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Isolation of antibodies specific to sickle hemoglobin by affinity chromatography using a synthetic peptide

(immunoabsorption/radioimmunoassay/solid-phase peptide synthesis)

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ABSTRACT **Antibodies to hemoglobin have been studied with a radioimmunoassay which employs [^{14}C]carbamylated (= carbamoylated) hemoglobin S. An antiserum raised against hemoglobin S, which initially discriminated poorly between hemoglobins S and A, was fractionated by absorption to a column of Sepharose to which a synthetic peptide corresponding to the first 13 amino-acid residues of the β chain of sickle hemoglobin had been covalently bound. A subpopulation of the antiserum was eluted from this column with 4 M guanidine · HCl. These antibodies showed binding to hemoglobin S but not to hemoglobin A and this interaction could be inhibited by the synthetic peptide. These antibodies, of demonstrated fine structural specificity, may be useful in the detection of sickle hemoglobin and in the study of its structure in solution.**

Antibodies which bind specifically to limited, well-defined regions of protein molecules have been prepared by several methods. Animals have been immunized with polypeptide fragments of the protein, and, in some cases, the antibodies produced react with the intact protein (1–4). More generally, antisera raised against the whole protein have been fractionated by absorption to polypeptide fragments covalently bound to solid matrices (2, 5, 6). The specificity of these antibodies may be demonstrated by the ability of the polypeptide that served as immunogen or immunoabsorbent to inhibit specifically the reaction with the native protein.

In special instances, highly specific antibodies have been obtained by sequential absorption to proteins similar but not identical to the protein used for immunization (7, 8). This method has been used to isolate antibodies to hemoglobin S which do not crossreact with hemoglobin A (8, 9). Hemoglobins A and S differ by a single amino-acid substitution at the sixth residue of the β chain, and their conformations are believed to be similar (10). These antibodies to sickle hemoglobin are presumed, but not proven, to be specific for the amino-terminal region of the β chain containing the mutation. This region also contains binding sites for carbon dioxide and 2,3-diphosphoglyceric acid and is involved in conformational changes upon oxygen binding (11, 12).

Antibodies which specifically bind to hemoglobin S have potential usefulness for the detection and quantitation of sickle hemoglobin, as for example, the intrauterine diagnosis of sickle cell anemia. In addition, antibodies to the amino-terminal region of the β polypeptide of hemoglobin S (β^S) may be used to study the conformation of hemoglobin in solution, specifically as a probe for changes in the structure of the amino-terminal region related to binding of inorganic phosphates, the interaction between chains, and the state of liganding of the heme moiety.

In this paper we describe the isolation of specific antibodies to hemoglobin S from a goat antiserum by absorption to, and subsequent elution from, a Sepharose column to which the synthetic peptide β^S (1–13) had been covalently bound. Binding of this nonprecipitating antibody has been detected and quantitated in a radioimmunoassay which employs ^{14}C-labeled carbamylated (= carbamoylated) hemoglobin and precipitation by a second antibody. This method may be of general use in isolating antibodies specific for mutant proteins.

Abbreviations: β^S, the β polypeptide of hemoglobin S; β^S(1–13), a polypeptide corresponding to residues 1–13 of β^S globin. The recommended name for the carbamyl group, $-CONH_2$, is *carbamoyl* [Fletcher, J. H., Dermer, O. C. & Fox, R. B., eds. (1974) *Nomenclature of Organic Compounds* (American Chemical Society, Washington, D.C.)].

MATERIALS AND METHODS

Antigens. Erythrocytes obtained from normal volunteers and patients with sickle cell anemia were washed three times with 0.15 M saline and lysed in distilled water. The lysate was made 0.5 M in NaCl and centrifuged at 30,000 × *g* for 90 min to remove cell membranes. After conversion to the carbonmonoxy form, hemoglobins were purified chromatographically on DEAE-Sephadex by the method of Huisman and Dozy (13).

Immunizations. Goats were innoculated at biweekly intervals with 10 mg of hemoglobin S in 1 ml of 0.05 M Tris·HCl, pH 8, emulsified in 1.2 ml of Freund's adjuvant. The immunological response was monitored periodically by immunodiffusion and quantitative precipitin analysis. All studies reported in this paper were performed on a single large blood sample from one animal after 30 weeks of immunization. Antiserum was fractionated by precipitation three times at 35% ammonium sulfate saturation, dialyzed against 0.15 M NaCl, and stored frozen at −15°.

Immunological Techniques. Immunodiffusion was performed by the method of Ouchterlony (14) on pattern B agar plates (Hyland). Quantitative precipitation was performed by the method described by Sachs *et al.* (6), with corrections for the absorbance of the hemoglobin. Passive hemagglutination was done by a modification of the standard technique (6). Micro-complement fixation was performed using the method described by Wasserman and Levine (15).

Radioactive Labeling of Hemoglobin. Chromatographically purified carbonomonoxy hemoglobins S and A were carbamylated preferentially at the α-amino termini by a modification of the method of Kilmartin and Rossi-Bernardi (16). Buffers were saturated with CO and all procedures were performed at 4° unless stated otherwise. Hemoglobin in 0.05 M sodium phosphate, pH 8.5, was reacted with cystamine·HCl (Sigma) at a 15-fold molar excess over dimer concentration to protect cysteine sulfhydryl groups. After removal of free cystamine by Sephadex G-25 column chro-

matography, the protected hemoglobin in 0.2 M sodium phosphate, pH 6.0, was reacted with $K^{14}CNO$ (New England Nuclear, 6.185 mCi/mmol) at a 50-fold molar excess over hemoglobin dimer for 2 hr at 20°. Free cyanate was removed by Sephadex G-25 chromatography. The protecting cystamine was removed by treatment with an excess of dithiothreitol (Calbiochem) overnight. The carbamylated hemoglobin had a specific activity of 1×10^{14} cpm/mol of heme; carbamylation to the extent of 1.9 CNO/dimer with 98% yield of hemoglobin was obtained. The derivative was stored as the carbonomonoxy form in 0.025 M sodium phosphate buffer, pH 8.5.

Radioimmunoassay for Hemoglobin Antibody. Antibody to hemoglobin was assayed in a double antibody radioimmunoassay. Antibody, ^{14}C-labeled hemoglobin, and competitive inhibitors when appropriate, in 0.04 M Tris·HCl–0.09 M NaCl, pH 7.4, to a total volume of 100–110 μl were incubated in 400 μl Microfuge tubes (Beckman) for 1 hr at 20°. Additional buffer, normal carrier goat gamma globulin (Pentex), and anti-goat gamma globulin (Miles) were then added to a total volume of 300 μl, and incubated for 1 hr at 20° and 2 days at 4°. Normal goat gamma globulin was used as a carrier protein to increase the size of the precipitate which formed when very small amounts of antibody to hemoglobin were being tested. The quantity of carrier gamma globulin and anti-goat gamma globulin to be added were determined by quantitative precipitation and a point of antibody excess was chosen. After incubation, assay tubes were centrifuged in a Model 152 Microfuge (Beckman) for 5 min. A 100 μl aliquot of the supernatant was pipetted into 15 ml of counting solution (Aquasol, New England Nuclear) to determine the unbound, or free, labeled hemoglobin. The small white precipitate remaining in the assay tubes was washed once with cold 0.01 M Tris·HCl–0.15 M NaCl, pH 7.4, dissolved in 200 μl of 40% acetic acid, and quantitatively transferred to 10 ml of scintillation fluid; the radioactivity was used to determine the amount of bound labeled hemoglobin. In some experiments, antibody binding to various concentrations of radioactive hemoglobins A and S was determined. Nonspecific binding of normal goat gamma globulin, equivalent in concentration to the first antibody, was determined at each hemoglobin concentration. Antibody-bound radioactivity was corrected by subtraction of the nonspecifically bound radioactivity prior to calculation of the bound/free ratio. In other experiments, small amounts of radioactively labeled hemoglobin were displaced by nonradioactive competitors; nonspecific binding of label was 5% or less of total binding, and it was not necessary to correct the bound/free ratios. In all experiments, duplicate samples usually agreed within 10% of total binding and bound/free ratios were reproducible among experiments. Counting was performed in a Searle Mark III instrument with an efficiency of 98%; correction was made for quenching at the highest concentration of hemoglobin used.

Peptide Synthesis. A peptide of 13 residues corresponding to amino acids 1 through 13 of the β chain of sickle hemoglobin [β^S(1–13)] (Fig. 1) was synthesized by a previously described modification of the Merrifield method (17). This peptide was separated from smaller synthetic fragment contaminants by column chromatography on Bio-Gel P-6 in ammonium acetate 0.05 M, pH 5. The peptide was hydrolyzed after evacuation of air in 6 N HCl at 110° for 20 hr and amino-acid analysis was performed on a Beckman 121 automated amino-acid analyzer. The amino-acid analysis of the purified peptide is shown in Table 1. A complete description of the synthesis and isolation of this peptide is in preparation (Eastlake, Curd, and Schechter.)

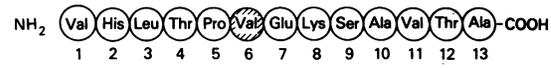

FIG. 1. Amino-acid sequence of β^S(1–13).

Affinity Chromatography. The synthetic peptide β^S(1–13), 0.5 μmol, was covalently bound to 6 ml of Sepharose 2B (Pharmacia) by cyanogen bromide activation at pH 11, and coupling in 0.1 M sodium bicarbonate, pH 7.4 (18). The Sepharose was washed sequentially with phosphate-buffered saline (0.15 M NaCl, 0.015 M Na_2HPO_4, 0.004 M KH_2PO_4), 1% ethanolamine, and 4 M guanidine. A total of 3 mg of β^S(1–13) was bound to the Sepharose as determined by amino-acid analysis of the resin. The column was stored in phosphate-buffered saline at 4°. Gamma globulin preparations fractionated by ammonium sulfate and applied to the column were eluted with 4 M guanidine·HCl in phosphate-buffered saline. Antibody eluted in guanidine was immediately dialyzed into 0.15 M NaCl at 4° and concentrated by either vacuum dialysis or lyophilization.

RESULTS

Characterization of the Antiserum. A goat immunized with hemoglobin S produced precipitating antibodies to hemoglobin in 6 weeks. Serum obtained from a large blood sample at week 30 was precipitated with ammonium sulfate and dialyzed into 0.15 M NaCl. This gamma globulin enriched fraction contained a titer of 3 mg/ml of antibody to hemoglobin by quantitative precipitin analysis. Quantitative precipitation and passive hemagglutination showed no difference between the reactivity of the gamma globulin preparation with hemoglobins A and S. The characteristics of this antibody could not be studied in the micro-complement fixation assay because of anticomplementary activity of the goat gamma globulin.

Radioimmunoassay. To detect possible small differences in binding of this gamma globulin to hemoglobins A and S, a radioimmunoassay employing carbamylated hemoglobins A and S was developed. Binding of antibody to hemoglobin was determined by the presence of radioactivity in the precipitate formed by the test antibody and the second antibody to goat gamma globulin. Hemoglobin S and nonradioactive carbamylated hemoglobin S were equally effective in displacing labeled carbamylated hemoglobin S from the antibody.

As assessed by this radioimmunoassay, the gamma globulin preparation of antibody to hemoglobin S showed, at most, a small but reproducible difference in its binding to

Table 1. Amino-acid analysis of β^S(1–13)

Residue	Theoretical	Found*
Lysine	1	1.00
Histidine	1	0.59
Threonine	2	2.17
Serine	1	1.00
Glutamic acid	1	1.06
Proline	1	1.03
Alanine	2	2.15
Valine	3	2.74
Leucine	1	0.86

* Molar ratio based on 13 residue average.

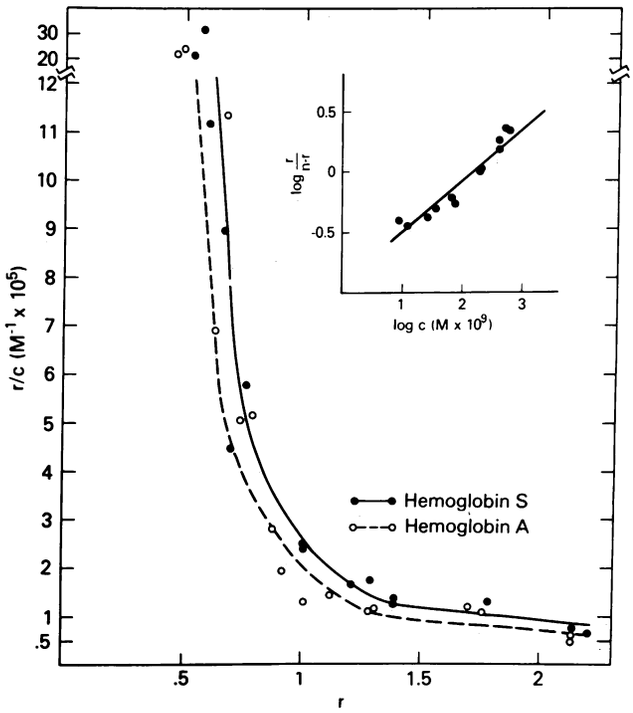

FIG. 2. Scatchard analysis of the binding of hemoglobins A (O- - -O) and S (●—●) to the gamma globulin prepared from antiserum raised against hemoglobin S. The incubation mixture consisted of this gamma globulin (1.3×10^{-9} M) and [^{14}C]hemoglobin A or S (1.9×10^{-6} M to 3.8×10^{-5} M) in 0.04 M Tris·HCl–0.09 M NaCl, pH 7.4, in a volume of 80 μl. After incubation at 20° for 1 hr, 160 μl of rabbit anti-goat gamma globulin and buffer were added to a final volume of 300 μl. After 1 hr at 20° and 48 hr at 4°, the precipitates and supernatants were harvested as described in the *text*. The data for hemoglobin S, plotted according to Sips analysis, are shown in the insert. The slope, or heterogeneity index, a, was 0.42.

hemoglobins A and S; a typical Scatchard analysis (19) is illustrated in Fig. 2. The concentration of antibody to hemoglobin was estimated from quantitative precipitin analysis. A 10% error in this determination would not be unlikely and would explain why the curve approaches 2.2, rather than 2.0 antigen molecules bound per antibody molecule. A Sips plot (20) of these data (insert, Fig. 2) showed a slope of 0.42, indicating marked heterogeneity of the antibody population. The Scatchard plot shows that about half of the total antibody population has a high affinity for either hemoglobin. An average affinity constant derived from the slope of the linear portion of the curve representing the higher affinity antibodies was estimated to be 6.5×10^6 M^{-1}.

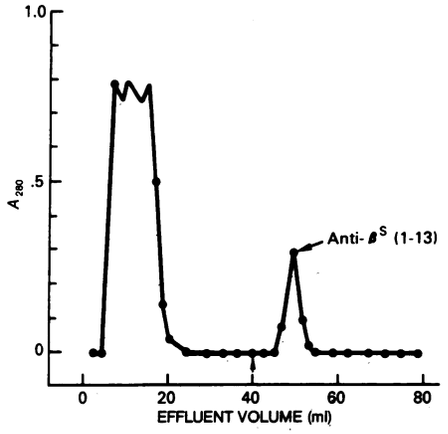

FIG. 3. Affinity chromatography on Sepharose-β^S(1–13) of gamma globulin prepared from an antiserum to hemoglobin S. Seven milliliters of gamma globulin was applied to the column (0.5 × 6 cm) in phosphate-buffered saline; at the arrow, elution with 4 M guanidine·HCl was begun.

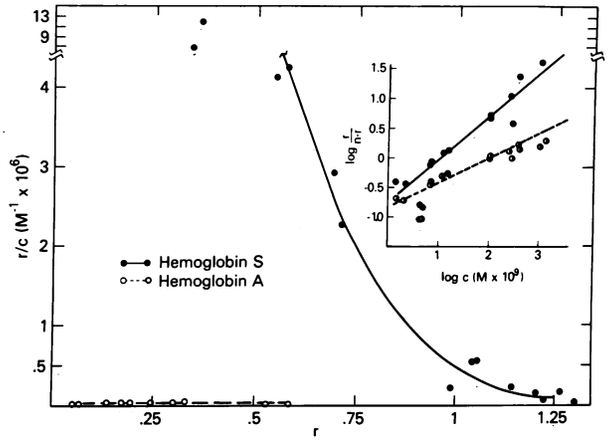

FIG. 4. Scatchard analysis of the binding of hemoglobins A (O- - -O) and S (●—●) to anti-β^S(1–13). The incubation mixture consisted of anti-β^S(1–13) (1.6×10^{-9} M) and hemoglobins A or S (7.5×10^{-7} to 6×10^{-5} M) in 0.04 M Tris·HCl–0.09 M NaCl, pH 7.4, in a volume of 110 μl. After incubation at 20° for 1 hr, 160 μl of rabbit anti-goat gamma globulin, 10 μl of normal goat gamma globulin (2 mg/ml), and buffer were added to a total volume of 300 μl. After 1 hr at 20° and 48 hr at 4°, the precipitates and supernatants were harvested as described in the *text*. The data for hemoglobin S, plotted according to Sips analysis, are shown in the insert. The functions based on the experimentally observed value n = 1.25 (●—●) (heterogeneity index, a = 0.72) and the theoretical value n = 2.0 (◑- - - -◑) (a = 0.40) are shown for comparison in the insert.

Affinity Chromatography. A Sepharose affinity column with the synthetic peptide (Fig. 1) corresponding to the first 13 residues of the β^S chain of hemoglobin was prepared. This sequence is centered about the mutation at residue 6 which characterizes hemoglobin S. When 7 ml of the gamma globulin preparation was applied to this column and eluted with phosphate-buffered saline, less than 1% of the starting protein and about 5% of the precipitating antibody to hemoglobin was absorbed (Fig. 3). The absorbed protein, anti-β^S(1–13), was eluted from the Sepharose column with 4 M guanidine·HCl and dialyzed into 0.15 M NaCl. Greater than 95% of this protein was absorbed when reapplied to the β^S(1–13) column. In general, after a single application to the affinity column, dialysis, and concentration, the yield of antibody was 0.18 mg/ml of the starting gamma globulin preparation. Recovery of activity was greater with concentration by vacuum dialysis than with lyophilization. Although the eluted gamma globulin was stable at 4° for several weeks, precipitation was observed with repeated freezing and thawing.

Characterization of Anti-β^S(1–13). The protein obtained by elution with guanidine from the Sepharose-β^S(1–13) column, denoted as anti-β^S(1–13), when concentrated 10-fold, did not precipitate with hemoglobin S in Ouchterlony immunodiffusion analysis. Anti-β^S(1–13) was analyzed in the radioimmunoassay for its ability to bind to hemoglobins A and S. Fig. 4 demonstrates that anti-β^S(1–13) binds to hemoglobin S and not to hemoglobin A. An average affinity constant for the higher affinity population, derived from the slope of the linear portion of the curve, was estimated to be

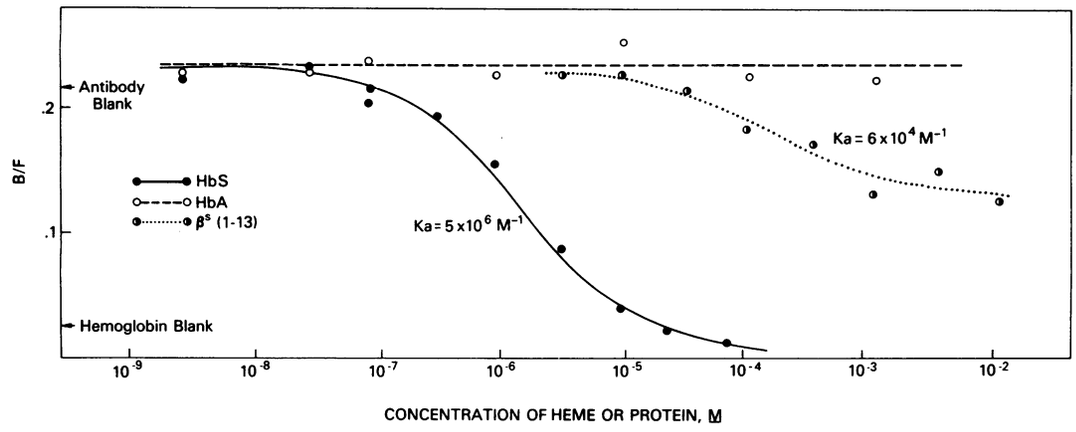

FIG. 5. Displacement of [^{14}C]hemoglobin S from anti-β^S(1–13) by hemoglobin A (O- - -O), hemoglobin S (●—●), and β^S(1–13) (◑ · · · · ◑). Each tube contained anti-β^S(1–13) (1.2×10^{-9} M), [^{14}C]hemoglobin S (9.8×10^{-7} M), and unlabeled hemoglobin A, or S, or peptide β^S(1–13) at final concentrations noted on the abscissa; the total volume was 110 μl. Tubes were incubated as described in the legend for Fig. 2 and harvested as described in the text. Antibody blank refers to binding of [^{14}C]hemoglobin S to anti-β^S(1–13) in the absence of displacer. Hemoglobin blank refers to [^{14}C]hemoglobin S precipitated nonspecifically by normal goat gamma globulin.

1.5×10^7 M^{-1}. Binding of hemoglobin A did not differ significantly from its nonspecific binding to gamma globulin. Approximately 50% of the total antibody population was of a relatively high affinity. The concentration of antibody was estimated from the absorbance at 280 nm of the anti-β^S(1–13) solution. The binding curve asymptotically approaches a value of 1.25 antibody binding sites for each $\alpha\beta$ dimer. This result is consistent with inactivation of about 40% of the antibody secondary to affinity chromatography. The Sips analysis (20) of these data, depending upon the value of n used (insert, Fig. 4), may indicate decreased heterogeneity. Isoelectric focusing results*, however, clearly indicate restriction of heterogeneity of the anti-β^S(1–13) as compared to the starting gamma globulin preparation.

The ability of hemoglobins A and S to displace carbamylated hemoglobin S from anti-β^S(1–13) was determined. A typical displacement experiment is shown in Fig. 5. By this method, the approximate, average affinity constant for hemoglobin S was calculated as 5×10^6 M^{-1}. This value is in reasonable agreement with that obtained from the Scatchard analysis (1.5×10^7 M^{-1}), which does not include some of the low affinity population of antibody. HbA did not significantly inhibit the binding to HbS in this range of concentrations.

The ability of the peptide β^S(1–13) to displace labeled hemoglobin S was also assessed. At the highest concentration of the peptide used (10^{-2} M), approximately 40% of the label was displaced. The shape of the curve was indeterminant at these concentrations and it was unclear whether this degree of inhibition was in fact maximal for the peptide. The affinity of anti-β^S(1–13) was several orders of magnitude less for the peptide than for hemoglobin S.

DISCUSSION

We here report the isolation of specific antibodies to hemoglobin S by immunoabsorption to a synthetic peptide. The antiserum from which these antibodies were obtained poorly discriminated between hemoglobins A and S by conventional immunological and radioimmunoassay techniques. A synthetic peptide, β^S(1–13), which includes the amino-terminal residues of the β chain exposed to solvent in the hemoglobin tetramer and is centered at the mutation at position 6, was covalently bound to Sepharose and used to fractionate a gamma globulin preparation of the antiserum. Approximately 5% of the antibody to hemoglobin, as determined by quantitative precipitation, was bound to the column and could be eluted with 4 M guanidine·HCl. This antibody, anti-β^S(1–13), bound [^{14}C]carbamylated hemoglobin S but not hemoglobin A. Unlabeled hemoglobin S completely displaced labeled hemoglobin S from anti-β^S(1–13). Hemoglobin A did not displace the labeled hemoglobin S at concentrations as high as 10^3 molar excess over the concentration of labeled hemoglobin S. The synthetic peptide β^S(1–13) displaced 40% of the labeled hemoglobin at the highest concentration of peptide used.

Fractionation of antibody populations by immunoabsorption to proteolytic fragments covalently bound to solid matrices has been reported for human albumin (5), hen egg white lysozyme (2), and staphylococcal nuclease (6). Nonprecipitating antibodies, produced by such fractionation procedures, directed against one or a few antigenic determinants of the loop of lysozyme (residues 60–83) (2) and of the cyanogen bromide fragment E of nuclease (residues 99–149) (3) are sensitive to chemical alterations of the conformation of the protein. Synthetic polymers have been used as antigens to raise antisera which crossreact with large native molecules, for example DNA (21) and collagen (22). A synthetic peptide corresponding to the loop region of lysozyme has been used as an antigen to produce an antiserum which recognized the loop region of the native protein, but did not recognize the performic acid-oxidized open chain peptide (23).

Antibodies to staphylococcal nuclease have been fractionated by absorption to a synthetic peptide corresponding to the region 53–69 of the protein, which was covalently bound to Sepharose. The antibodies, eluted by acid or by an excess of free peptide, were able to bind and inactivate the enzyme. Their failure to precipitate the antigen was suggestive of specificity to a limited region of the molecule, but specificity for the region 53–69 has not been demonstrated (24).

In the present study, the marked enrichment of the population of high affinity antibodies directed against sickle hemoglobin is strong evidence for the specificity of absorption of antibodies on the synthetic peptide column. The peptide

* N. S. Young, A. Eastlake, A. N. Schechter, manuscript in preparation.

β^S(1–13) inhibited binding of anti-β^S(1–13) to hemoglobin S, but this inhibition was not complete at the highest concentration of peptide that it was practical to employ. It is possible that higher concentrations would completely inhibit binding to hemoglobin. Less likely, a fraction of the antibodies may absorb nonspecifically, possibly by an ion-exchange effect, and these antibodies might not recognize the peptide in solution. It is significant that relatively weak interactions, as indicated by the low binding constant to the peptide, are useful in purifying specific antibody by affinity chromatography.

Antibodies to hemoglobin have been shown to be capable of discriminating between different states of liganding of the molecule and among abnormal mutant species (25, 26). Antibodies which are specific to hemoglobin S have been produced by sequential immunoabsorption to remove antibodies which react with hemoglobin A. Fab fragments obtained by this method and assayed by fluorescence quenching are bound only by sickle hemoglobin (8). Similar results have been reported in which anti-hemoglobin S antibodies have been measured in a radioimmunoassay using hemoglobin lebeled with ^{125}I (9). Antibodies to hemoglobin S have been characterized as having restricted heterogeneity by the kinetics of interaction with hemoglobin and by polyacrylamide gel electrophoresis; an association constant of 2.1×10^{10} M^{-1} has been reported (27).

The present study differs from previous reports concerning antibodies to hemoglobin S in methods of preparation and characterization. First, the method of immunoabsorption utilized here selected a population directed against a specific region of the hemoglobin molecule, the amino-terminus of the sickle β chain. This method is not dependent upon possible differences in the tertiary or quaternary structure between hemoglobins A and S in regions of the molecule distant from the mutation. The ability to compare the reactivity of anti-β^S(1–13) with hemoglobins S and A allowed us to determine directly that immunoabsorption to a synthetic peptide has resulted in purification of an antibody population with the desired specificity. However, the specificity of these antibodies to hemoglobin S, as compared to hemoglobin A, is similar to that reported for antibodies prepared by sequential immunoabsorption with hemoglobin A (8, 9). The conformational nature of the antigenic site(s) of the β^S(1–13) region will be described in a subsequent publication (Young, Eastlake, and Schechter). Second, carbamylation with $K^{14}CNO$ who chosen to label the hemoglobin because of its well-defined and limited alterations of protein structure, in contrast to iodination of hemoglobin (27). The lower specific activity of this label, however, limits accurate measurements of antibody affinity constants to the intermediate range (10^6–10^8 M^{-1}).

Antibodies with high affinity and specificity for sickle hemoglobin have potential use in medicine, both in detection of the abnormal protein and in the study of its properties. Anti-β^S(1–13) will have to be studied to characterize it further immunologically and to localize the antigentic determinant against which it is directed, and which determines its binding to other mutant hemoglobins and to various liganded states of the molecule. The use of synthetic peptides for fractionation by affinity chromatography may have broad application in the production of antibodies with specificities to limited regions of other normal and mutant proteins.

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