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Oligopeptides as potential antiaggregation agents for deoxyhemoglobin S

(sickle cell hemoglobin/inhibitors/minimum gelling concentration)

SHIGEO KUBOTA AND JEN TSI YANG*

Cardiovascular Research Institute and Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

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ABSTRACT **Oligopeptides that mimic segments of the amino acid sequence of hemoglobin S at potential contact sites can be used to inhibit aggregation. These oligopeptide inhibitors raise the minimum gelling concentration of deoxyhemoglobin S so that chemical modification does not have to be used. The hexapeptide amides of both β^S1–6, which is believed to be one of the contact areas among aggregates, and β^A1–6 of hemoglobin A increase the minimum gelling concentration by more than 70%. The hexapeptide amide β79–84 behaves like β1–6 (β being β^S or β^A). Shorter oligopeptides, such as β^S3–6, are less effective as an inhibitor but longer ones, such as β^S1–8, are no more effective than β1–6. Permutations of the sequence, such as β^S125634, do not alter the percent increase in the minimum gelling concentration. Leu- and Met-enkephalin increase the minimum gelling concentration just as β1–6 does, but $(Pro)_6$ is not very effective. Thus, the use of complementary oligopeptides as inhibitors is extended to include certain "flexible" peptides, which can adapt themselves to interfere with the molecular contacts and thereby gelation of deoxyhemoglobin S.**

The primary sequence of hemoglobin (Hb) S at the NH_2 terminus of the two β chains is

1 5 10
Val-His-Leu-Thr-Pro-*Val*-Glu-Lys-Ser-Ala . . .

Out of 574 amino acid residues of the normal adult Hb A molecule, only two glutamic acid residues in the 6 position of the β chains are replaced by two valine residues in Hb S. The β6 region lies on the surface of the Hb molecule. Residues β1–3 are nonhelical; residues β4–19 form helix A. The specificity of the gelation of deoxyHb S suggests some minor conformational difference between Hb S and Hb A in solution. Intuitively we might suspect that the β1–6 region is a possible contact site between neighboring molecules. One of us further suggested that the first turn of helix A in Hb S might be unwound to adapt the first six residues for aggregation between neighboring Hb molecules (1). (Proline is a helix breaker, but β5 Pro is tolerated in Hb A at the beginning of a helix that does not have hydrogen bonds.) This possible change in local conformation could occur before or during gelation of deoxyHb S in solution. However, x-ray diffraction of Hb S crystals may not be able to detect this small difference, which might be present only in the aggregate.

Our working hypothesis (1) is that oligopeptides mimicking portions of the amino acid sequence at the contact sites of the protein molecule might be used to inhibit aggregation. DeoxyHb S molecules aggregate to form nuclei (nucleation), which rapidly grow into a fiber-like gel (polymerization) (2). The complementary oligopeptides might compete for the binding sites on the protein molecule, thus acting as noncovalent terminators to block further aggregation. This inhibition is possible if the protein–oligopeptide interaction is energetically favorable or if the oligopeptide concentration is sufficiently high. The size of the nuclei is not known at present and numerous equilibria such as $P_i + P_j \rightleftarrows P_{i+j}$ can exist (P refers to the Hb molecule). These reactions do not change the thermodynamic results of gelation although they will affect its kinetics.

We have shown elsewhere (2) that oligopeptides such as β-6 hexapeptide interfere with the gelation of deoxyHb S. Addition of these peptides raises the minimum Hb concentration at which the protein gels; this is commonly referred to as the minimum gelling concentration (MGC). The maximum increase amounts to about 75% at a molar ratio of about 2.5 peptides per heme. A similar concept of using complementary peptides as inhibitors of Hb S gelation has also been put forward by Schechter (3).

Our working hypothesis (1) also implies that only one of the two β^S1–6 regions is involved in the formation of fibers. Upon deoxygenation the $\alpha_1\beta_1$ and $\alpha_2\beta_2$ halves in the Hb molecule slide past each other. As a result, their regions of the helices E and F and nonhelix EF of the two β chains shift away from each other in the deoxy-state. Because deoxygenation trigers the formation of Hb S fibers, it is tempting to suggest that the nonhelix EF (β77–84) region

77 80 84
. . . His-Leu-Asp-Asn-Leu-Lys-Gly-Thr . . .

is a candidate for contact with β^S1–6 of another molecule. The geometric arrangement is such that the deoxy-form (the T conformer according to some allosteric models) makes such a contact possible. Originally the contacts were thought to exist in the same strand rather than in the adjacent strand as the x-ray crystallographers have now proposed (4, 5). For a molecule as large as Hb, the number of contact sites between neighboring molecules of both intra- and inter-strands must be large, but the mutation site (β1–6 region) might be an important contact area for the formation of fibers.

It is worth recalling that in arterial blood the Hb is 95–98% saturated with oxygen. The so-called deoxygenated state in the venous blood of a normal adult retains about 70% saturation of oxygen. Even though the binding affinity of oxygen by Hb S is lower than that by Hb A (6, 7), the deoxyHb S *in vivo* still has a considerable amount of bound oxygen. Thus, the fibrous structure of deoxyHb S that is completely devoid of oxygen, as postulated from x-ray diffraction studies (4, 5), may differ from that of Hb S inside the sickle cell. L. Peller (personal communication) points out that the aggregates of deoxyHb S *in vivo* resemble a copolymer of Hb S molecules having various

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Abbreviations: Hb, hemoglobin; MGC, minimum gelling concentration.

* To whom correspondence should be addressed.

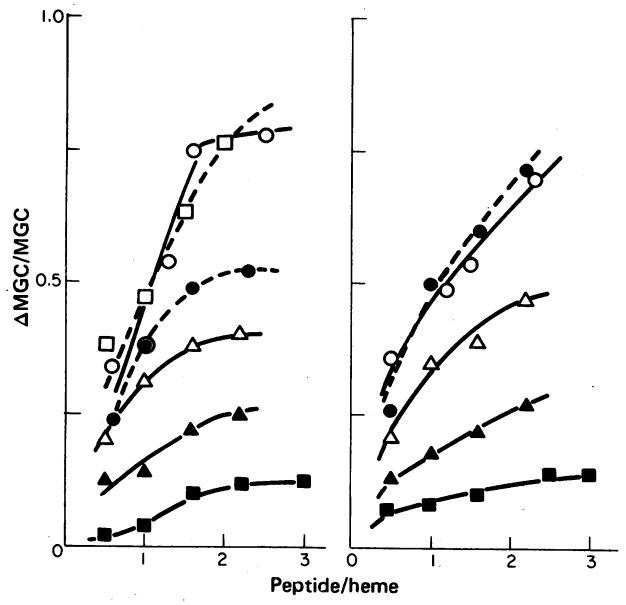

FIG. 1. Increase in MGC of deoxyHb S in the presence of oligopeptide amides and amino acid amides mimicking the NH_2 termini of the β chains of (*Left*) Hb S and (*Right*) Hb A. Solvent: phosphate buffer (pH 6.8, ionic strength 0.1) at 37°. □, β1–8; ○, β1–6; ●, β3–6; △, β4–6; ▲, β5–6; ■, β6.

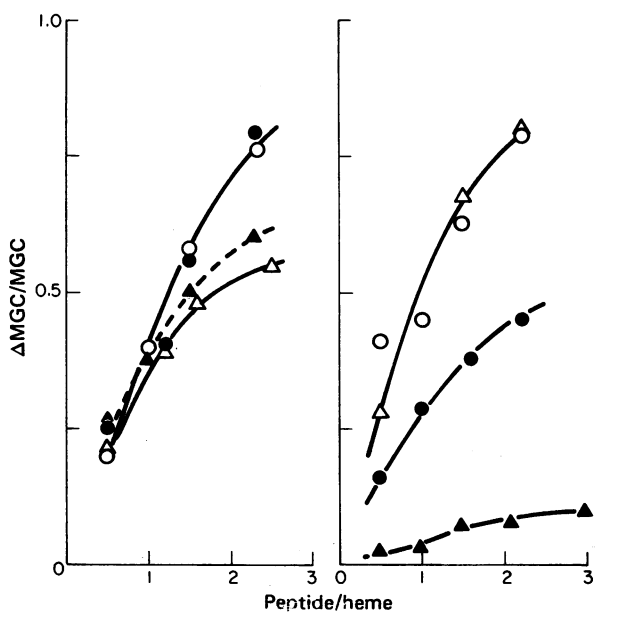

FIG. 2. Increase in MGC of deoxyHb S in the presence of various oligopeptides. (*Left*) Parts of the amides of the β chain and permuted β^S1–6 and β^S3–6 of Hb S. ●, β79–84; ○, β^S125634; ▲, β81–84; △, β^S5634. (*Right*) Non-Hb peptides and (▲) homoserine. The enkephalins (△, Leu-enkephalin; ○, Met-enkephalin) have a carboxyl group and (●) $(Pro)_6$ has an amide group at the COOH terminus. Solvent: same as for Fig. 1.

amounts of bound oxygen. At any percentage of saturation, the distribution of unaggregated Hb molecules with zero to four oxygen molecules depends on the four equilibrium association constants. From electron micrographs Edelstein *et al.* (8) found that fibers of Hb S obtained by direct lysis of sickle cells with negative staining solution had several forms of Hb S aggregates. These included the eight-stranded fibers and also thick cable-like assemblies of many monofilaments, two-dimensional sheets, and six-stranded fibers in stack-disk arrangement as reported by Finch *et al.* (9).

This work further tests the use of oligopeptides as inhibitors for aggregation without resorting to chemical modification. We report an increase in the MGC of deoxyHb S by the addition of various oligopeptides and the degree of specificity, or the lack of it, of these additives.

MATERIALS AND METHODS

Preparation of Hb S. Blood obtained from a patient having sickle cell trait was used for preparing Hb S by the procedures of Huisman and his associates (10, 11). We used carbon tetrachloride instead of toluene during hemolysis to facilitate the removal of the hemolysates from the upper layer of the mixed solvents. Disk electrophoresis on polyacrylamide gel gave a single band for the Hb S fraction. The pooled solution of Hb S at 4° was concentrated through a Bio-Fiber 50 Beaker, which was attached to a Bio-Fiber Pump Module. The solution was then dialyzed against phosphate buffer (pH 6.8 and ionic strength 0.1) at 4° and further concentrated through a Bio-Fiber Minibeaker to about 25% Hb S, which contained a small amount of metHb S (less than 5%) as checked spectroscopically (12).

Synthesis of Oligopeptides. To insure purity we synthesized a series of oligopeptide amides by conventional organic chemistry instead of solid-phase synthesis. The general strategy was: (*i*) to prepare *t*-butyloxycarbonyl amino acids (13) and their active esters, *N*-hydroxysuccinimides (14), with all functional groups protected by benzyl and carbobenzoxy groups; (*ii*) to couple an active ester with another amino acid in 1:1 dioxane/water at room temperature (except for β1–2 dipeptide, which was converted into an azide in ethylacetate at 4° before being coupled with other peptides because it contained a histidine residue); (*iii*) to convert the COOH-terminal amino acid into an amide; (*iv*) to prepare longer peptides by coupling shorter ones; and (*v*) to remove the protecting groups in boron tris(trifluoroacetate) (15). The final products were passed through a Dowex-1 chloride column.

Determination of Minimum Gelling Concentration. The MGC of deoxyHb S was followed visually in a test tube (16). Deoxgenation was done by nitrogen flush over the solution and its completion was checked spectroscopically (12). To avoid a thin film forming at the gas–liquid surface during deoxygenation, we used a small magnetic bar inside the tube and slowly moved it at intervals with another magnetic bar outside the tube. (The omission of this step would give a false high MGC.) For protein concentrations above MGC, Hb S usually gelled within 5–10 min after nitrogen flush at 37°. Near MGC, deoxyHb S gelled in about 20–25 min. If gelation did not occur after 1.5–2 hr, we considered the Hb S concentration below its MGC. Each experimental point was the average of three measurements; its MGC varied by about ±0.5%. Since the MGC is slightly dependent on ionic strength, appropriate amounts of NaCl were added to the control experiments (Hb S alone) to compensate for the change in ionic strength due to the added oligopeptides.

RESULTS

Figs. 1 and 2 summarize the effectiveness of various oligopeptide amides on raising the MGC of deoxyHb S in phosphate buffer (pH 6.8; ionic strength 0.1). The MGC of Hb S alone (as the control) was 9.5 g/dl at 0.1 ionic strength. (The MGC is sensitive to solvent composition and temperature of the solution; see, for example, refs. 6 and 17.) Because the MGC also increases slowly with the ionic strength of solution (2, 18), addition of NaCl raised the MGC to about 11% at 0.36 ionic strength. (The imidazole·HCl in the oligopeptide was neutralized with con-

centrated NaOH, which produced additional NaCl.) A pH lower than that of arterial plasma was chosen for two reasons: the pH inside the erythrocytes is slightly lower than 7.4 and the lower MGC at low pH is easier to handle experimentally.

Several features emerge from our results in Fig. 1. First, in all cases the oligopeptide additives increase the MGC of deoxyHb S, more so at higher molar ratios of the peptide to heme. The increase seems to gradually level off above peptide/heme $\cong$ 2. Second, β^A1–6 amide (Fig. 1 *right*) is essentially as effective as β^S1–6 amide (Fig. 1 *left*). For instance, at peptide/heme = 2.5, both hexapeptides raise the MGC of deoxyHb S by more than 70%. This is not surprising if several amino acid residues are involved in contacts and the end residue (Glu or Val) may not have much effect on the MGC. (The curves for β^A1–6 and β^A3–6 virtually coincide; the curve for β^A1–6 increases more gradually than that of β^S1–6; the tetrapeptide β^A3–6 appears to raise the MGC more effectively than β^S3–6.) Third, the shorter peptides, β4–6 and β5–6 amides, are less effective than the β1–6 amide. Valine (β^S6) amide has almost no effect on the MGC at all. On the other hand, β^S1–8 octapeptide amide appears to have the same effect as β^S1–6.

Surprisingly, permutation of the sequence of an oligopeptide additive does not seem to affect the increase in the MGC of deoxyHb S. Thus, β^S125634 hexapeptide amide (Fig. 2 *left*) has about the same MGC as β^S1–6. Likewise, β^S5634 tetrapeptide amide is about as effective as β^S3–6. The hexapeptide amide β79–84 (Fig. 2 *left*) is as effective as β1–6 amide in raising the MGC of deoxyHb S. Here again, shorter oligopeptides such as the tetrapeptide amide β81–84 are less effective. (We also synthesized the octapeptide amide β77–84, but it was insoluble in phosphate buffer at pH 6.8.) Perhaps the nonhelix EF region is indeed another contact area between the Hb S molecules. The results of the permutation experiments suggest the lack of specificity of these oligopeptides as inhibitors for aggregation. To further test this point, we studied the effect of two enkephalins, Tyr-Gly-Gly-Phe-Met and Tyr-Gly-Gly-Phe-Leu, as additives; they are believed to inhibit the binding of morphine to the synaptic plasma membrane and therefore are of current interest in pharmacology. Their effects on ΔMGC/MGC are about the same as the effect of β1–6 hexapeptide amide (Fig. 2 *right*). At present it is difficult to say where the binding sites for the two pentapeptides are.

The conformation of free oligopeptides is largely "random" in solution, but they may adopt a fixed conformation once attached to a protein. In fact this is necessary if they supplant a region of the β-chain sequence of the Hb molecule. Hexa-L-proline amide is less flexible than any other hexapeptide studied; possibly $(Pro)_6$ is less adaptable and thus less able to interfere at the contact area of the Hb aggregates (Fig. 2 *right*).

Certain amino acids, such as L-homoserine, L-glutamine, and L-asparagine, have been reported to inhibit and reverse the sickling of erythrocytes *in vitro* (except for irreversible sickle cells) (19–21). However, L-homoserine, for instance, has little or no effect on the increase in MGC (Fig. 2 *right*). That the shape of red cells can be altered by certain amino acids which have no effect on gelation is intriguing. A satisfactory explanation is still lacking.

DISCUSSION

Segments of the peptide chain in a protein molecule are fixed in a right conformation, but isolated fragments such as the oligopeptides may be random in solution. The binding, if any, of these compounds to proteins would result in a loss of conformational entropy, which must be compensated by a decrease in enthalpy through hydrogen bonding or hydrophobic interaction or both (although the exact locations of these interactions are not known). Of course these oligopeptides are competing with sites on Hb S where there is also an enthalpy decrease on aggregation. The significant point is that the protein–protein association is reduced in the presence of the oligopeptides. In general the equilibrium association constant of proteins is expected to be larger than the equilibrium binding constant between a protein and an oligopeptide. The standard free energy change for the equilibrium involving proteins and oligopeptides would determine the effectiveness of these antiaggregation agents. Raising their concentrations could help shift the equilibrium toward the protein–oligopeptide complex. Recently, Hofrichter *et al.* (22) reported that the changes in free energy, enthalpy, and entropy accompanying the Hb S gelation were very small. This finding could be favorable for the disaggregation of deoxyHb S by oligopeptides. The idea that is implicit in deoxyHb S–oligopeptide interaction can equally well be applied to other biological aggregates if the binding of the antiaggregation agent to a biopolymer is energetically favorable, as for instance, in the dissociation of the insulin dimer (2). Although the evidence is not yet conclusive, the proposed working hypothesis merits further examination of its possible relevance to other biological systems.

The ultimate objective of studying Hb S is of course to find a therapy for sickle cell anemia. Our idea of using noncovalently bound oligopeptides as potential inhibitors is prompted by the belief that any chemical modification, such as carbamoylation by cyanate, will equally modify other proteins that are exposed to the reagent. Clinically, this may cause serious side effects. For the same reason we avoid the use of general denaturing agents that drastically alter the conformation of the proteins.

The function of an antisickling agent is essentially to raise the MGC of deoxyHb S so high that the protein will not gel at physiological concentrations. In our experiments *in vitro* the Hb molecules are completely deoxygenated, unlike the so-called deoxyHb S inside the erythrocytes, which retains a considerable amount of bound oxygen. However, our MGC values in the presence of oligopeptides (Figs. 1 and 2) are much lower than the mean intracellular Hb concentration. (The pH used in our experiments was also lower than that inside the erythrocytes.) However, Minton (23, 24) found that the tendency of Hb S to aggregate only slightly decreased with increasing oxygen saturation until the fractional saturation of nonaggregated Hb S exceeded one-half.

However potent any antiaggregation agent for Hb S may be found to be, its transport across the cell membrane is a serious problem. New techniques must be developed to overcome this difficulty. One approach is to search for an oligopeptide permease like the carrier Ames *et al.* (25) have found for *Escherichia coli*. Another approach is to partially lyse the erythrocytes and subsequently reseal them (26, 27). This allows the oligopeptides to enter the membrane before reclosure. The drawback is that not all the Hb molecules return to the interior of the cells. The same is true for compounds other than Hb. The extent to which erythrocytes are damaged during this cycle of opening and resealing is still unknown. Frequently we found that the reconstituted erythrocytes did not possess the biconcave shape (J. T. Yang, unpublished data). Perhaps the use of liposomes as potential carriers of oligopeptides is a promising approach. This is an area of expanding interest (28–30) with perhaps a potential applicability to the entry problem.

Addendum. After completing this work we were gratified to learn that Votano *et al.* (31) also used certain tri- and tetrapeptides as noncovalently bound inhibitors for the gelation of deoxyHb S.

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