Partial Characterization of the Copolymerization Reaction of Erythrocyte Membrane Band 3 with Hemichromes†

Stephen M. Waugh,‡ Joseph A. Walder,§,‖ and Philip S. Low*,‡

Department of Chemistry, Purdue University, West Lafayette, Indiana 47907, and Department of Biochemistry, University of Iowa, Iowa City, Iowa 52242

Received September 4, 1986; Revised Manuscript Received November 10, 1986

ABSTRACT: Early intermediates in the denaturation of hemoglobin, termed hemichromes, have been found previously to associate with the cytoplasmic domain of erythrocyte membrane band 3 in a manner which rapidly propagates into an insoluble, macroscopic copolymer. Because this interaction is thought to force a redistribution of band 3 in situ, the properties of the copolymerization reaction were investigated in greater detail. The band 3–hemichrome coaggregate was found to be stabilized largely by ionic interactions since elevation of either ionic strength or pH led to dissolution of the complex. The pH dependence, however, shifted to a more alkaline pH with increasing hemichrome concentration, suggesting a strong linkage between band 3 or hemichrome protonation and copolymer formation. The stoichiometry of the copolymer was measured at five globin chains per band 3 chain whenever underivatized dimer–tetramer hemichrome mixtures were employed. However, cross-linking of the hemichromes at either the α or the β chains to form the stabilized tetramer yielded a copolymer stoichiometry of approximately eight globin chains per band 3 chain, i.e., two hemichrome sites per band 3 subunit. While underivatized hemichromes exhibited both a fast and slow phase of copolymerization, the cross-link-stabilized tetrameric hemichromes displayed predominantly the fast phase kinetics. Naturally occurring disulfide cross-linked hemichromes also reacted more avidly with band 3 than their reduced counterparts; however, the copolymerization process also proceeded to completion with totally reduced components. It is concluded that copolymerization of band 3 with hemichromes should occur under normal cellular conditions and at an accelerated velocity when the intracellular reducing power is low.

Heinz bodies are aggregates of denatured hemoglobin, termed hemichromes, which are commonly seen by phase-contrast microscopy as dark spots free in the cytoplasm or attached to the cytoplasmic surface of the erythrocyte membrane. Heinz bodies occur in red cells naturally under three distinct conditions. First, erythrocytes containing unstable hemoglobins, especially hemoglobins with mutations near the heme site in the β chain (Jacob & Winterhalter, 1970), show elevated levels of Heinz bodies. In this situation, the altered bonding at the heme is believed to contribute to the structural instability of the variant, causing its denaturation, precipitation on the membrane, and ultimately the resulting Heinz body hemolytic anemia (Winterbourn & Carrell, 1974). Second, Heinz bodies are seen in cells containing normal hemoglobin under conditions of oxidant stress. This oxidant stress can arise either in cells suffering from a diminished capacity to maintain intracellular reducing power, e.g., in glucose-6-phosphate dehydrogenase and glutathione peroxidase deficiency diseases (Jandl et al., 1960; Tudhope & Hopkins, 1974; Hopkins & Tudhope, 1974), or in cells subjected to oxidant-generating drugs, e.g., phenylhydrazine (Rifkind, 1965; Itano et al., 1977; French et al., 1978; Ortiz de Montellano & Kunze, 1981). Third, Heinz bodies often appear in normal cells as they age (Sear et al., 1975; Campwala & Desforges, 1982). This age-related appearance of Heinz bodies is especially pronounced in splenoectomized individuals where a major organ of senescent red cell removal has been excised (Selwyn, 1955).

Heinz body formation, whether natural or drug induced, is believed to occur by a similar mechanism. Methemoglobin present in the red cell is converted first to a reversible hemichrome, then to an irreversible hemichrome, and finally into an aggregate called a Heinz body (Winterbourn & Carrell, 1974; Peisach et al., 1975). The intermediate hemichromes are partially denatured ferrihemoglobin derivatives in which the sixth coordination position of the heme is occupied by a ligand endogenous to the protein, such as the imidazole of the distal histidine.

Recently, we demonstrated that the cytoplasmic domain of the major integral erythrocyte membrane protein, band 3, provides a high-affinity site for hemichrome binding to the membrane (Waugh & Low, 1985). The binding was shown to propagate rapidly into a macroscopic copolymer upon mixing the isolated cytoplasmic domain of band 3 (cdb3)[1] with a solution of hemichromes. Significantly, no polymerization was found to occur when cdb3 was reacted with nondenaturated forms of hemoglobin, e.g., methemoglobin , (carbonmonoxy)hemoglobin, etc. (Waugh & Low, 1985). The copolymerization was also shown to manifest itself in vivo as a redistribution of band 3 in the plane of the membrane (Low et al., 1985; Waugh et al., 1986). This redistribution occurred whether the Heinz bodies were formed by phenylhydrazine treatment of normal cells (Low et al., 1985) or by natural processes as in the case of sickle cell hemoglobinopathies (Waugh et al., 1986). We have suggested that the Heinz body promoted aggregation of band 3 may contribute to the abnormalities associated with Heinz body containing cells, e.g.,

† Supported by National Institutes of Health Grants GM24417 and HL 33555.

‡ Purdue University.

§ University of Iowa.

‖ Recipient of an established investigatorship award of the American Heart Association.

[1] Abbreviations: cdb3, 43 000-dalton, dimeric cytoplasmic domain of band 3; SDS, sodium dodecyl sulfate; kDa, kilodalton(s); BSA, bovine serum albumin.

0006-2960/87/0426-1777$01.50/0

mechanical instability and altered permeability (Waugh & Low, 1985).

The possible relevance of the band 3–hemichrome interaction to red cell biochemistry, as exemplified by the Heinz body promoted aggregation of band 3 in sickle cells, suggests that a more thorough understanding of this interaction is warranted. The present investigation was undertaken to explore in greater detail the structure, stoichiometry, and stability of the hemichrome–band 3 complex as well as the kinetics of copolymer formation.

Experimental Procedures

Materials. Fresh human blood was obtained from the Central Indiana Regional Blood Bank in citrate/dextrose solution, and the blood was used before it became officially outdated. Ribonuclease A, myoglobin, carbonic anhydrase, ovalbumin, and bovine serum albumin were all obtained from Sigma Chemical Co. Molecular weight standards for SDS–polyacrylamide gel electrophoresis were also from Sigma. Phenylhydrazine was from MCB.

Preparation of Hemoglobin and the Cytoplasmic Domain of Band 3. Erythrocyte membranes were prepared by hypotonic lysis of washed erythrocytes in 5 mM sodium phosphate buffer, pH 8.0, as described by Dodge et al. (1963). The dimeric, water-soluble cytoplasmic domain of band 3 was prepared by mild chymotryptic digestion of erythrocyte membranes (Appell & Low, 1981). Hemoglobin destined for hemichrome preparation was prepared as described by Salhany et al. (1972). Hemoglobin dervatives covalently cross-linked between α or β subunits were prepared by using bis(3,5-dibromosalicyl) fumarate as the cross-linking agent. The cross-link between the β subunits lies between Lys-82β_1 and Lys-82β_2, and is formed in the reaction of bis(3,5-dibromosalicyl) fumarate with oxyhemoglobin (Walder et al., 1980; Chatterjee et al., 1982). The cross-link between the α subunits is from Lys-99α_1 to Lys-99α_2 and is formed when the reaction is carried out with deoxyhemoglobin (Chatterjee et al., 1986).

Preparation of Hemichromes. Hemoglobin which had been dialyzed overnight in 10 mM sodium phosphate, pH 7.5, was adjusted to a concentration of 3.5×10^{-4} M tetramer. To this hemoglobin solution was added an equal volume of 30 mM phenylhydrazine in the same buffer. The resulting dark brown solution was incubated at 37 °C for 90 min with occasional mixing. The precipitated protein, which began forming soon after the start of incubation, was removed by a brief centrifugation, and the brown supernatant was immediately passed down a Sephadex G-25 column to remove the unwanted phenylhydrazine. The most concentrated fractions were pooled and stored at 4 °C until use, generally within 48 h.

Copolymer Formation. Addition of the cytoplasmic domain of band 3 to a solution of hemichromes under the appropriate conditions resulted in the immediate formation of a turbid suspension. The amount of insoluble copolymer formed was quantitated by measuring the turbidity of the sample at 700 nm in black side-walled cuvettes. These turbidity values were routinely corrected to give true turbidity by subtracting out a minor absorbance contribution of the hemichromes at 700 nm. A standard curve relating turbidity to the mass of insoluble isolated copolymer per 1 mL of reaction mixture demonstrated that the two parameters were directly proportional over a 10-fold range of turbidity values (0.2–2.0). Gradual deviation from linearity was observed only below turbidity values of 0.2. Taking care to allow for this deviation wherever necessary, the standard curve was used to convert turbidity values to copolymer mass, and the data are presented as such (see Figures 3 and 4). Most experiments were done on ice to avoid any unstimulated precipitation of hemichromes which was found to gradually occur at higher temperatures.

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. Turbidity measurements were obtained on a IBM recording spectrophotometer. Protein concentrations were determined according to Lowry et al. (1951) using bovine serum albumin as the standard.

Results

Kinetics of Polymerization and Band 3–Hemichrome Stoichiometry. In an earlier study, we demonstrated that the major, integral protein of the human erythrocyte membrane, band 3, provided a high-affinity binding site for hemichromes (Waugh & Low, 1985). When the isolated 43-kDa water-soluble, cytoplasmic domain of band 3 (cdb3) was mixed with a solution of hemichromes, a water-insoluble copolymer of defined stoichiometry resulted (five hemichrome chains per band 3 chain). Importantly, the stoichiometry remained invariant regardless of the molar ratio of band 3 to hemichromes in the initial reaction mixture. However, because of the unusual value of this stoichiometry, a subsequent question was raised as to whether the ratio remains constant during the entire course of the reaction, or alternatively, is the apparent 5/1 stoichiometry an artifact of competing reactions with different stoichiometries. The kinetic data addressing this concern are shown in Figure 1, where the amount of 43-kDa band 3 fragment and 16-kDa hemichrome monomer in the water-insoluble copolymer is evaluated as a function of time after mixing the two soluble reactants. At each time point, the insoluble product was collected, solubilized in SDS, and run on an SDS–polyacrylamide gel (Figure 1, lanes d–i). The hemichrome to cdb3 ratio, quantitated by densitometry of the Coomassie blue stained proteins in the gel, ranged from 5.6 hemichrome chains per cdb3 chain at the 20-min time point to 4.7 at the 6-h time point. The stoichiometries at each time point are plotted in Figure 2 (see closed triangles). While there is some variation in the values, clearly the variations are not time dependent, and the stoichiometries all agree well with the ratio reported previously (Waugh & Low, 1985).

Information on the rate of polymerization was also obtained from the data in Figure 1. Since the copolymer generated at each time point was quantitatively collected from identical reaction volumes, the amount of sample visualized in the SDS–polyacrylamide gel should correspond to the amount of copolymer present at that time point. When the lanes of the gel were scanned for Coomassie blue staining intensity and the combined hemichrome–cdb3 staining intensities for each lane were plotted vs. time, a biphasic curve resulted (Figure 2, closed circles). The initial phase of the reaction was very rapid and complete in less than 0.5 h. The latter, much slower phase, however, progressed in a linear fashion for at least 24 h. The same biphasic kinetics were confirmed in turbidimetric studies of the polymerization process under conditions where the progress of the reaction was monitored continually (vide infra). During the slower phase, the entire complement of soluble cdb3 was gradually consumed (Figure 1, lanes j–m), leaving no detectable band 3 in the unpolymerized supernatant by the 24-h time point (lane m). Thus, in the presence of excess hemichromes, the reaction eventually proceeds to completion.

Stabilization of Copolymer by Ionic Interactions. Previous investigations of the interactions of band 3 with native hemoglobin and several of the glycolytic enzymes have demonstrated that the complexes are stabilized largely by ionic in-

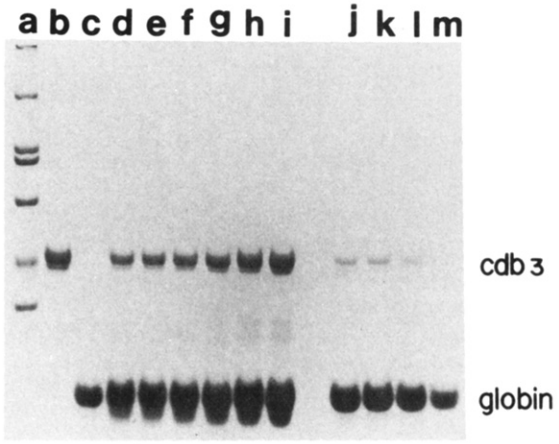

FIGURE 1: Determination of hemichrome–cdb3 stoichiometry in the insoluble copolymer as a function of reaction time. The cytoplasmic domain of band 3 (0.6 mg/mL, final) and hemichromes (3.0 mg/mL, final) in 10 mM sodium phosphate, pH 6.0, were mixed and incubated on ice. At different times, 0.30 mL of the reaction mixtures was centrifuged (5 min at 12000*g*). The precipitates, which appeared as thin films on the bottom of the centrifuge tubes, were washed once with cold buffer and dissolved in 0.10 mL of solubilization buffer in preparation for SDS–polyacrylamide gel electrophoresis. All samples contained 50 mM dithiothreitol. An equal volume of the solubilized protein from each time point was loaded onto the gel. Therefore, the Coomassie blue staining intensity in each lane is proportional to the amount of precipitate formed at each time point. The lanes are (a) molecular weight standards, (b) the cytoplasmic domain of band 3, (c) hemichromes (d–i) precipitate from the 5-min, 20-min, 1.5-h, 6.0-h, 16.0-h, and 24.0-h time points, and (j–m) supernatants, after precpitate removal, from the 5-min, 20-min, 16.0-h, and 24.0-h time points. The actual stoichiometries are plotted in Figure 2. The molecular weight standards (lane a) are (from top to bottom) the following: myosin, M_r 205 000; β-galactosidase, M_r 116 000; phosphorylase *b*, M_r 97 400; BSA, M_r 66 000; ovalbumin, M_r 45 000; and carbonic anhydrase, M_r 29 000.

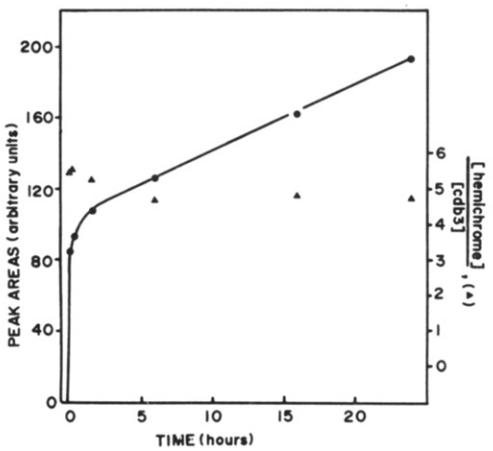

FIGURE 2: Copolymer formation as a function of time. The hemichrome–cdb3 stoichiometry was determined from integrated scans of the Coomassie blue stained gels in Figure 1. Stoichiometries (▲) are expressed in terms of the 16-kDa globin subunit and the 40-kDa cdb3 subunit. The total amount of protein recovered in the precipitate, plotted as "peak areas", was generated from the same gel scans by integrating the area under each densitometry peak (●).

teractions (Higashi et al., 1979; Kant & Steck, 1973; Prasanna Murthy et al., 1984; Strapazon & Steck, 1977; Fung et al., 1983; Low, 1986; Shaklai et al., 1977). It was, therefore, of interest to determine whether the hemichrome–cdb3 association was also controlled by such forces. Figure 3 shows the effect of increasing ionic strength on the amount of copolymer formed after 1 h, i.e., in the initial polymerization reaction. As the NaCl concentration is raised, copolymer precipitation is inhibited, suggesting the involvement of charge interactions

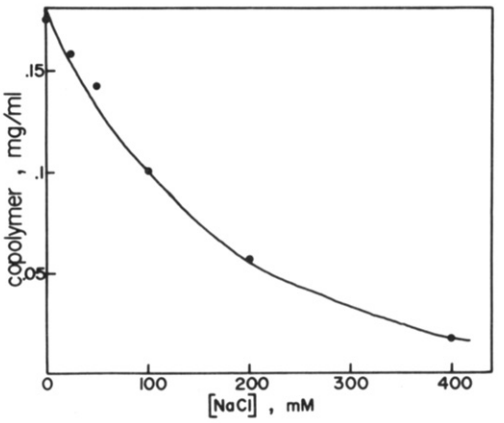

FIGURE 3: Ionic strength dependence of the interaction between the cytoplasmic domain of band 3 and hemichromes. The proteins, in 10 mM sodium phosphate buffer, pH 6.0, at the indicated salt concentrations, were mixed and incubated on ice for 60 min. The turbidity was then measured at 700 nm and converted to copolymer concentration by using the standard curve as described under Experimental Procedures. The final concentrations of hemichromes and cdb3 in the reaction mixtures were 1.30 and 0.38 mg/mL, respectively.

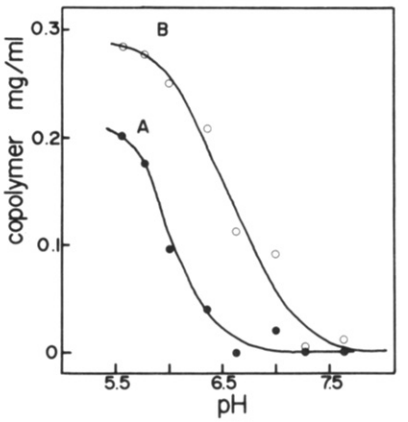

FIGURE 4: pH dependence of the interaction between the cytoplasmic domain of band 3 and hemichromes. Aliquots of the cytoplasmic domain of band 3 and hemichromes were dialyzed against 5 mM sodium phosphate–5 mM sodium citrate at the indicated pH values. The proteins were then mixed and allowed to incubate on ice for 1 h. Turbidity in each reaction mixture was then measured at 700 nm and converted to copolymer concentration by using the standard curve as described under Experimental Procedures. For curves A and B, the final band 3 concentration in each reaction mixture was 0.25 mg/mL. For curve A, the hemichrome concentration was 1.02 mg/mL, and for curve B, it was 5.10 mg/mL. The computer-derived pH midpoints obtained by nonlinear least-squares fit of the data to the Hill equation are 6.0 (curve A) and 6.6 (curve B).

in stabilizing the complex. When the concentration of NaCl was raised to 120 mM, the amount of aggregate generated was seen to decrease ~50%, and by 400 mM NaCl, formation of the complex was nearly halted.

The dependence of the band 3–hemichrome interaction on salt bridges is further suggested by the data in Figure 4. In this study, the copolymerization reaction was examined as a function of pH at two different but constant hemichrome concentrations. With increasing pH, the affinity of hemichromes for cdb3 decreases markedly, as also occurs in the binding of native hemoglobin and the glycolytic enzymes (Higashi et al., 1979; Kant & Steck, 1973; Prasanna Murthy et al., 1984; Strapezon & Steck, 1977; Fung et al., 1983; Low, 1986; Shaklai et al., 1977). This behavior indicates that there must be a net uptake of protons on formation of the complex. Assuming that the initial polymerization reaction is governed by the solubility product:

$$[\text{band 3}][\text{hemichrome}]^{1.25} = K \qquad (1)$$

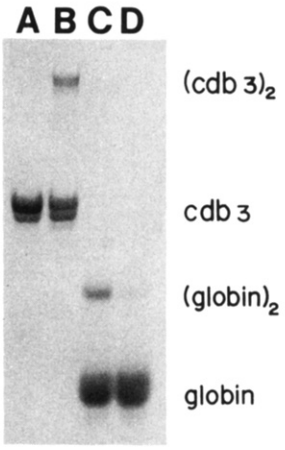

FIGURE 5: Reduction of disulfide bonds in samples of hemichromes and the cytoplasmic domain of band 3. Solutions of the cytoplasmic domain of band 3 (A and B) and separate solutions of hemichromes (C and D) were dialyzed overnight at 4 °C against 10 mM sodium phosphate buffer, pH 6.0 (B and C), or against the same buffer containing 2 mM dithiothreitol (A and D).

where the exponent 1.25 corresponds to the stoichiometry of five globin chains per band 3 monomer in the copolymer and assumes that the hemichrome tetramer is the predominant reacting species, then the pH dependence of the reaction is given by (Wyman, 1964; Laskowski & Finkenstadt, 1972)

$$\text{d log } K/\text{d pH} = n \qquad (2)$$

where n is the number of protons bound per cdb3 subunit in forming the polymer. Equation 2 predicts that the pH midpoint of the transition (pH_m) will increase with increasing concentrations of hemichrome (HE) according to the following expression:

$$pH_m = 1.25/n \log [HE] + C$$

where C is a constant dependent on the total band 3 concentration. In agreement with this relationship, a 5-fold increase in hemichrome concentration elevated the pH midpoint from 6.0 (curve A in Figure 4) to 6.6 (curve B). This result suggests that there are approximately 1.5 protons taken up per molecule of cdb3 incorporated in the copolymer.

Importance of Disulfide Bonding. Hemichromes isolated from cells in vivo frequently contain some disulfide-cross-linked subunits (Winterbourn & Carrell, 1974). When our phenylhydrazine-generated hemichromes were examined by SDS–polyacrylamide gel electrophoresis, a significant fraction of oligomeric species was also observed (Figure 5). Thus, while approximately 20% of the globin chains migrated as a 32-kDa dimer in the absence of a reducing agent (lane C), essentially all of the globin converted to the 16-kDa monomer in the presence of dithiothreitol (lane D). The isolated cytoplasmic domain of band 3 also gradually formed disulfide cross-bridges when stored in the absence of a reducing agent (compare lanes A and B), but these cross-bridges occur between juxtaposed sulfhydryls within the native dimeric molecule and are thought to have little influence on the structure of the isolated cytoplasmic domain (Low et al., 1984; Low, 1986).

To determine whether the cross-linked hemichromes participate more or less actively in the copolymerization process, the ratios of cross-linked to un-cross-linked globin chains were examined in both the copolymer pellet and the soluble supernatant (Table I). As noted above, the ratio in unreacted hemichromes remained at ≈0.22–0.25 for at least 42 h, i.e., the time required to complete the reaction. However, addition of the cytoplasmic domain of band 3 to the hemichrome solution gradually depleted the soluble supernatant of cross-linked globin chains, enriching the flocculant coprecipitate in the same species. Thus, the ratio of disulfide-stabilized dimeric to unoxidized monomeric globin chains in the pellet was measured at 0.6–0.65 over the entire course of the reaction, while the same ratio in the supernatant decreased from ≈0.22 to ≈0.11 during the 42-h incubation (Table I). This mild preference of cdb3 for the cross-linked hemichrome fraction is probably responsible for the small but reproducible increase in copolymer yield (≤5%) obtained when reducing agents are omitted from the reaction solution (data not shown). However, the fact that totally reduced components still avidly copolymerize demonstrates that disulfide cross-bridging is not essential for formation of the macroscopic coaggregate.

Table I: Disulfide-Cross-Linked Subunits in Hemichromes Involved in Copolymer Formation

incubation time[a]	$(globin)_2$[c] globin
5-min copolymer	0.65
20-min copolymer	0.60
1.5-h copolymer	0.61
6-h copolymer	0.61
24-h copolymer	0.65
42-h copolymer	0.63
5-min hemichrome[b]	0.22
42-h hemichrome[b]	0.25
42-h supernatant after copolymer removal	0.11

[a] Copolymer formation was initiated by combination of hemichromes (3.0 mg/mL) and cdb3 (0.6 mg/mL) on ice. [b] The 5-min and 42-h hemichrome samples were incubated in the absence of cdb3. [c] Determined from peak heights in densitometric scans of the Coomassie blue stained proteins in the polyacrylamide gel.

Studies of the Site on Hemichromes Involved in Band 3 Binding. In previous work, it was shown that hemichromes attach to the red cell membrane predominantly at the extreme N-terminus of the cytoplasmic domain of band 3 (Waugh & Low, 1985). Importantly, this acidic N-terminal sequence of band 3 is also involved in binding several glycolytic enzymes and native hemoglobin, the latter largely in its deoxygenated state (Prasanna Murthy et al., 1981; Walder et al., 1984; Jenkins et al., 1985; Tsai et al., 1982; Cassoly & Salhany, 1983; Chetrite & Cassoly, 1985). The interaction between deoxyhemoglobin and cdb3 has been further characterized by X-ray analysis, where it has been demontrated that the extreme N-terminus of band 3 protrudes ≈18 Å into the central diphosphoglycerate binding cavity of the hemoglobin molecule (Walder et al., 1984). In another study (Cassoly & Salhany, 1983), it was demonstrated that native hemoglobin can also form cross-bridges between band 3 dimers, although unlike the situation with hemichromes no macroscopic polymerization occurs. Finally, hemoglobin binding to the membrane appears to induce some degree of membrane restructuring, albeit not as extensive as the hemichromes (Salhany et al., 1980; Wiedenmann & Elbaum, 1983).

Because of the apparent similarities between the band 3–hemoglobin and band 3–hemichrome interactions, it was of interest to determine whether a central cationic cavity in hemichrome, analogous to the diphosphoglycerate site in deoxyhemoglobin, provides the binding site for band 3. To evaluate this possibility, two chemically cross-linked derivaives of hemichromes were prepared. In the first derivative, the cross-link occurs between Lys-82β_1 and Lys-82β_2, spanning the central cavity between the β subunits. This blocks the entrance to the β cleft and prevents the binding of 2,3-diphosphoglycerate as well as band 3 (Walder et al., 1980, 1984). It was reasoned that if cdb3 requires access to this same region of the hemichrome for binding, then reaction of cdb3 with β

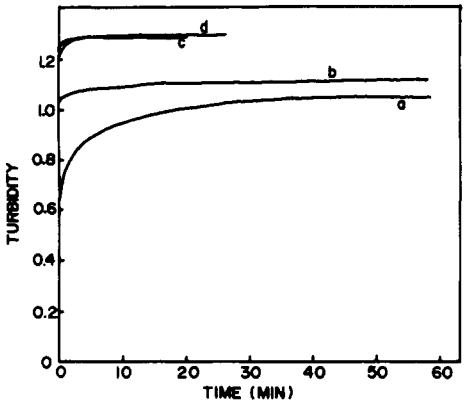

FIGURE 6: Turbidity as a function of time upon mixing hemichromes with the cytoplasmic domain of band 3. All proteins were in 10 mM sodium phosphate buffer, pH 6.0, equilibrated to room temperature. Hemichrome solutions were kept on ice until just before use at which time the solutions were equilibrated to room temperature by immersion in a water bath. The hemichrome solution was added to the cuvette and the reaction intiated by addition of the cytoplasmic domain of band 3. Monitoring of turbidity was begun after a 20-s mixing period. All reaction mixtures contained 0.3 mg/mL cytoplasmic domain of band 3. The hemichrome concentrations were (a) 1.0 mg/mL hemichromes (b) 2.0 mg/mL hemichromes, (c) 1.0 mg/mL α-hemichromes, and (d) 1.0 mg/mL β-hemichromes. The α and β designate hemichrome derivatives covalently cross-linked between α and β subunits as described in the text.

cross-linked hemichrome should not lead to copolymer formation. As a control, a second cross-linked hemichrome was generated in which the cross-link lies between the α chains, from Lys-99α_1 to Lys-99α_2, and does not block access to the β cleft.

Addition of the cytoplasmic domain of band 3 to either the α- or the β-hemichrome derivative led to rapid formation of a turbid suspension (Figure 6, curves c and d). As judged by turbidimetry, both reactions were nearly complete within the $\approx$20-s mixing time of the technique. Since no diminution of copolymerization was observed with the derivative cross-linked between the β chains, it is evident that hemichromes associate differently at the N-terminus of band 3 than native deoxyhemoglobin. Comparison of the kinetics of copolymerization of derivatized and unmodified hemichromes further emphasizes this conclusion. When examined at the same concentration, the unmodified hemichromes formed the copolymer at a much slower rate, requiring about 20 min for the fast phase to reach completion (Figure 6, curve a). Even increasing the hemichrome concentration to twice its level used in curves a, c, and d did not promote as rapid or extensive a polymerization reaction as the α- or β-chain cross-linking did (compare curve b). Thus, rather than inhibit the interaction between band 3 and hemichromes due to occlusion of the central cavity, covalent cross-bridging of the protein's β chains (or α chains) actually enhanced the fast phase polymerization process.

It was reported previously that hemichromes exist as a mixture of stable dimers and presumed tetramers (Waugh & Low, 1985). Because of this oligomeric heterogeneity, it was difficult to estimate the number of potential hemichrome sites on each cdb3 dimer from the copolymer's stoichiometry. Reexamination of this issue using the α- or β-chain cross-linked hemichromes, however, obviates this problem, since the cross-linked hemichromes were found to exist almost exclusively as stable tetramers (data not shown). Thus, like the β-chian cross-linked hemoglobin, the two cross-bridged hemichromes eluted from a Sephadex G-100 column with an apparent molecular weight of $\sim$56 400, i.e., the approximate molecular weight value of the native tetramer. Determination of the stoichiometry obtained with these cross-linked hemichromes was conducted as described in Figure 2. For the α derivative, the stoichiometries ranged from 3.2 to 3.7 hemichrome tetramers per band 3 dimer, while the same ratio for the β cross-linked hemichrome varied from 3.5 to 4.0. Importantly, under no conditions did the copolymer stoichiometry of the modified hemichromes exceed four hemichrome tetramers per cdb3 dimer. Therefore, it would appear that band 3 provides maximally two hemichrome sites per cdb3 subunit.

Discussion

Hemoglobin, glyceraldehyde-3-phosphate dehydrogenase, phosphofructokinase, and aldolase all bind to the highly acidic N-terminus of the major integral erythrocyte membrane protein, band 3 (Murthy et al., 1981, 1984; Tsai et al., 1982; Jenkins et al., 1985; Walder et al., 1984). It is likely that hemichromes also bind at this acidic site since (a) the binding is highly dependent upon pH and ionic strength, as is the binding of native hemoglobin and the glycolytic enzymes, (b) glyceraldehyde-3-phosphate dehydrogenase blocks hemichrome binding to the erythrocyte membrane (Waugh & Low, 1985), and (c) native hemoglobin can compete with hemichromes for their interaction with the isolated cytoplasmic domain of band 3 in solution (Waugh & Low, 1985). The strong pH dependence of binding of hemichromes to band 3 (see Figure 4) demonstrates a significant contribution from titratable groups in stabilizing the band 3–hemichrome complex. The linkage relationship in eq 2 demands that there be a net uptake of protons when binding occurs. This must also be true for the binding of native hemoglobin and the glycolytic enzymes to band 3 (Walder et al., 1984). In each case, the binding of protons is probably associated directly with the salt bridges that are formed between the acidic N-terminal sequence of band 3 and basic residues within the binding site of the target protein. For deoxyhemoglobin, the affinity of a synthetic peptide comprising just the first 11 residues of band 3 is virtually identical with that of the entire cytoplasmic domain (Walder et al., 1984; Chetrite & Cassoly, 1985). X-ray crystallographic studies showed that the binding site for the peptide includes the cluster of cationic residues within the diphosphoglycerate site as well as basic residues further inside the central cavity between the β subunits (Walder et al., 1984). An uptake of protons has also been shown to occur with the binding of other polyanions in this region. For inositol hexaphosphate, this process makes a major contribution to the overall binding energy (Gill & Brill, 1982).

The sharp decrease in the affinity of hemoglobin and the glycolytic enzymes for band 3 with increasing pH and ionic strength has made it difficult to demonstrate the binding of these proteins under physiological conditions. However, some evidence for the in vivo association of the glycolytic enzymes with band 3 has come from an examination of the kinetics of enzyme release from red cells during hemolysis (Kliman & Steck, 1980; Jenkins et al., 1984). A more direct approach was recently employed by Chetrite and Cassoly (1985) to demonstrate the interaction of hemoglobin with band 3 under physiological conditions. This approach, which used immobilized cdb3 as the stationary ligand, was successful largely because the binding could be studied at high, near-intracellular hemoglobin concentrations. Likewise, the data in Figure 4 show that a complex between hemichromes and cdb3 becomes more stable at higher pH values as the hemichrome concentration is increased. Additionally, within the red cell, the thermodynamic activity of soluble proteins is greatly enhanced due to the excluded volume effect resulting from the high concentration of hemoglobin (Ross & Minton, 1977). Hem-

ichromes, having a size and shape similar to those of native hemoglobin, would have an activity coefficient of approximately 50. These considerations suggest that the interaction of hemichromes with band 3 can occur under normal conditions within the erythrocyte. The coclustering of hemichromes and band 3 in vivo (Waugh et al., 1986) provides firm evidence in support of this hypothesis.

It was shown previously (Waugh & Low, 1985) that both dimeric and tetrameric hemichromes can copolymerize with the cytoplasmic domain of band 3. However, the results in Table I and Figure 6 suggest that the tetramer is incorporated preferentially into the insoluble copolymer. Significantly, copolymers with the β hemichrome dervative formed at a similar rate and to the same extent as observed with the α–α cross-linked hemichrome. This result indicates that the binding of hemichromes to cdb3 does not occur by insertion of the acidic N-terminal peptide of band 3 into the central cavity between the β subunits, as in the case of the binding to deoxyhemoglobin (Walder et al., 1984). The binding of the N-terminal peptide at an alternative site off of the dyad axis would guarantee that there are at least two binding sites per tetramer at equivalent positions of each α–β dimer. This is clearly a necessary condition for the copolymerization of hemichromes with band 3 to form higher molecular weight aggregates.

The simplest model for the structure of the complex between hemichromes and band 3 is an alternating copolymer between the two proteins. This would predict a stoichiometry of two globin subunits for each 43-kDa cdb3 monomer in an extended polymer chain. The values observed experimentally are much greater, 5 for native hemichromes and as high as 8 for the cross-linked derivatives. These higher values may be due to the existence of two distinct hemichrome binding sites per cdb3 subunit. Within the hemichrome binding region at the N-terminus of band 3 are two homologous, adjacent 12 amino acid sequences (Kaul et al., 1983). If these two highly acidic sequences were allowed to fully extend, they would expose a length of nearly 90 Å along which two hemichromes could bind. The preferential incorporation of disulfide-cross-linked tetramers within the polymer may also contribute to the increase in the globin to cdb3 ratio. Values of the disulfide-cross-linked globin dimer to monomer ratio above 0.5 observed in the precipitates (see Table I) suggest that extended chains of intermolecularly cross-linked hemichromes may be formed. (The most likely site of cross-linking is from Cys-93β of one molecule to Cys-93β of a second, rather than intramolecularly.) These observations may have particular significance for Heinz body–band 3 interactions within the red cell since hemichrome formation in vivo occurs in a environment of low intracellular reducing power, i.e., a situation where disulfide cross-links between globin subunits are likely to occur.

References

Appell, K. C., & Low, P. S. (1981) *J. Biol. Chem. 256*, 11104–11111.

Campwala, H. Q., & Desforges, J. F. (1982) *J. Lab. Clin. Med. 99*, 25–28.

Cassoly, R., & Salhany, J. M. (1983) *Biochim. Biophys. Acta 745*, 134–139.

Chatterjee, R., Walder, R. Y., Arnone, A., & Walder, J. A. (1982) *Biochemistry 21*, 5901–5909.

Chetrite, G., & Cassoly, R. (1985) *J. Mol. Biol. 185*, 639–644.

Dodge, J. T., Mitchell, C., & Hanahan, D. J. (1963) *Arch. Biochem. Biophys. 100*, 119–130.

Fairbanks, G., Steck, T. L., & Wallach, D. F. H. (1971) *Biochemistry 10*, 2606–2617.

French, J. K., Winterbourn, C. C., & Carrell, R. W. (1978) *Biochem. J. 173*, 19–26.

Fung, L. W.-M., Litvin, S. D., & Reid, T. M. (1983) *Biochemistry 22*, 864–869.

Gill, S. J., & Brill, R. V. (1982) in *Hemoglobin and Oxygen Binding* (Chien Ho, Ed.) pp 165–168, Elsevier Biomedical, Amsterdam.

Higashi, T., Richards, C. S., & Uyeda, K. (1979) *J. Biol. Chem. 254*, 9542–9550.

Hopkins, J., & Tudhope, G. R. (1974) *Clin. Sci. Mol. Med. 47*, 643–647.

Itano, H. A., Hirota, K., & Vedvick, T. S. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 2556–2560.

Jacob, H. S., & Winterhalter, K. H. (1972) in *VI International Symposium uber Struktur und Funktion der Erythrocyten* (Rapoport, S., & Jung, F., Eds.) pp 93–96, Akademie-Verlag, West Berlin.

Jandl, J. H., Engle, L. K., & Allen, D. W. (1960) *J. Clin. Invest. 39*, 1818–1836.

Jenkins, J. D., Madden, D. P., & Steck, T. L. (1984) *J. Biol. Chem. 259*, 9374–9378.

Jenkins, J. D., Kezdy, F. J., & Steck, T. L. (1985) *J. Biol. Chem. 260*, 10426–10433.

Jennings, M. L., Adams-Lackey, M., & Denney, G. H. (1984) *J. Biol. Chem. 259*, 4652–4660.

Kant, J. A., & Steck, T. L. (1973) *J. Biol. Chem. 252*, 7418–7420.

Kaul, R. K., Prasanna Murthy, S. N., Reddy, A. G., Steck, T. L., & Kohler, H. (1983) *J. Biol. Chem. 258*, 7981–7990.

Kliman, H. J., & Steck, T. L. (1980) *J. Biol. Chem. 255*, 6314–6321.

Laskowski, M., Jr., & Finkenstadt, W. R. (1972) *Methods Enzymol. 26*, 198–227.

Low, P. S. (1986) *Biochim. Biophys. Acta 864*, 145–167.

Low, P. S., Westfall, M. A., Allen, D. P., & Appell, K. C. (1984) *J. Biol. Chem. 259*, 13070–13076.

Low, P. S., Waugh, S. M., Zinke, K., & Drenckhahn, D. (1985) *Science (Washington, D.C.) 227*, 531–533.

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

Ortiz de Montellano, P. R., & Kunze, K. L. (1981) *J. Am. Chem. Soc. 103*, 6534–6536.

Osgood, E. E. (1935) *Arch. Intern. Med. 56*, 849.

Peisach, J., Blumberg, W. E., & Rachmilewitz, E. A. (1975) *Biochim. Biophys. Acta 393*, 404–418.

Prasanna Murthy, S. N., Liu, T., Kaul, R. K., Kohler, H., & Steck, T. L. (1981) *J. Biol. Chem. 256*, 11203–11208.

Prasanna Murthy, S. N., Kaul, R. K., & Kohler, H. (1984) *Hoppe-Seyler's Z. Physiol. Chem. 365*, 9–17.

Rifkind, R. A. (1965) *Blood 26*, 433–448.

Ross, P. D., & Minton, A. P. (1977) *J. Mol. Biol. 112*, 437–452.

Salhany, J. M., Mathers, D. H., & Eliot, R. S. (1972) *J. Biol. Chem. 247*, 6985–6990.

Salhany, J. M., Cordes, K. A., & Gaines, E. D. (1980) *Biochemistry 19*, 1447–1454.

Sears, D. A., Friedman, J. M., & White, D. R. (1975) *J. Lab. Clin. Invest. 86*, 722–732.

Selwyn, J. G. (1955) *Br. J. Haematol. 1*, 173.

Shaklai, N., Yguerabide, J., & Ranney, H. M. (1977) *Biochemistry 16*, 5585–5592.

Strapezon, E., & Steck, T. L. (1976) *Biochemistry 15*, 1421–1424.

Strapezon, E., & Steck, T. L. (1977) *Biochemistry 16*, 2966–2971.

Tsai, I.-H., Prasanna Murthy, S. N., & Steck, T. L. (1982) *J. Biol. Chem. 257*, 1438–1442.
Tudhope, G. R., & Hopkins, J. (1974) *Clin. Sci. Mol. Med. 46*, 635–645.
Walder, J. A., Walder, R. Y., & Arnone, A. (1980) *J. Mol. Biol. 141*, 195–216.
Walder, J. A., Chatterjee, R., Steck, T. L., Low, P. S., Musso, G. F., Kaiser, E. T., Rogers, P. H., & Arnone, A. (1984) *J. Biol. Chem. 259*, 10238–10246.
Waugh, S. M., & Low, P. S. (1985) *Biochemistry 24*, 34–39.
Waugh, S. M., Willardson, B. M., Kannan, R., Labotka, R. J., & Low, P. S. (1986) *J. Clin. Invest. 78*, 1155–1160.
Wiedenmann, B., & Elbaum, D. (1983) *J. Biol. Chem. 258*, 5483–5489.
Winterbourn, C. C., & Carrell, R. W. (1974) *J. Clin. Invest. 54*, 678–689.
Wyman, J. (1964) *Adv. Protein Chem. 19*, 223–286.
Zilber, I., & Shaklai, N. (1982) *Biochem. Int. 4*, 297–303.

Determination of the Depth of Bromine Atoms in Bilayers Formed from Bromolipid Probes†

T. J. McIntosh*
Anatomy Department, Duke University Medical Center, Durham, North Carolina 27710
Peter W. Holloway*
Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22908
Received June 10, 1986; Revised Manuscript Received November 18, 1986

ABSTRACT: X-ray diffraction analysis has been performed on a series of 1-palmitoyl-2-dibromostearoylphosphatidylcholines (BRPCs) with bromine atoms at the 6,7-, the 11,12-, or the 15,16-positions on the *sn*-2 acyl chains. The diffraction patterns indicate that, when hydrated, each of these lipids forms liquid-crystalline bilayers at 20 °C. For each lipid, electron density profiles and continuous Fourier transforms were calculated by the use of swelling experiments. In the electron density profiles, high-density peaks, due to the bromine atoms, are observed. The separation between these bromine peaks in the profile decreases as the bromine atoms are moved toward the terminal methyl of the acyl chain. For the 6,7- and 11,12-bromolipids, experimental Fourier transforms can be approximated by the sum of the transform of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) and the transform of two symmetrically placed peaks of electron density (the bromines). For the case of the 15,16-bromolipids, a better fit is obtained for the transforms of a model bilayer where the thickness of the methylene chain region of the bilayer is 3 Å greater than that of POPC. Our analysis indicates the following: (1) for each of these bromolipids, the bromines are well localized in the bilayer; (2) the distance of the bromines from the head-group–hydrocarbon boundary are 3.5, 8.0, and 14 Å, for 6,7-, 11,12-, and 15,16-BRPC, respectively; (3) the bilayer thickness and perturbation to bilayer hydrocarbon chain packing caused by the bromine atoms depend on the position of the bromines on the hydrocarbon chain.

Although many important functions are associated with cell membranes, our understanding of these processes has been hampered by the lack of high-resolution techniques for determining the three-dimensional structure of membrane proteins (Eisenberg, 1984), and only recently has there been a complete X-ray crystallographic analysis of an integral membrane protein (Deisenhofer et al., 1985). Both X-ray and neutron diffraction have been applied to membrane proteins in partially ordered structures, for example, cytochrome b_5 in oriented stacks of membranes, and in these studies, the resolution is sufficient to locate the protein mass within the bilayer (Gogol & Engelman, 1984; Rzepecki et al., 1986). Low-resolution structural information on membrane proteins can also be obtained by nuclear magnetic resonance (Smith & Oldfield, 1984) and by fluorescence techniques, the latter having the advantage that fluorescence is inherently very sensitive. Three recent reports and reviews (London, 1982; Kleinfeld, 1985; Blatt & Sawyer, 1985) have described the use of fluorescence quenching or fluorescence energy transfer to determine the structure, and other properties, of membrane proteins. These techniques have been most commonly used to determine the depth of protein-bound fluorophores within the membrane bilayer.

To determine the depth of the fluorophore, a series of probes which have a quencher or energy acceptor attached to different positions of a hydrocarbon backbone is used. The spin-labeled fatty acids (London, 1982) or the anthroyloxy fatty acids (Kleinfeld, 1985; Blatt & Sawyer, 1985) are commonly used. These probes are incorporated into membranes which also contain the protein under investigation. The simple assumption is that the most efficient quenching or energy transfer will occur when the quencher or acceptor and the protein fluorophore are at the same depth in the membrane. The extrapolation of this type of observation to a prediction of the depth of the protein-bound fluorophore requires that the depth of the quencher or acceptor be precisely known. The depths, in the membrane, of the spin-labeled fatty acid quenchers and the anthroyloxy-labeled fatty acid acceptors have been determined by a variety of techniques [reviewed in London

†This work was supported by NIH Grants GM23858 (to P.W.H.) and GM27278 (to T.J.M.).

0006-2960/87/0426-1783$01.50/0