triton X-100, pH 7.5. For the absorption spectrum in (B) the band 3 cytoplasmic fragment was added to a solution of hemichromes and oxyhemoglobin giving final concentrations in the mixture of 0.57 mg/mL fragment, 1.72 mg/mL hemichromes, and 17.5 mg/mL oxyhemoglobin. All proteins were in 10 mM sodium phosphate buffer, pH 6.0. After a 15-min incubation the resulting precipitate was isolated by centrifugation, washed with buffer, and dissolved in a minimal amount of 10 mM sodium phosphate buffer and 0.5% Triton X-100, pH 7.5, and the absorption spectrum was recorded. (C) The absorption spectrum of 1.4×10^{-5} M oxyhemoglobin in 10 mM sodium phosphate and 0.5% Triton X-100, pH 7.5.

preference of band 3 for denatured over native hemoglobin.

A final, perhaps more quantitative evaluation of the relative affinities of hemichromes and native hemoglobin for the cytoplasmic domain of band 3 was obtained by examining the ability of native hemoglobin to block band 3–hemichrome copolymer formation. The results of this experiment are shown in Figure 5, where the turbidity of a band 3–hemichrome suspension was measured in the presence of increasing concentrations of either oxy- or deoxyhemoglobin. The results demonstrate that a 20-fold excess of oxyhemoglobin over the denatured hemoglobin is required to reduce the turbidity by 50%. Under deoxygenated conditions, the added hemoglobin had an even lower ability to block coprecipitate formation and

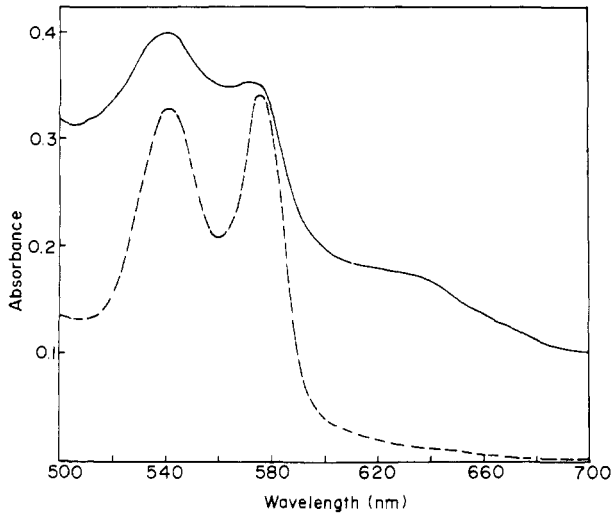

FIGURE 4: Ability of oxyhemoglobin to block hemichrome binding to the red cell membrane. All proteins and membranes were in 10 mM sodium phosphate, pH 6.0. Red cell membranes (1.0 mg/mL) were mixed with a solution containing hemichromes and oxyhemoglobin at final concentrations of 1.0 and 20.0 mg/mL, respectively. This mixture was allowed to incubate on ice for 90 min. The membranes were then collected by centrifugation and washed 4 times with 15 volumes of 10 mM sodium phosphate, pH 6.0, until the supernatant was visibly clear of protein. The membranes were solubilized in approximately 2 volumes of 20 mM sodium phosphate and 125 mM sodium chloride, pH 7.5, containing 1.5% Triton X-100 and the absorption spectrum recorded (solid line). The instrument was zeroed for this spectrum by using a sample of Triton X-100 solubilized ghosts containing no bound hemichromes or hemoglobin. The broken line is the absorption spectrum of a 40-fold dilution into Triton X-100 buffer of the supernatant from the original incubation mixture.

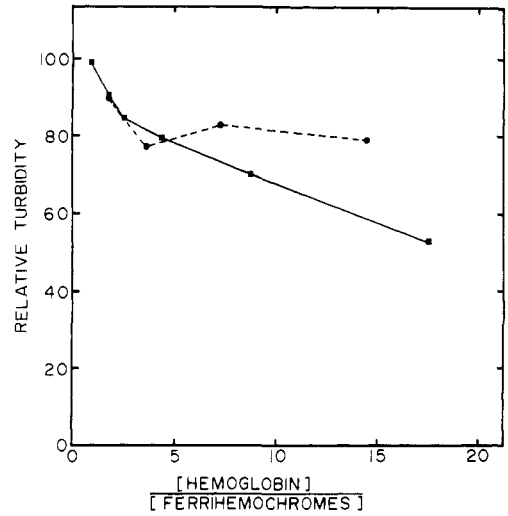

FIGURE 5: Comparison of the ability of oxyhemoglobin and deoxyhemoglobin to block formation of the coprecipitate of hemichromes and the cytoplasmic domain of band 3. All proteins were in 5 mM sodium citrate and 5 mM sodium phosphate, pH 6.0. Hemichromes (final concentration 2.35 mg/mL) were mixed with various concentrations of either oxyhemoglobin (■) or deoxyhemoglobin (●), and copolymerization was initiated by addition of 0.31 mg/mL band 3 (final concentration). After incubation on ice for 60 min the turbidity of each suspension was measured at 700 nm as described under Methods. Because hemoglobin absorption at high hemoglobin concentrations contributed to the apparent turbidity at 700 nm, the suspensions were invariably pelleted, and the absorbance at 700 nm of the clear supernatant was measured and subtracted from the apparent turbidity to yield the corrected turbidity. This corrected turbidity is plotted as a function of the weight ratio of hemoglobin to ferrihemochromes in the figure. For the reaction with deoxyhemoglobin, oxyhemoglobin and hemichromes were mixed in a septum-covered tube penetrated by a syringe needle. The suspension was repeatedly degassed under vacuum and reequilibrated with N_2. After deoxygenating the cytoplasmic domain of band 3 similarly, it was added through the syringe needle to the hemichrome–deoxyhemoglobin mixture. This suspension was allowed to incubate as described above, transferred through a septum to a N_2-flushed cuvette, and measured for turbidity. Analysis of the degassed hemoglobin solution by the method of Benesch et al. (1973) indicated that greater than 90% of the hemoglobin was in the deoxygenated form.

did not show the clear downward trend seen for oxyhemoglobin (Figure 5).

DISCUSSION

Heinz bodies are aggregates of denatured hemoglobin that bind to the inner surface of the erythrocyte membrane. They occur most frequently in red cells carrying genetically unstable hemoglobins including variants such as HbS or HbC (Schneider et al., 1972). Heinz body formation in these cells can be induced by subjecting the cells to oxidative stresses. These stresses may arise as part of the body's normal defense mechanism against infection and disease (Winterbourn et al., 1981), and they are also commonly found in cells deficient in enzymes necessary for maintaining intracellular reducing power (e.g., glucose-6-phosphate dehydrogenase and glutathione peroxidase) (Hopkins & Tudhope, 1974). In addition, the presence of Heinz bodies in cells of postspleenectomy patients suggests that these precipitates may have a function in senescent red cell removal and degradation (Jandl et al., 1960). Hemoglobin precipitation, rather than secondary oxidation events (i.e., lipid peroxidation), is believed to be the major cause of cell fragility which accompanies Heinz body formation (Bates & Winterbourn, 1984).

Our results demonstrate that a high-affinity binding site for hemichromes exists at the N-terminus of the cytoplasmic domain of the major erythrocyte transmembrane protein, band 3. Thus, band 3 on the membrane and in solution greatly prefers hemichromes to native hemoglobin, forming an insoluble complex with the hemichromes. While our study was conducted at low pH, ionic strength, and protein concentration, we have observed that the complex is still stable near physiological pH if the hemichrome concentration is also raised.[2] This fact, coupled with the known existence of membrane-bound hemichromes in vivo, suggests that the above reported hemichrome–band 3 interaction is physiologically relevant.

Probably the most unique property of the band 3–hemichrome interaction is its ability to propagate beyond the stoichiometric unit complex. The oligomeric, i.e., multivalent, nature of both band 3 and hemichromes provides a reasonable mechanism for the polymerization process to occur. Thus, the cytoplasmic domain of band 3 is a tight dimer under all native solution conditions (Appell & Low, 1981; Low et al., 1984), and the hemichromes are apparently a mixture of dimers and tetramers. While several structural models of the copolymer are possible, any acceptable model must clearly provide for the ability of hemichrome dimers/tetramers to cross-link or bridge between band 3 dimers. We believe this single property of the band 3–hemichrome complex is highly significant, since it can provide an explanation for several unusual characteristics of cells containing unstable or denatured hemoglobins. For example, cells containing denatured hemoglobins (e.g., aged cells and cells containing unstable hemoglobins) are generally found to be less deformable than normal erythrocytes (Lubin & Desforges, 1972; Rice-Evans & Dunn, 1982; Rifkind, 1965). Since the lipid matrix has little or no capacity to influence gross cell rigidity, this abnormal behavior must be due at least in part to a less flexible proteinaceous reticulum supporting the lipid bilayer. It is conceivable that cross bridges of denatured hemoglobin between adjacent band 3 molecules could provide

[2] Waugh and Low, unpublished results.

the additional, unwanted rigidity in these membranes. Since red cell flexibility/morphology is largely stabilized by $\sim 10^5$ bridges between band 3 and the cytoskeleton (Hargreaves et al., 1980; Bennett & Stenbuck, 1979), it is possible that only a small fraction of the $\sim 10^6$ copies of band 3 per cell must be cross-linked by denatured hemoglobin to modify erythrocyte flexibility/morphology.

The cross-linking of band 3 by hemichromes can also account for the enhanced fragility of the membrane commonly observed in cells containing elevated levels of denatured hemoglobin. Thus, cells containing denatured hemoglobin frequently undergo isotonic hemolysis in vivo. In other cases, where isotonic hemolysis is not observed, the cells are found to experience hypotonic hemolysis at weaker osmotic stresses than normal cells (Goldberg & Stern, 1977). While oxidative damage remains a possible cause of this hemolysis (Hebbel et al., 1982), the lateral tethering and aggregation of band 3 molecules by hemichromes could also generate the mechanical strain in the membrane which leads to membrane weakening and ultimately to the hemolytic anemia.

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