Band 3 Peptides Inhibit Deoxy S Polymerization:  
Viscosity Studies

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We have previously obtained evidence that N-terminal band 3 peptides inhibited deoxyhemoglobin S (deoxy S) polymerization as determined by equilibrium solubility assays. An N:1-15AA fragment binds to the 2,3-diphosphoglycerate (2,3-DPG) receptor locus of deoxy S with five to seven amino acids (AA) extending internally, while ten to eight AA remained external to deoxy S and inhibited polymerization by steric hindrance. A true mirror-image peptide, corresponding to two N:1-8AA + lysine (K) linked by coupler, binds to the 2,3-DPG loci of two deoxy S molecules, tethering them together to form "binary complexes" incapable of entering the polymer chains. The reduction in the concentration of deoxy S available for extended chain formation decreased polymerization. We now report time:viscosity profiles of the sol-gel transformation of purified solutions of deoxy S with and without peptides and studies of the gel solidity at equilibrium. Samples with peptides had longer lag times than controls of similar deoxy S concentrations. The mirror-image peptide was a more effective inhibitor than the N:1-15AA peptide. When the mirror-image peptide was present in peptide:hemoglobin molar ratios of 0.2:1:1, the increases in lag time were equivalent to decreasing the deoxy S concentrations by 15-25%, comparable to projected major therapeutic effects. Gel solidity, determined by yield temperature, was less in the sample with mirror-image peptide compared to control. These results support the proposed mechanisms of inhibition of deoxy S polymerization by band 3 peptides. © 1993 Wiley-Liss, Inc.

Key words: band 3 peptides; sickle hemoglobin; 2,3-DPG, receptor locus

INTRODUCTION

We have previously obtained evidence that synthetic N-terminal band 3 peptides bind to the 2,3-diphosphoglycerate (2,3-DPG) receptor locus of deoxyhemoglobin S (deoxy S), as demonstrated by a rightward shift in the hemoglobin binding curves and, are inhibitory of deoxy S polymerization as measured by equilibrium solubility assays [1-5]. We now describe the inhibitory action on deoxy S polymerization by two of the peptides as measured by continuous viscosity monitoring of the sol-gel transformation and by the physical properties of the deoxy S gels. One peptide, corresponding to the N-terminal 15 amino acids (AA) of the cytoplasmic domain of erythrocyte band 3, was designed to bind to the 2,3-DPG receptor locus of deoxy S with five to seven AA extending into the central cavity along the dyad axis, while the remaining ten to eight AA would be expected to inhibit polymerization through steric hindrance by the protruding portion of the peptide. A second true mirror-image peptide, in which duplicate peptides comparable to the N-terminus of band 3 were linked by a coupler, was designed to bind to the 2,3-DPG loci of two deoxy S molecules tethering them to form "binary complexes" that could not properly enter the polymer chains. Decreased polymerization would then result from the reduction in the concentration of deoxy S available for extended chain formation. The following experiments characterize the interactions of such designed synthetic peptides with purified solutions of stripped deoxy S.

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MATERIALS AND METHODS
Peptide Synthesis and Purification

Two peptides corresponding to the N-terminal cytoplasmic domain of band 3 were synthesized, purified to homogeneity, and neutralized as previously described [5]. One was N:1-15AA, corresponding to the N-terminal one to fifteen AA of band 3 (Ac-M-E-E-L-Q-D-D-Y-E-D-D-M-E-E-N; Mr 1947). The other was a true mirror-image peptide corresponding to two N-terminal eight AA of band 3 coupled through an added lysine using Bis(sulfosuccinimidyl)suberate (BS3) (Ac-M-E-E-L-Q-D-D-Y-K-COUGLER-K-Y-D-D-Q-L-E-E-M-cA; Mr 2564).

Hemoglobin Preparation

The methods used for hemoglobin purification, concentration, dialysis with 5 mM Bis Tris, pH 7.40, and deoxygenation with N2 and Na2S2O4 to PO2 = 0 mm Hg were as previously published [1-5].

Controls for Excluded Volume Occupied by Peptide

Because the presence of unbound peptide would enhance the polymerization of deoxy S by increasing the deoxy S activity through excluded volume effects, controls using bovine serum albumin (BSA) were also studied. The amount of BSA was calculated after consideration of the size of the peptide and molar amounts present in the experiment.

Time:Viscosity Profiles

The effects of addition of the two peptides on the kinetics of deoxy S polymerization were monitored by viscometry using a Wells-Brookfield HBT Digital microviscometer (1.565° cone) operating at a shear rate of 38.4 sec−1 (Brookfield Engineering Laboratories, Inc., Stoughton, MA). Maintenance of temperature, hydration, anaerobic conditions, and initiation of the sol-gel transformation by temperature jump were as previously published [6,7]. In our previous studies, the coefficient of variation of lag times between duplicate runs was 9% [7]. The length of the lag phase and the time to peak, and peak viscosity values for samples with peptide were compared to those for samples without peptide. The lag times of the few control runs of deoxy S dialyzed with 5 mM Bis Tris buffer were equivalent to those previously reported using 150 mM K phosphate buffer in the same system [7-9]. The more extensive data with phosphate buffer were therefore used to compare the lag times of runs with and without peptides. Lag times of samples with peptide were superimposed on the regression line for log 1/lag (min) vs. log [Hb] for deoxy S (Fig. 1 in Danish et al. [7,8]).

Figure 1. Time: viscosity profiles of two solutions of deoxy S of initial hemoglobin concentration of 20.2 g/dl with and without N:1-15AA peptide. Hemoglobin:peptide molar ratio was 1:2. Solution conditions were PO2 0 mm Hg, pH 6.50, at 37°C.

RESULTS

Kinetic (Time:Viscosity) Studies

N:1-15AA. This peptide, when present in 2:1 peptide:hemoglobin molar ratio, was associated with minimal prolongation of the lag time and lower peak viscosity compared to control (Fig. 1). A sample of initial hemoglobin concentration of 20.2 g/dl had a lag time of 261 sec and a peak viscosity of 56 cps at 390 sec. The run with peptide:hemoglobin of 2:1 of the same initial concentration had a lag time of 346 sec and a peak viscosity of 44 cps at 450 sec. This degree of prolongation of lag time is equivalent to decreasing the hemoglobin concentration to 19.9 g/dl (2%).

Mirror-image BS3 peptide. Time:viscosity profiles of three studies of deoxy S of different initial hemoglobin concentrations and different peptide:hemoglobin molar ratios are shown in Figures 2-4. Figure 2 includes a control for excluded volume effect using an amount of...
Fig. 2. Time:viscosity profiles of deoxy S of initial hemoglobin concentrations of 21.1-21.3 g/dl. The BSA sample was the control for the excluded volume of the peptide, assuming no binding of peptide to deoxy S. Peptide was mirror image BS5 peptide in 1:1 hemoglobin:peptide molar ratio. Solution conditions were PO2 0 mm Hg, pH 6.88, at 37°C.

Fig. 3. Time:viscosity profiles of deoxy S of initial hemoglobin concentrations of 20.9-21.1 g/dl with and without mirror-image BS3 peptide in hemoglobin:peptide molar ratios of 1:0.25 and 1:0.5. Solution conditions were PO2 0 mm Hg, pH 6.95, at 37°C.

BSA calculated to occupy the same volume as the peptide, assuming no binding of peptide to hemoglobin. The lag time of the control deoxy S sample of 21.3 g/dl was 118 sec and that of the S-BSA sample of 21.1 g/dl was 135 sec; the deoxy S sample of 21.3 g/dl with peptide present in 1:1 peptide:hemoglobin molar ratio did not gel during the 3,900 sec of monitoring. This is equivalent to decreasing the deoxy S concentration to at least 16.9 g/dl (>20%). Figure 3 shows time:viscosity profiles of a deoxy S sample of initial hemoglobin concentration of 20.9 g/dl with a lag time of 394 sec and two runs of deoxy S of 21.2 and 21.0 g/dl with peptide present in peptide:hemoglobin ratios of 0.25:1 and 0.5:1, respectively. The lag times of the latter runs were 2,070 and 6,200 sec. The lengths of these lag times are comparable to decreasing the hemoglobin concentrations to 17.8 (15%) and 15.7 g/dl (25%), respectively. The times to peak viscosities were longer, and the peak viscosity values were lower as more peptide was present in the initial sample. The studies shown in Figure 4 were done with higher initial hemoglobin concentrations of 26.0 g/dl. When peptide:hemoglobin ratio was 0.5:1, lag time was 72 sec compared to 8 sec in the sample without peptide. This degree of prolongation of lag time by peptide is comparable to decreasing the deoxy S concentration to 22.9 g/dl (12%).

Gel Studies

The yield temperatures of gels formed of deoxy S of initial concentration of 26.0 g/dl at 37°C were determined after annealing the gels in the absence of stress or strain for 30 min. The yield temperature of the sample with mirror-image BS3 peptide (0.5:1 peptide:hemoglobin) was 19.6°C, whereas the control gel had a yield temperature of 16.9°C. The gel formed with peptide present had less solid behavior than the gel without peptide. Since the polymer fraction of the gel with peptide (.50) was less than that of control (.54), the less solid gel behavior most likely is due to lower polymer fraction rather than to differences in gel structures.

DISCUSSION

Both synthetic peptides were associated with increases in lag times of deoxy S gel formation when compared to controls of similar initial hemoglobin concentrations and solution conditions. The N:1-15AA, when present in peptide:hemoglobin molar ratio of 2:1, was associated with a minimal increase in lag time (Fig. 1), whereas the mirror-image peptide was the more effective inhibitor of deoxy S polymerization. Significant increases in lag times were evident at peptide:hemoglobin ratios as low as 1:0.25:1 (Fig. 3); at ratios of 1:1, no gelation occurred during the 65 min of monitoring of the sample (Fig. 2). The difference in effectiveness of these two peptides is consistent with the proposed mechanisms of inhibition of
deoxy S polymerization. The N:1-15AA fragment binds to the 2,3-DPG receptor locus (as previously demonstrated by rightward shifts in the hemoglobin binding curves [5]), with ten to eight AA remaining external to the deoxy S molecule, inhibiting polymerization by steric hindrance. This protruding portion, which if fully extended would project 24–30 Å from the surface of the hemoglobin, also increases the activity of the unbound deoxy S molecules. The effect of this peptide on deoxy S polymerization reflects the contributions of the deoxy S molecules bound to peptide that are unable to enter the polymer and the increased activity of the unbound deoxy S molecules (brought about by that portion of peptide extending into the solution) that would enhance polymerization. The mirror-image peptide, by binding two deoxy S molecules (as demonstrated by decreased osmolality and viscosity [5]), decreases the concentration of polymerizable molecules to a greater extent than the N:1-15AA fragment. Since the excluded volume occupied by the portion of the mirror-image peptide in solution between molecules by calculation closely approximates that of the N:1-15AA fragment, this peptide would be expected to have comparable effects on the activity of unbound deoxy S molecules.

The inhibition of deoxy S polymerization by other compounds has been studied mainly by equilibrium solubility assays, where solubility ratios (i.e., ratio of solubility of deoxy S plus reagent to that of pure deoxy S) of 1.3 are indicative of those believed necessary for a major therapeutic effect [10,11]. Our previously reported solubility ratios with the mirror image peptide reach 1.3 with peptide:hemoglobin ratios as low as 1:1 [5]. Only p-aminobenzoylpolyglutamyldiphenylalanines [12] and macro-molecular polyanions [13], noncovalent antisickling compounds that bind to the 2,3-DPG receptor locus, have been reported to be effective in such low effector:hemoglobin ratios.

Our present kinetic assays of the inhibition of the sol-gel transformation by peptides are the first studies using time:viscosity profiles of deoxy S solutions to monitor effects of therapeutic agents. Somewhat analogously to Sunshine et al. [10] and Eaton and Hofrichter [11], we have developed criteria for obtaining a therapeutic effect when inhibition of deoxy S polymerization is monitored by viscometry. Our calculations are based on lag times (using shear rates comparable to those of microcirculation [14]) of mixtures of deoxy S and A and deoxy S and F of initial total hemoglobin concentrations and temperatures close to in vivo [8,9] (Table I). For a therapeutic effect to occur (comparable to patients with Hb S-β-thalassemia with 20% Hb A), lag time ratios (ratio of lag time for the mixture to lag time for pure deoxy S) of 3–15 are necessary. For a major therapeutic effect (e.g., comparable to patients with Hb S-hereditary persistence of fetal hemoglobin, in which 30% Hb F is present in a

<table>
<thead>
<tr>
<th>Table 1. Lag Time Ratios for Different Mixtures of Deoxy SA and Deoxy SF Calculated Using Initial Total Hemoglobin Concentrations of 20 and 33.5 g/dl at 37°C From Danish et al. [8–9]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mixture</td>
</tr>
<tr>
<td>SA</td>
</tr>
<tr>
<td>SF</td>
</tr>
<tr>
<td>AS</td>
</tr>
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</table>

TABLE II. Lag Time Ratios for Deoxy S With Mirror-Image Peptide at Different Peptide: Hemoglobin Molar Ratios at 37°C

<table>
<thead>
<tr>
<th>[Hb] (g/dl)</th>
<th>Pep:Hb</th>
<th>Lag time ratio</th>
<th>Therapeutic effect</th>
</tr>
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<tbody>
<tr>
<td>21.2</td>
<td>0.25:1</td>
<td>5</td>
<td>Present</td>
</tr>
<tr>
<td>21.0</td>
<td>0.5:1</td>
<td>16</td>
<td>Major</td>
</tr>
<tr>
<td>21.0</td>
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<td>16</td>
<td>Present</td>
</tr>
<tr>
<td>21.3</td>
<td>1:1</td>
<td>&gt;33</td>
<td>Major</td>
</tr>
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REFERENCES
