HUMAN ERYTHROCYTE MEMBRANE BAND 3 PROTEIN INFLUENCES HEMOGLOBIN COOPERATIVITY. POSSIBLE EFFECT ON OXYGEN TRANSPORT

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Running Title: Erythrocyte Membrane Band 3 and Hemoglobin Function
Abbreviations

Hb: hemoglobin; DPG: 2,3-diphosphoglyceric acid; R-state: liganded (oxy) conformation; T-state: unliganded (deoxy) conformation

Dedication

This paper is dedicated to the memory of Eraldo Antonini on the occasion of the twentieth anniversary of his premature death. Interpreting the results in this paper has been greatly aided by the many contributions to hemoglobin function made by him and his colleagues.
Abstract

Hemoglobin function can be modulated by the red cell membrane but some mechanistic details are incomplete. For example, the 43 kDa chymotryptic fragment of the cytoplasmic portion of red cell membrane Band 3 protein and its corresponding N-terminal 11-residue synthetic peptide lower the oxygen affinity of hemoglobin but effects on cooperativity are unclear. Using highly purified preparations, we also find a lowered Hill coefficient (n values < 2) at sub-equivalent ratios of Band 3 fragment or of synthetic peptide to Hb, resulting in an oxygen affinity that is moderately decreased and a partially hyperbolic shape for the O₂ binding curve. Both normal HbA and sickle HbS display this property. Thus, the determinant responsible for the Hb cooperativity decrease by the 43 kDa fragment resides within its first 11 N-terminal residues. This effect is observed in the absence of chloride and is reversed by its addition. As effector : Hb ratios approach equivalence or with saturating chloride, normal cooperativity is restored and oxygen affinity is further lowered because the shape of the oxygen binding curve becomes completely sigmoidal. The relative efficiencies of DPG, the 43 kDa Band 3 fragment, and the 11-residue synthetic peptide in lowering cooperativity are very similar. The findings are explained based on the stereochemical mechanism of cooperativity by Perutz as due to two populations of T-state hemoglobin tetramers – one with bound effector and the other free. As a result of this property, hemoglobin at the membrane inner surface in contact with the N-terminal region of Band 3 could preferentially bind O₂ at low oxygen tension and then release it upon saturation with DPG in the interior of the red cell. Membrane modulation of hemoglobin oxygen affinity has particularly interesting implications for the polymerization of hemoglobin S in the sickle red cell.


Introduction

Interactions of red cell membrane components with each other and with intracellular molecules play important physiological roles in both normal and sickle cells (1, 2). The 43kDa cytoplasmic fragment of red cell membrane Band 3 (also called the anion exchanger protein, AE1) is in contact with red cell components, both membrane-bound and free in the cytoplasm. Among these are several glycolytic enzymes including aldolase, glyceraldehyde-3-phosphate dehydrogenase and phosphofructokinase as well as other membrane components such as ankyrin and protein 4.1. The structure of the Band 3 cytoplasmic fragment, which is obtained by selective chymotryptic cleavage, has recently been reported (3) and shows that its N-terminal region protrudes from the main body of the structure presumably enabling it to interact with such a variety of other red cell components. There have also been a number of reports that it interacts with hemoglobin to a significant extent (4-7). An x-ray crystallographic structure of a complex between deoxy Hb and a synthetic 11-residue peptide corresponding to the N-terminus of the cytoplasmic fragment has been published (8); a functional consequence of this interaction was to lower the oxygen affinity of hemoglobin but no effect on cooperativity was reported in the presence of the peptide although a reduced n value was observed in the presence of the intact Band 3 chymotryptic fragment. No correlation of this effect with the relative concentrations of either was made. Intact red cell membrane preparations (ghosts) also lower Hb oxygen affinity and lead to reduced cooperativity (9). However, this report did not focus on the Band 3 – Hb relationship nor directly address the specific source of the reduced cooperativity in the membrane preparation. In the present communication we describe how hemoglobin cooperativity as well as oxygen affinity are both influenced by highly purified Band 3 cytoplasmic fragment and its corresponding synthetic N-terminal peptide and we establish the stoichiometry for this relationship. We also investigate the competing effects of chloride with both Band 3 43kDa fragment and its N-terminal peptide on Hb functional properties.

Hemoglobin subunits within the tetramer exhibit cooperative behavior during the transition between the oxy (R-state) and deoxy (T-state) conformations giving rise to the sigmoidal O2 binding curve. This sigmoidal shape endows hemoglobin with important physiological properties since it maximizes binding or release of O2 in the steepest part of the curve where the Hill coefficient is highest. The R and T states by themselves (at either end of the curve) are not cooperative but cooperativity arises
when both states are in the process of interconverting, i.e. at intermediate levels of oxygenation. Cooperativity, as expressed by the Hill coefficient, ranges from 1 (non-cooperative) to approximately 3 for Hb and is maximal when the [T]/[R] ratio is unity (10-14); for hemoglobin it has been studied by many investigators particularly Perutz (10, 11), who has established its basis as due to the re-arrangement of subunit contacts at the mobile allosteric interface and of salt bridges near the allosteric interface. The allosteric regulatory molecules DPG, chloride, and protons facilitate O₂ release by binding preferentially to the T-state, e.g. DPG binds very efficiently to a single site on deoxy Hb (15) whereas chloride binds to several sites, some of which are shared with DPG but others are not (16-20). Hence, these two allosteric regulators are mutually competitive. The displacement of the O₂ binding curve to the right by allosteric regulators does not affect the degree of cooperativity when they are present in amounts equal to or greater than Hb so that all Hb tetramers would contain bound effector. However, Kister et al (21) reported that sub-equivalent ratios of DPG:Hb resulted in lowered Hb cooperativity, decreasing from an n value of 2.6 to about 2.0. They explained this result as due to two different populations of T-state tetramers - some with bound DPG and others free. They analyzed their data in terms of an expanded Monod-Wyman-Changeux (MWC) model and concluded that it had physiological relevance.

In this report we present evidence for a similar lowered cooperativity for HbA and HbS in the presence of sub-equivalent amounts, relative to Hb, of either the purified 43 kDa chymotryptic cytoplasmic fragment of membrane Band 3 or a synthetic 11-residue peptide corresponding to its N-terminus sequence. Since the decreased cooperativity occurs with a moderate decrease in oxygen affinity in contrast to a greater decrease in the presence of saturating DPG, we consider the possibility that the cooperativity differences for the hemoglobin in direct contact with Band 3 in the red cell membrane and the hemoglobin that is intracellular and saturated with DPG could have a role in O₂ transport due to a gradient of Hb tetramers with varying degrees of O₂-saturation. Thus, in this context, Band 3 cytoplasmic fragment might be considered an allosteric regulator of hemoglobin function.

**Experimental Procedures**

**Hemoglobin Desalting** – Oxy Hb (~ 5 ml of 0.8 mM in 0.1 M NaCl) was dialyzed extensively against 2 liters of de-gassed distilled water at 4°C with four changes. The dialysate was then passed through a 1.5 x 16 cm column of mixed-bed ion exchange resin (AG501-X8(D), 20-50 mesh, Bio-Rad, Hercules, CA)
that had been washed with de-gassed distilled water to remove the entrapped air (22). This procedure was repeated until the conductivity value of the Hb did not change before and after passing through the column; usually three passages sufficed. Conductivity of the hemoglobin solution as well as the 43 kDa fragment and the synthetic peptide, both of which are described below, was measured with a CDM 2e Conductivity Meter (Radiometer Copenhagen). The amount of residual chloride in the samples was determined with a chloride electrode as described below. Hb concentration was determined by amino acid analysis on a Beckman 6300.

**Determination of Chloride** - Chloride ion content of the de-salted hemoglobin, the 43 kDa Band 3 fragment and the human peptide solutions was measured using a MI-200 Micro-Chloride Electrode with MI-402 Micro-Reference Electrode (Microelectrodes, Inc., Bedford, NH) on a model 84 pH Meter (Radiometer Copenhagen). Standard sodium chloride solutions (10 µM to 0.1M) in 100 mM sodium acetate were used to construct a calibration curve by plotting the chloride concentrations versus the millivolt reading. The chloride concentrations of the samples were then read from this graph.

**43kDa Cytoplasmic Domain of Band 3** – The 43 kDa chymotryptic fragment of the cytoplasmic portion of human erythrocyte membrane Band 3 was prepared at 4° (23) except that the final ion exchange chromatography was performed on a Pharmacia-Amersham Mono Q column using a 0 – 0.6 M NaCl gradient in 10 mM sodium phosphate, pH 7.5. Since significant losses were encountered in the gel filtration step, it was omitted. Furthermore, we found this step to be unnecessary since both SDS-PAGE and amino acid analysis showed that the chymotryptic fragment was pure after the Mono Q step (Table 1). Chloride was removed from this sample first by dialysis against several changes of bis-Tris acetate, pH 7.5 (2 liters each), followed by continuous buffer exchange and concentration using Centriprep-10 and Centricon-10 devices successively. Residual chloride usually amounted to about 1.5 moles per mole of Band 3 chymotryptic fragment, which is far less than the amount needed to reverse the lowered cooperativity effect described below. Finally, the sample was concentrated with a Microcon YM-10 (500 µL). A single band in the expected position was found by SDS-PAGE electrophoresis. The pH of the samples was carefully adjusted to 7.5 with NaOH. The protein concentration of the Band 3 fragment was determined by amino acid analysis, which agreed with published values (24) as shown in Table 1.
Synthetic Peptides – These were synthesized using Fmoc blocking groups either at Rockefeller University Protein/DNA Facility or by Bio-Synthesis, Inc. (Lewisville, TX). Each peptide was purified by HPLC. The three N-terminal 11 amino acid peptides used in this study were de-salted twice using Sephadex G-25 (medium), which was eluted with distilled water. After drying in a Savant concentrator, each peptide was re-dissolved in 50 mM BisTrisAc or 50 mM potassium phosphate and pH was adjusted to 7.5. Amino acid analysis, performed on a Beckman 6300, agreed with the theoretical values (Table 1) and was used to determine concentration.

Functional Properties – Oxygen binding experiments were performed on a modified Hem O Scan instrument as described previously (25, 26).

Measurement of HbS Polymerization – The Csat/dextran assay of Bookchin, which was performed under completely anaerobic conditions, was used (27); control values for HbS are 34 +/− 1 mg/ml.

Results

Effects of Human Red Cell Membrane Band 3 Cytoplasmic Fragment and its Synthetic N-Terminal Peptide Analog on Hemoglobin Cooperativity – When the oxygen binding curve of either sickle hemoglobin (HbS) or normal adult hemoglobin (HbA) was measured in the presence of either the purified natural 43 kDa cytoplasmic N-terminal chymotryptic fragment of human red cell membrane Band 3 protein or an 11-residue synthetic peptide corresponding to its N-terminal sequence (each present in sub-equivalent ratios with respect to Hb and in chloride-free buffer), the shape of the curve had a pronounced hyperbolic profile indicative of a lowered cooperativity (see curve B of Fig. 1A where the ratio of the 11-residue peptide to HbS was 0.5). This result is highly reproducible (see below). Usually, cooperativity values are given for the middle section of the Hill plot, e.g. 30-70% O2 saturation where the slopes are the steepest (nmax). P50 is defined as the oxygen pressure on the X-axis corresponding to 50% saturation. P50 and nmax values are shown in the inset of Fig. 1A. A reduced Hill coefficient at sub-equivalent effector to Hb ratios was found with the 43 kDa chymotryptic cytoplasmic Band 3 fragment as well as with its 11-residue synthetic peptide N-terminal analog. The fractional O2 saturation at the same sub-equivalent ratio of peptide : Hb in the presence or absence of saturating 2,3-DPG is shown in Fig. 1B. Curve B shows that oxygen saturation initially rises more steeply at low oxygen tension in the absence of DPG than it does in
its presence (curve C). Arrows have been added before, at, and after the midpoints of O\textsubscript{2} saturation in Fig. 1B to emphasize this difference. The presence of excess 2,3-DPG, as shown in curve C of Fig. 1A and Fig. 1B, restored cooperativity to normal as demonstrated by its sigmoidal character; O\textsubscript{2} affinity was further lowered. Note that at higher oxygen tensions, curve B and curve C of Fig. 1B tend to merge. This is due to mutual competition for the same binding site so that the total effector : Hb ratio is \( > 1 \). It is more clearly shown in the separate titration experiments for each of these three effector molecules described below.

With either the synthetic 11-amino acid N-terminal peptide (Fig. 2A) or the 43 kDa human cytoplasmic fragment of band 3 (Fig. 3A) in varying ratios with respect to Hb, cooperativity is first lowered reaching a minimum value at an effector : Hb ratio about 0.5. At higher ratios the profiles of the \( n \) vs effector : Hb ratio are reversed and cooperativity returns to normal. The overall parabolic profiles shown in Figs. 2A and 3A describe this effect. At the sub-equivalent ratios, the shape of the individual oxygen binding curves are like those of curve B of Fig. 1A. The extent of the cooperativity decrease is very similar in magnitude for both the 43 kDa fragment and the 11-residue peptide. These results strongly suggest that the determinant responsible for the lowering of the \( n \) value by the 43 kDa chymotryptic fragment resides in the first 11 amino acids of its sequence. In these experiments, Hb, as well as the 43 kDa fragment and the synthetic peptide are virtually devoid of chloride except for residual amounts presumably representing very tightly bound, non-exchangeable chloride anions. These amounted to 1.5 and 0.6 mole/mole for the Band 3 fragment and for the synthetic peptide to Hb, respectively. The effects of added chloride are described below. The results in Figs. 1, 2 and 3 have been reproduced by two of the co-authors at different times using different preparations of Hb, peptide, and 43 kDa fragment.

**Effects on Oxygen Affinity** - As mentioned above, lowering of oxygen affinity by Band 3 has already been reported (8). Our results are included here since they show that this effect is not linear, i.e. the plots of oxygen affinity vs effector : Hb ratio indicate that the oxygen affinity continually decreases in the presence of either the 11-residue peptide (Fig. 2B) or the 43 kDa fragment (Fig. 3B) with the largest changes occurring at an effector : Hb ratios between 0 - 0.5. At higher ratios, oxygen affinity decreases more gradually than in the initial phase. These decreases are similar to those found when Hb is titrated with 2,3-DPG (see below).
Effects of Other Additives - In order to evaluate the specificity of the lowered cooperativity in the presence of sub-equivalent effector : Hb ratios, we studied the effects of two related peptides. Peptide 1 (Table 2) has two basic amino acid substitutions compared to the highly acidic 11-residue peptide described above; peptide 2 corresponds to the mouse Band 3 N-terminal sequence. Both of the peptides differ from the natural human N-terminal sequence in the number and location of acidic residues, i.e. the sequence of the natural human N-terminal 11-residue peptide has 7 acidic residues whereas peptide 1 in Table 2 has one of the acidic residues replaced by a basic Arg residue and a neutral residue replaced by a His. Some smaller unrelated peptides and amino acids were also tested (Table 3). None of these additives had any effect on the \( n \) value of human Hb, thus demonstrating the specificity of the effect of the natural 11-residue human synthetic peptide with human Hb.

Effects of Added Chloride - At a constant ratio of 0.5:1 of synthetic Band 3 human peptide : Hb, increasing amounts of added NaCl gradually restored the lowered Hill coefficient to normal values (Fig. 4). At a NaCl/peptide ratio of 100 corresponding to 25 mM added chloride, the \( n \) value increased to 2.7 with no further change at higher ratios. The finding that relatively high amounts of NaCl are required to restore cooperativity to normal values is an indication of its relatively low binding affinity with Hb compared with the synthetic peptide. As described above (16-20), chloride is a relatively non-specific regulator of Hb function since it binds to multiple sites. This result explains why a lowered cooperativity due to binding of the 11-residue N-terminal peptide was not reported (8) since buffers containing 0.1M chloride were used. However, these investigators (8) did note a lowered cooperativity with Band 3 when phosphate buffers were used.

Effect of 2,3-DPG on Cooperativity - A decreased Hill coefficient at sub-equivalent ratios of DPG to Hb has been reported (21). We compared the relative efficiencies of 2,3-DPG, the 11 residue synthetic peptide and the 43 kDa fragment in achieving this effect. With 2,3-DPG, the Hill coefficient was first lowered and then increased; the largest decrease occurred at a ratio of 0.43 followed by an increase to normal at a ratio of 1.25 (Figs. 5A, 5B, 5C). The partially hyperbolic shapes of the \( O_2 \) binding curves at the sub-equivalent DPG/Hb ratios resemble those found with the synthetic 11-residue peptide (Fig. 1A, Curve B) and the 43 kDa fragment. Likewise, the parabolic profile of the plot of \( n \) vs varying DPG/Hb ratios with a minimum at a ratio of about 0.5 (Fig. 5C) resembles the shapes of the corresponding plots found with both the 43 kDa Band 3 N-terminal fragment (Fig. 3A) and the 11-residue...
synthetic peptide (Fig. 2A). $P_{50}$ values also increase in a similar manner. Thus, it appears likely that these three effectors act by a similar mechanism to influence Hb cooperativity.

**Effect of N-Terminal Band 3 Peptide on HbS Polymerization** – Danish et al. (28) reported that a crosslinked peptide made up of two 8-residue segments corresponding to the N-terminal segment of Band 3 inhibited HbS polymerization. The rationale for using a crosslinked peptide was that one 8-residue segment would tether to the 2,3-DPG binding site and the second 8-residue fragment would extend from the HbS tetramer and prevent contacts between HbS tetramers (polymerization) by steric interference. No direct evidence was provided to support this model. The uncrosslinked peptide did not inhibit HbS polymerization although a 15-residue uncrosslinked peptide did inhibit HbS polymerization slightly. For all the experiments reported by Danish et al. (28), peptide/HbS ratios did not exceed 4:1. Since the rationale for using a crosslinked peptide was not convincing to us and the control uncrosslinked 8-residue peptide had no effect on HbS polymerization, we decided to study the effect of our 11-residue peptide on HbS polymerization in the complete absence of O$_2$ (27). As shown in Fig. 6, low ratios had little effect perhaps due to incomplete binding of peptide, but ratios of peptide : HbS higher than 2 showed a pronounced decrease of HbS polymerization; the largest effect occurred at a 5:1 ratio of peptide : HbS followed by a more modest decrease at higher ratios. We conclude that crosslinking of such peptides is not necessary to inhibit HbS polymerization.

**Discussion**

Cooperativity during oxygenation/deoxygenation of Hb occurs with changes in subunit contacts at the allosteric interface between the oxy (R) and deoxy (T) conformational states and at a set of salt bridges in the T-state that are absent in the R-state (10). Although the locations of these sites are known (10, 11, 14), their exact individual strengths relative to each other are not. Some recent findings that we have reported show that overall subunit contact strength at the allosteric interface is influenced even by small changes in its side chains, e.g. Asp-43($\gamma$) in HbF provides more subunit contact strength than does Glu-43($\beta$) in HbA (26). In addition, contributions to the overall strength of the allosteric interface of Hb arises from other parts of the tetramer, as we have shown by fusing the A-helix of the $\gamma$-subunit to helices B – H of the $\beta$-subunit to produce recombinant Hb Felix which accounts for a significant part of the 70-fold higher allosteric interface strength of HbF compared to HbA in the liganded state (25).
The established principles of Hb cooperativity as well as its less well understood and its controversial aspects have recently been reviewed (29). Maximum cooperativity occurs when the R/T equilibrium is as far towards the T-state as it is towards the R-state (10, 11, 14). Perutz (10) described a stereochemical mechanism involving the allosteric interface contacts and salt bridges (some involving DPG) that were either rearranged or broken during deoxygenation or oxygenation, respectively. The effectors described here also involve salt bridge formation with Hb so that this additional set of salt bridges requires a higher O2 tension to break. At a sub-equivalent ratio of 0.5, only one-half of the tetramers would have this additional set of salt bridges in the T-state. Thus, there would be two populations of Hb tetramers in the T-state. In such cases, the overall course of the cooperativity changes may not be a smooth transition to achieve maximum cooperativity due to this additional variability in response to O2 for those tetramers either with or without effector bound, as described next.

The results described above for sub-equivalent ratios of DPG : Hb, Band 3 : Hb, and synthetic peptide : Hb are consistent with the expanded two-state allosteric model of Kister et al. (21). Since the \( n \) value is at a minimum when the peptide : Hb ratio is about 0.5, \( \beta \)-subunit inequivalence in binding of the 43 kDa fragment, the 11-residue peptide, as well as DPG could be responsible for the lower cooperativity observed. The Monod-Wyman-Changeux (MWC) model for normal cooperativity indicates that all like subunits are affected equally during the switch between the two conformational states i.e. there is a smooth transition between the R and T states leading to a sigmoidal curve with maximal cooperativity. If some \( \beta \)-subunits had fragment bound and others did not, this condition would not be met resulting in a mixture of R state and two types of T-states leading to a heterogeneous binding curve having a decreased sigmoidal, partially hyperbolic shape affecting O2 binding. Analysis of Fig. 1B and Fig. 1A below is consistent with this explanation.

For Fig. 1B the T-state without bound peptide would be oxygenated first up to 50% saturation. Since no peptide is bound to this type of tetramer, the decrease in oxygen affinity (shift to the right) up to 0.5 fractional O2 saturation is less than that beyond 0.5 fractional O2 saturation (see distance between horizontal arrows). In the latter phase the second type of T-state tetramer with bound peptide would be oxygenated with a more pronounced decrease in oxygen affinity. When excess peptide is present only one type of T-state tetramer is present since all sites are saturated, i.e. all \( \beta \)-sites are occupied by effector and no longer inequivalent, thus restoring full cooperativity. Since chloride ion binds to several sites on
Hb (see above), at ratios of Band 3 : Hb ratios of 0.5 but with saturating chloride, all sites would be occupied by one type of effector or the other and cooperativity would be normal. These findings as well as the effects on oxygen affinity, which are similar for each of the effector molecules described here, are consistent with the possibility that the cytoplasmic portion of Band 3 that penetrates the red cell membrane interacts with intracellular Hb to influence its functional properties (see below). Earlier reports on the Band 3-hemoglobin interaction (cited in the Introduction) are consistent with this possibility.

Band 3 is the most abundant protein in the red cell membrane comprising about 25% of the total membrane protein (1, 2, 30). We used the pure cytoplasmic fragment of Band 3 as well as its corresponding N-terminal peptide prepared by synthesis to study effects on cooperativity. Thus, the report that intact red cell membranes can lower Hb cooperativity (9) can now be attributed mainly to the Band 3 that is present. Since the oxygen binding curve in the presence of sub-equivalent amounts of peptide to Hb (Fig. 1A, curve B) has a distinct hyperbolic character with an n value of 1.5, Hb binds more O\textsubscript{2} at a low O\textsubscript{2} tension (< 25 mm Hg) than it would at the same O\textsubscript{2} tension if the curve were sigmoidal, e.g. in the presence of DPG (Fig. 1A, curve C). For example, the sigmoidal character of curve C in Fig. 1A between 20 – 25 mm Hg (the physiological O\textsubscript{2} tension in the venous circulation) corresponds to 42 – 58% O\textsubscript{2} saturation compared to the 66 – 75% O\textsubscript{2} saturation in Curve B in the same O\textsubscript{2} tension range. If those Hb tetramers that adhere to the inner surface of the red cell membrane contain Band 3 cytoplasmic fragment bound in some fractional amount, such tetramers would have a higher capacity for oxygen due to their reduced cooperativity than would intracellular tetramers completely bound with DPG. The mutual competition between these two effectors could thus lead to a gradient of varying amounts of oxygen saturation among Hb tetramers, i.e. those in direct contact with the membrane with higher O\textsubscript{2}-saturation compared to the less O\textsubscript{2}-saturated tetramers in the interior of the red cell. Since the effects on cooperativity by purified Band 3 cytoplasmic fragment and its N-terminal peptide closely reflect that reported for intact membranes (9), a physiological role is possible.

The results on the effects of the 11-residue peptide on HbS polymerization under completely anaerobic conditions clarify a report on the effects of a similar peptide on polymerization (28). Our findings also suggest that HbS polymerization could be modulated by the red cell membrane under physiological conditions, and may be heterogeneous within the cell. Hence, an increased degree of O\textsubscript{2}-saturation of HbS at the membrane surface would also contribute to decreased HbS polymerization due to
a shift in the R/T equilibrium to the R-state, which does not polymerize. Whether events such as membrane contributions to O$_2$-transport and its consequent effects on HbS polymerization actually occur remain for future study.

**Acknowledgements**

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References


References (continued)


References (continued)


**Figure Legends**

**Fig. 1.** Oxygen Binding Curves of Hemoglobin S in the Presence of the 11-Residue Synthetic Peptide. The HbS concentration was 0.5 mM (tetramer) in 50 mM bis-Tris-Ac, pH 7.5, for each experiment. Part A - Curve A – HbS alone, Curve B – HbS with synthetic 11-residue peptide at a ratio to Hb of 0.5, Curve C – same as Curve B but including 2,3-DPG at a 1:1 ratio with Hb. The experiments were performed at 37° in a Hem O Scan instrument as described previously (see Experimental Procedures). The n value in the inset is $n_{\text{max}}$ calculated between 30-70% $O_2$ saturation. Part B – Plot of fractional $O_2$ saturation vs log $pO_2$. The individual data points were measured from the original graph of the Hem O Scan recording (having a grid background) of Fig. 1A. The horizontal double-headed arrows indicate the differences between curves A and B, B and C. Oxygen saturation changes are greater below 0.5 saturation than they are above this value, consistent with the two types of T-state tetramers described in the text.

**Fig. 2.** Effect of 11-Residue N-Terminal Band 3 Synthetic Peptide on Hemoglobin Functional Properties. Part A – Cooperativity changes. Part B – Oxygen affinity ($P_{50}$) changes. The Hb concentration (0.5 mM) and the peptide concentration were determined by amino acid analysis. The amount of peptide was varied to achieve the ratios shown.

**Fig. 3.** Effect of the 43 kDa Band 3 Fragment on Hemoglobin Functional Properties. Part A – Cooperativity changes. Part B – Oxygen affinity ($P_{50}$) changes. The concentrations of Hb and of the 43 kDa fragment were determined by amino acid analysis. The Hb concentration was 0.5 mM and the 43 kDa fragment concentration was varied to achieve the ratios shown.

**Fig. 4.** Effect of Chloride on Hemoglobin Cooperativity in the Presence of the 11-Residue Peptide. The Hb and peptide concentrations were constant at 0.5 mM and 0.25 mM, respectively. Chloride concentration varied from 0.5 – 50 mM.
**Figure Legends (continued)**

**Fig. 5.** Oxygen Binding Curves of Hemoglobin with Varying Ratios of 2,3-DPG. The experimental conditions were the same as those in Fig. 1. The DPG/Hb ratios are shown in the inset to the figure. Part A – Oxygen binding curves. Part B – Hill plots. Part C – Plot of $n$ vs DPG/Hb ratio (open circles) and $n$ vs $P_{50}$ (closed circles).

**Fig. 6.** Effect of the 11-Residue Peptide on HbS Polymerization. The $C_{sat}$/dextran method of Bookchin as described in Himanen et al. (27) was used.
Table 1
Amino Acid Composition of 43 KD Band 3 Fragment and Synthetic Human N-Terminal Peptide

**43 kDa Band 3 chymotryptic cytoplasmic fragment peptide**

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<th>Amino Acid</th>
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<tr>
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<td>Arg</td>
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The amount of Thr, Ser, Met, Tyr and Trp were not included since these amino acids are partially or completely destroyed during acid hydrolysis.

**Band 3 N-terminal human peptide: AcMEELQDDYDE**

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<th>Amino Acid</th>
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<td>Glx</td>
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<tr>
<td>Tyr</td>
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<tr>
<td>Met</td>
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The amino acid analysis values are an average of 3 determinations. Met is partially destroyed. The values are expressed relative to Asp, which was set to 3.0.


Table 2

Effect of Other Band 3 Peptides on Cooperativity and Oxygen Affinity

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Ratio to Hb</th>
<th>n</th>
<th>P_{50}</th>
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<td>1\textsuperscript{a} AcMEELRDHYEDE</td>
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<td>2\textsuperscript{b} AcMGDMRDHEEVL</td>
<td>0</td>
<td>2.6</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>2.6</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>0.50</td>
<td>2.6</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>2.8</td>
<td>8</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Analog of natural human peptide.

\textsuperscript{b} N-terminal sequence of Band 3 from mouse red cell membrane.
Table 3

Effects of Other Additives on Cooperativity and Oxygen Affinity

<table>
<thead>
<tr>
<th>Additive</th>
<th>Ratio to Hb</th>
<th>n</th>
<th>P&lt;sub&gt;50&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>N-Ac-Asp-Glu</td>
<td>0</td>
<td>3.0</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>2.8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0.64</td>
<td>3.0</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>3.22</td>
<td>2.9</td>
<td>11</td>
</tr>
<tr>
<td>N-Ac-Asp</td>
<td>0</td>
<td>3.0</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>2.9</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0.64</td>
<td>3.0</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>3.22</td>
<td>2.9</td>
<td>10</td>
</tr>
<tr>
<td>N-Ac-(Ala)&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0</td>
<td>3.0</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>2.8</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>0.64</td>
<td>3.1</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td>3.22</td>
<td>3.1</td>
<td>10</td>
</tr>
</tbody>
</table>
Fig. 1A

% \( \text{O}_2 \) Saturation

\( \text{pO}_2 \) (mmHg)

<table>
<thead>
<tr>
<th>Curve</th>
<th>Description</th>
<th>n</th>
<th>( P_{50} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Hb alone</td>
<td>2.7</td>
<td>8</td>
</tr>
<tr>
<td>B</td>
<td>Hb + Peptide</td>
<td>1.5</td>
<td>12</td>
</tr>
<tr>
<td>C</td>
<td>Hb + Peptide + DPG</td>
<td>2.3</td>
<td>20</td>
</tr>
</tbody>
</table>
Fig. 3A

Hill Coefficient (n)

Band 3 N-Terminal Fragment: Hb Ratio
Fig. 3B

Band 3 N-Terminal Fragment: Hb Ratio

$P_{50}$ (mmMg)
Human erythrocyte membrane band 3 protein influences hemoglobin cooperativity.
Possible effect on oxygen transport
Yuxun Zhang, Lois R. Manning, Jill Falcone, Orah Platt and James M. Manning

J. Biol. Chem. published online July 31, 2003

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