and his encouragement throughout this work. We also acknowledge the excellent technical assistance of Nancy Guman Genk and Ruth MacAlpine.

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Studies on Antibodies Directed toward Single Antigenic Sites on Globular Proteins†

Robert W. Noble,* Morris Reichlin,‡ and Robert D. Schreiber§

ABSTRACT: We have isolated an antibody population which reacts with hemoglobin S but not with hemoglobin A. These proteins differ by a single amino acid substitution. We have isolated an antibody population which reacts with human cytochrome *c* but not with monkey cytochrome *c*. These proteins also differ by a single amino acid residue. These antibody populations react with their specific antigens with a stoichiometry of one antibody combining site per asymetrical antigenic unit. A second, different antibody population which also reacts with human cytochrome *c* with a stoichiometry of unity has been isolated by virtue of its excess production. The kinetics of reaction of these site specific antibody populations indicates considerable homogeneity. This study represents the first isolation of antibodies specific to single chemical modifications of globular proteins and the first demonstration of a one to one binding stoichiometry between such an antigen and an antibody population.

From classical studies in immunology we know that antisera to proteins show great specificity. Interspecies immunochemical differences between homologous proteins are the rule; and genetically controlled intraspecies differences are frequently detectable (Margoliash *et al.* (1970) and Sarich and Wilson (1967). The immunochemical differences are related to differences in the amino acid sequences of the homologous proteins and can be viewed as modifications of the haptenic

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structures on the antigen. Often, single amino acid differences or a single chemical modification are detectable immunologically (Atassi and Thomas, 1969; Nisonoff *et al.*, 1970). The study of the immunochemical effects of single amino acid differences or chemical modifications of a single group has led to the suggestion that the antigenicity of a protein is confined to a limited number of discrete regions on the surface of the molecule (Reichlin, 1972).

Since single amino acid alterations in an antigen molecule often produce significant precipitin differences (Nisonoff *et al.*, 1970), it is possible to isolate antibodies whose reaction with antigen is dependent on the particular alterations being considered. If these antibodies, which are sensitive to single amino acid alterations, interact with the antigen at the site of the alteration, one would expect that only one such antibody should be able to react with each antigen molecule. It is of interest to examine these antibodies for their degree of homogeneity.

The stoichiometry of the reaction of antibodies to heme protein antigens such as hemoglobin (Hb)[1] or cytochrome *c* (cyt *c*) can be measured directly by fluorescence quenching titrations as described by Noble *et al.* (1969). Furthermore, homogeneity of reaction kinetics can be assessed by means of the same assay when a stopped-flow apparatus is used. In this paper we report some of our attempts to isolate site-specific antibodies to Hb and cyt *c*, and some of the properties of such antibodies.

Materials and Methods

Hemoglobin. Hemolysates were prepared by the method of Drabkin (1964). HbS was obtained from the blood of a patient who was homozygous for HbS. HbS for immunization was separated from the small amount of fetal hemoglobin and HbA_2 in the hemolysate by the method of Reichlin *et al.* (1964).

Cytochrome c. Monomeric cyt *c*, prepared as previously described by Margoliash *et al.* (1970) was kindly supplied by E. Margoliash. Polymeric cyt *c* was used for immunization as described by Reichlin *et al.* (1970).

Immunizations. Rabbits were immunized with polymeric hyman cyt *c* as previously described by Reichlin *et al.* (1970). Goats were immunized with HbS by a schedule and dose identical with that described for polymeric human cyt *c* (Reichlin *et al.*, 1970).

Quantitative Precipitin Analysis. This assay was performed according to the method of Heidelberger and Kendall (1935).

Purification of Specific Antibodies. Specific anti-human or anti-monkey cyt *c* antibodies were purified as previously described by Noble *et al* (1969). A specific antigen–antibody precipitate was formed at equivalence as judged by quantitative precipitin data. This precipitate was washed and then dissolved in 1.0 N acetic acid and applied to a Sephadex G-100 column also equilibrated with 1.00 N acetic acid. The cyt *c* antigen and the antibodies were well resolved on this column. 7S antibodies were converted to monovalent Fab fragments by digestion with pepsin at pH 4 (Nisonoff *et al.*, 1960) and alkylated with iodoacetamide as previously described by Noble *et al.* (1969).

For the purification of specific anti-HbS antibodies from goat sera, an immune precipitate was formed at equivalence as in the case of the anti-cyt *c* antibodies. The precipitate was washed with 0.85% saline and then digested with pepsin using a modification of the method of Nisonoff *et al.* (1960). A typical digestion is outlined as follows. The washed immune precipitate was suspended in 0.2 M acetate buffer (pH 4.3) and the suspension was made 0.04 M in cysteine. The pH was adjusted to 4.0, then pepsin was added in the amount of 2.0% of the total protein weight. The digestion was allowed to proceed for 24 hr at 37°. At the end of this time, the dark brown precipitate that has formed was spun down and discarded and the almost colorless supernatant was slowly titrated to pH 8.0 with 1.0 M sodium hydroxide. The resulting Fab fragments were alkylated as previously described (Noble *et al.*, 1969). Excess cysteine and iodoacetamide were removed by dialysis against 0.05 M phosphate–borate buffer (pH 8.0). The resulting Fab fragments were separated from other digestion products by gel filtration on Sephadex G-100 equilibrated with 1.0 M acetic acid.

Assay for Nonreactive Fab. The amount of nonreactive Fab present in a Fab preparation was determined as follows. A known amount of Fab solution (dialyzed against 0.01 M phosphate buffer, pH 6.5) was added to a small amount of CM-52 resin which had been saturated with the antigen and washed extensively with buffer. The amount of antigen present was sufficient to bind approximately ten times as much antibody as was added. Twenty-four hours later, the resin was spun down and the supernatant read at 280 nm to determine the amount of unbound Fab material. A fluorescence quenching titration with the antigen was performed on the supernatant to insure complete removal of all reactive Fab.

Reaction Stoichiometry. The stoichiometry of the combination of antigen and antibody was measured as previously described by Noble *et al.* (1969). A solution of antibodies or Fab fragments (2 ml at approximately 0.1 mg/ml) was titrated with a solution of antigen using an Agla syringe (Burroughs Welcome, Inc.) for the small volume additions. An antigen concentration was chosen so that the titration was complete after addition of about 50 μl of the antigen solution. After each addition of antigen, the fluorescence of the antibody solution was measured relative to that of a solution of nonimmune γ-globulin to which an equal amount of antigen was added. Thus shielding effects were eliminated and fluorescence quenching could be attributed to specific antigen–antibody interactions. Fluorescence measurements were performed with an Aminco-Bowman spectrophotofluorimeter equipped with a parabolic mirror attachment for increased sensitivity. All experiments were carried out at room temperature; and except where noted, the buffer used was 0.05 M phosphate plus 0.05 M borate (pH 8).

Reaction Kinetics. The time course of the reaction of antibodies with cyt *c* and Hb antigens was measured as previously described by Noble *et al.* (1969). A Gibson-Durrum stopped-flow apparatus was modified for fluorescence measurements as described by Gibson *et al.* (1966). The sources of illumination and the optical filters used were identical with those described by Nagel and Gibson (1967).

Spectrophotometric Measurements. These were carried out by means of a Zeiss Model PMQII spectrophotometer and a Cary Model 14 recording spectrophotometer. The extinction coefficients listed previously by Noble *et al.* (1969) were used for determining the concentrations of antibody and antigen solutions.

Results

Three different systems have been explored in an attempt to isolate and to study the properties of antibodies specific to

[1] Abbreviations used are: Hb, hemoglobin; cyt *c*, cytochrome *c*.

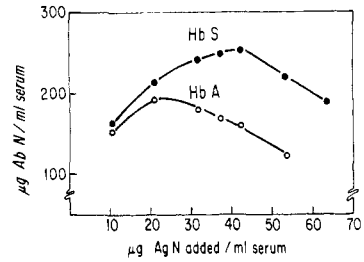

FIGURE 1: Quantitative precipitin curves for the reaction of hemoglobins S and A with goat antiserum to hemoglobin S.

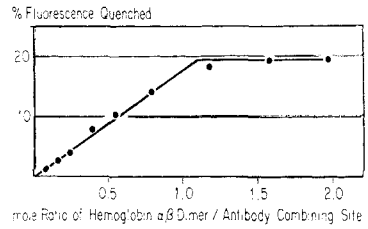

FIGURE 3: The titration of anti-Val Fab fragments (0.29×10^{-5} M) with hemoglobin S. The percentage fluorescence quenched is plotted against the ratio of $\alpha\beta$ dimer to the concentration of reactive Fab fragments.

single sites on a protein antigen. The first is the population of goat anti-human HbS antibodies which will not react with HbA. Since these two proteins differ only in the amino acid residue at position 6 of the β chain which is valine in HbS and glutamic acid in HbA, these will be termed anti-Val antibodies. The second is a population of rabbit anti-monkey cyt *c* antibodies which fails to precipitate from the antiserum upon addition of monomeric antigen. Because of their specific inhibitory effect on the reaction of cyt *c* with cytochrome oxidase they are termed anti-oxidase antibodies. The third system is the population of rabbit anti-human cyt *c* antibodies which will react with the human protein but not with monkey cyt *c*. Since these two proteins differ by a single amino acid residue at position 58 which is isoleucine in human cyt *c* and threonine in monkey cyt *c*, these are termed anti-Ile antibodies. The results that have been obtained on the different systems will be described separately.

Goat Anti-HbS Valine Antibodies. The anti-HbS serum obtained from the goat contained 1.58 mg of specific antibody/ml and exhibited a quantitative precipitin difference of 24.4% when HbS and HbA were used as antigens as shown in Figure 1.

A specific immune precipitate was formed at equivalence as judged by quantitative precipitin data. The precipitate was washed with 0.85% ice-cold saline, digested with pepsin, and alkylated (see Methods). The pH of the alkylated digest was adjusted to 2.5 and the digest was chromatographed on a 4.0×75 cm Sephadex G-100 column equilibrated with 1.0 M acetic acid. Two protein fractions were obtained, one containing 410-nm-absorbing material and the other free of 410-nm color and containing material with a sedimentation coefficient of 3.4 S at pH 7.00.

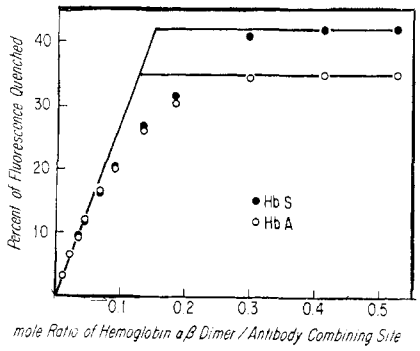

FIGURE 2: The titration of total anti-hemoglobin S Fab fragments (0.34×10^{-5} M) with hemoglobins S and A. The percentage of the fluorescence quenched is plotted against the ratio of $\alpha\beta$ dimer to total Fab concentration.

Using fluorescence quenching, the total goat anti-HbS Fab fragments were titrated with HbS and HbA to determine the stoichiometry of the reaction. Fab fragments at a concentra- of 0.151 mg/ml (0.34×10 M^{-5}) were titrated with hemoglobins S and A in concentrations of 0.50 mg/ml. The results are shown in Figure 2 where the percentage of fluorescence quenched is plotted as a function of the mole ratio of antigen to antibody. In the case of HbS, the total quenching observed at the end of the titration was 41.9% and the intersection of this line with the initial slope yields an average binding of 6.5 Fab molecules/hemoglobin $\alpha\beta$ dimer. In the case of HbA, the final total quenching observed was 37.5% and the initial slope was identical with that generated in the titration with HbS.

The following procedure was used to obtain anti-Val Fab fragments. The solution of purified Fab fragments was added to CM52 resin fully saturated with HbA and equilibrated with 0.01 M phosphate (pH 6.5). After incubation for 12 hr at 4°, the resin was spun down and the supernatant was collected. This supernatant contained appreciable amounts of 410-nm-absorbing material. The supernatant was concentrated and layered onto a 2.5×80 cm Sephadex G-100 column and eluted with 0.05 M phosphate–borate buffer (pH 8.0) at 4°. Two protein peaks were obtained. The first contained all the 410-nm color and was, therefore, complexes of hemoglobin and antibody. The second was free of color and was used as the pure anti-Val Fab fragments. The amount of nonreactive antibody present in the anti-Val Fab preparation was determined (see Methods) and found to be 33% of the total.

The final yield of anti-Val Fab fragments was 9% of the total anti-HbS Fab population. This correlates with the 10.5% difference in the maximum fluorescence quenching obtained when the total Fab population is titrated with HbA. However, it is much less than the 24.4% quantitative precipitin difference seen in Figure 1. This indicates that a significant fraction of antibody sensitive to the structural difference between the two antigens will still bind to HbA to a significant extent. This is consistent with the identity of the initial slopes of the titrations of the total anti-HbS Fab populations with HbS and HbA in Figure 2.

The anti-Val Fab fragments were titrated with HbS and HbA. There was no quenching with HbA. The results of the titration with HbS are shown in Figure 3. The abcissa in this figure (moles of Hb $\alpha\beta$ dimer/mole of Fab) has been corrected for the amount of nonreactive material in the anti-Val Fab preparation. With this correction for the amount of nonreactive material a stoichiometry of 0.92 mole of anti-Val Fab/mole of HbS $\alpha\beta$ dimer is obtained.

Kinetics of the reactions of the total anti-HbS Fab preparation with hemoglobins S and A and the anti-Val Fab preparation with HbS were studied by mixing the Fab preparations with HbS in a stopped-flow apparatus such that the final con-

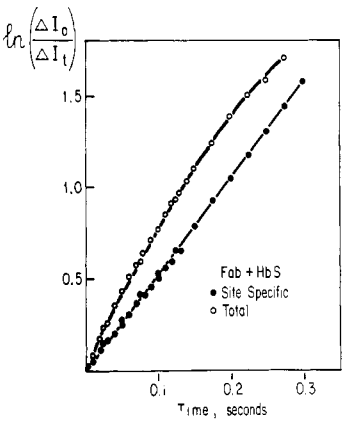

FIGURE 4: A pseudo-first-order kinetic plot of the reaction of total anti-hemoglobin S Fab and anti-Val (site specific) Fab fragments with hemoglobin S. The effective antibody concentrations (see text) were 1.53×10^{-6} M for the total Fab and 1.46×10^{-6} M for the anti-Val Fab while the effective antigen concentration was 9.23×10^{-6} M. The buffer was pH 8 phosphate–borate (0.05 M in both antions).

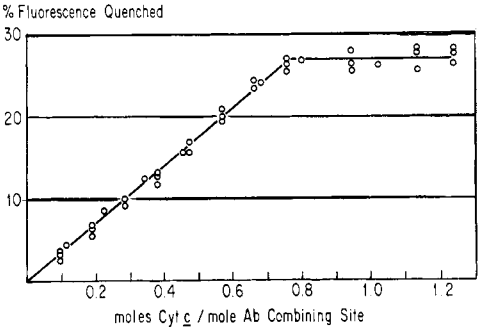

FIGURE 5: Titration of purified anti-oxidase Fab fragments with human cytochrome *c*. The per cent of the antibody fluorescence that is quenched is plotted as a function of the molar ratio of cytochrome *c* to Fab fragments.

centration of Hb was 9.23×10^{-6} M. The total anti-hemoglobin S Fab concentration after mixing was 1.53×10^{-6} M while the anti-Val concentration after mixing was 1.46×10^{-6} M. The kinetic data for these experiments are shown in Figure 4. The rates of the two reactions do not differ greatly, but while the reaction of the total anti-HbS Fab population is kinetically heterogeneous, the reaction of the anti-Val Fab fragments is homogeneous.

Anti-oxidase Antibody. When monkey cyt *c* is added to a rabbit anti-monkey cyt *c* serum to obtain maximum precipitation of the antibodies reactive with monkey cyt *c*, a fraction of antibody remains in the supernatant which binds to monkey cyt *c* but is not precipitated by it. An explanation for this phenomenon would be a disproportionately large production of antibodies directed to one of the antigenic sites on the cyt *c* molecule. Smith *et al.* (1970) reported that anti-cyt *c* Fab fragments stoichiometrically inhibit the oxidation of reduced human cyt *c* by beef heart cytochrome oxidase. They also reported that the nonprecipitating fraction is considerably enriched in the anti-oxidase activity, inhibiting the oxidase reaction at a stoichiometry slightly greater than 1 antibody combining site/cyt *c* molecule (Smith *et al.*, 1972).[2]

The nonprecipitating fraction was isolated as follows. Monomeric monkey or human cyt *c* was added to rabbit antiserum (50 ml), induced to *Macaca mulatta* cyt *c*, in equivalent amounts as determined by precipitin titration.[3] To the supernatant was added ammonium sulfate to 40% saturation at room temperature. The precipitate was dissolved in a minimal volume of distilled water and equilibrated by dialysis against 0.01 M sodium phosphate buffer (pH 6.5). The solution was mixed with 0.5 ml of CM52 (Whatman) equilibrated against the same buffer and to which 10 mg of human cyt *c* had been adsorbed. The CM cyt *c* served as a specific immunoadsorbent. Normal rabbit γ-globulin was not bound. The immunoadsorbant was washed three times with 10.0-ml aliquots of 0.01 M phosphate buffer (pH 6.5) to remove nonspecific globulin. The cyt *c*–antibody complex was eluted with 0.2 M Na_2HPO_4. The solution was concentrated to 3 ml by ultrafiltration (Diaflo apparatus, Amicon), adjusted to pH 2.9 with 2.0 N HCl and passed through a Sephadex G-100 column (2.5 × 50 cm) (Pharmacia), in 0.1 M acetic acid. The separated antibody appeared at the exclusion volume, was dialyzed against 100 volumes of phosphate–borate buffer (0.05 M, pH 8.0), and was concentrated by ultrafiltration.

When the nonprecipitating antibody was titrated with human cyt *c*, the titration had two distinct regions, the first region having a higher stoichiometry than the second. It was reasoned that this fraction contained predominantly antibodies directed toward one antigenic site on the cyt *c* molecule, plus a small amount of antibody material directed toward a second antigenic site. Therefore, an attempt was made to isolate a more homogeneous product. First the 7S material was digested with pepsin, reduced, and alkylated to yield Fab fragments. Then an amount of human cyt *c* equal to one-fifth the molar concentration of the Fab fragments was added. This amount of cyt *c* was sufficient to bind all of the contaminating Fab material directed toward the second antigenic site. It was of course insufficient to bind all of the major fraction, and it was therefore predicted that the unbound material would be composed of only anti-oxidase Fab fragments. The mixture was gel filtered on a Sephadex G-100 column at pH 8.0. The cyt *c* was found in the early peak at the exclusion volume (presumably as cyt *c* Fab_2), an intermediate form of cyt *c* was noted (probably cyt *c* Fab_1) and finally a colorless peak appeared at the elution volume characteristic of Fab and overlapping the intermediately eluting cyt *c* Fab complex.

This purified anti-oxidase antibody was then titrated with human cyt *c* and the results are shown in Figure 5. The data from four independent titrations are included. The titration is linear and the slope corresponds to an antibody to antigen ratio of 1.3 without any correction for the presence of nonreactive antibodies since this determination was not made on this sample.

The kinetics of the reactions of both the unpurified and the purified anti-oxidase antibodies with human cyt *c* were examined. The reaction of the unpurified anti-oxidase antibodies with human cyt c was not kinetically homogeneous, but was far less heterogeneous than the reaction of the total Fab population with cyt *c*. However, the reaction of the purified material with human cyt *c*, was very heterogeneous. Since the final product was more kinetically heterogeneous than the unpurified anti-oxidase fraction, the heterogeneity must have been induced. It may have resulted from the prolonged prep-

[2] Smith, L., Davis, H. C., Reichlin, M., and Margoliash, E. (1972), submitted for publication.

[3] With this serum (164) human and monkey cyt *c* are equivalent as antigens (Nisonoff *et al.*, 1970).

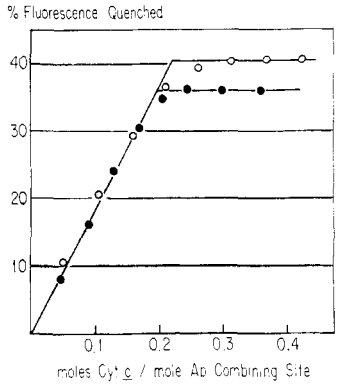

FIGURE 6: The titration of rabbit anti-human cytochrome *c* Fab fragments (total concentration 0.66 × 10^{-6}) with human (○) and monkey (●) cytochromes *c*.

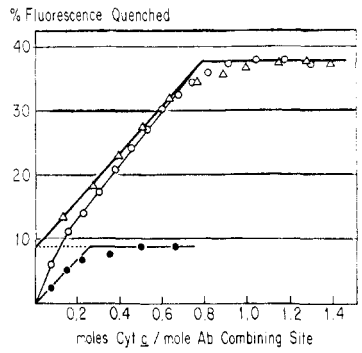

FIGURE 7: The titration the unreacted Fab fragments (0.44 × 10^{-6} M), isolated from an anti-human cyt *c*–monkey cyt *c* reaction mixture, with human cyt *c* (○), monkey cyt *c* (●), and with human cyt *c* after prior titration with monkey cyt *c* (△).

aration time or from one of the multiple steps used in the ultimate purification of this fraction.

Anti-Ile Antibody. Nisonoff *et al.* (1970) have reported that when rabbit antibodies produced against human cyt *c* are reacted with the monkey protein, a significant fraction, 25%, of the antibodies will not react in the precipitin test. Human and monkey cyt *c* differ by a single amino acid at position 58; in human cyt *c* it is isoleucine (Matsubara and Smith, 1962) while threonine is present in the monkey protein (Rothfus and Smith, 1965). The fraction of the antibodies that will not react with monkey cyt *c* will only react with human and kangaroo cyt *c*. Both of these cytochromes *c* have isoleucine at position 58 (Nolan and Margoliash, 1966).

Purified anti-human cyt *c* antibodies were prepared from an immune precipitate and then digested with pepsin to yield Fab fragments. When titrated with human cyt *c* and with monkey cyt *c*, the results shown in Figure 6 were obtained. The titration with the human protein gave a final fluorescence quenching of slightly over 40%. The intercept of the major slope of the titration and the final quenching level indicates a binding stoichiometry of 4.5 antibody combining sites/cyt *c* molecule assuming that all of the Fab fragments are reactive. The titration with monkey cyt *c* begins with approximately the same slope as that with the human protein, but it levels off at a lower final percent quenching. This is consistent with the titration beginning with the same antibody to antigen combining ratio, but with there being a fraction of antibody that reacts with the human but not with the monkey protein.

These anti-human cyt *c* Fab fragments were then equilibrated with 0.01 M phosphate buffer (pH 6.5) and mixed with 0.5 ml of CM52 resin coated with 7.0 mg of monkey cyt *c*. After equilibration, some antibody-bound monkey cyt *c* eluted from the resin and we gel filtered this material on a Sephadex G-100 column equilibrated with 0.05 M phosphate–borate buffer (pH 8.0). The elution pattern consisted of two peaks, an early one containing the cyt *c*–antibody complex and a later colorless one. The first peak represented the exclusion volume of the column and the second corresponded to an elution volume characteristic of Fab fragments of molecular weight 50,000. The activity of this latter fraction was assayed by its ability to bind to resin-bound human cyt *c* (see Methods). 30% of the fraction was found to be inactive.

When this material was titrated with human and with monkey cyt *c*, the results shown on Figure 7 were obtained. In this graph the abcissa (moles of cyt *c* per mole of Ab combining site) has been corrected for the amount of inactive antibody present in the system. The titration with human cyt *c* is biphasic indicating the presence of more than one antibody population. When the antibody was titrated with monkey cyt *c* a significant amount of binding was detected which plateaued at a quenching level of 8%. When the material titrated with the monkey protein was then titrated with the human protein, a linear titration was obtained with a slope corresponding to a stoichiometry of 0.96 antibody combining site/human cyt *c* molecule. Clearly, the original absorption with monkey cyt *c* was not complete and the preparation of site specific antibody contained 21% of material as judged by quenching that would bind both to human and to monkey cyt *c*.

The yield of the anti-Ile antibody was 7.0%. This is far less than the 30% precipitin difference that exists between the human and monkey cytochromes *c* when they react with this same serum. This strongly indicates that there is more than one type of antibody that distinguishes human from monkey cyt *c* in this anti-human cyt *c* serum. Our methods, particularly when immunoabsorbents are used, select for one antibody population that exhibits the property of binding to human *c* but not at all to monkey cyt *c* at the concentrations employed in the assay. There is obviously a significant fraction of antibody sensitive to the structure difference between human and monkey cyt *c* which binds monkey cyt *c* to a finite and significant extent.

For kinetic studies, anti-Ile antibody was prepared by a slightly different procedure. The first step was the precipitation of the total purified 7S material with the equivalent amount of monomeric monkey cyt *c* as determined by a quantitative precipitin test with monkey cyt *c*. Following that step there was further treatment of the nonprecipitating antibody with 2 ml of CM resin equilibrated at pH 6.5 with 0.01 M phosphate buffer to which 5.0 mg of monkey cyt *c* had been adsorbed. Antibody treated in this way bound to human cyt *c* but not to monkey cyt *c* in radioactive binding tests performed as follows. Using a method previously described (Noble *et al.*, 1969) human cyt *c* was radioiodinated and could be effectively displaced from the Ile antibody by an equivalent amount of cold human cyt *c*. However, with cold monkey cyt *c* in 50-fold molar excess no displacement of hot human cyt *c* from this Ile antibody could be achieved. Overall yield of anti-Ile prepared in this way was about 5% of the original purified antibody.

The kinetic properties of the reaction of this anti-Ile 7S

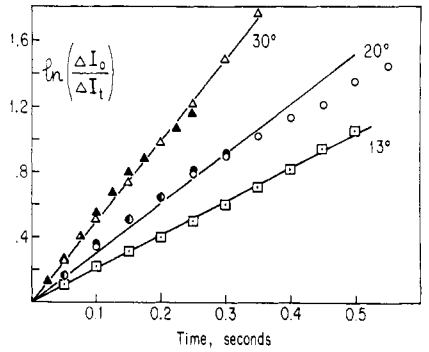

FIGURE 8: The kinetics of the reaction of purified anti-Ile 7S antibodies (0.67 μM) with human cytochrome *c* (6.7 μM) as measured in the stopped-flow apparatus. The reactions were treated as pseudo-first order and ln ($\Delta I_0/\Delta I_t$), is plotted as a function of time. Results obtained at three temperatures are shown. The buffer used was 0.1 M phosphate (pH 7).

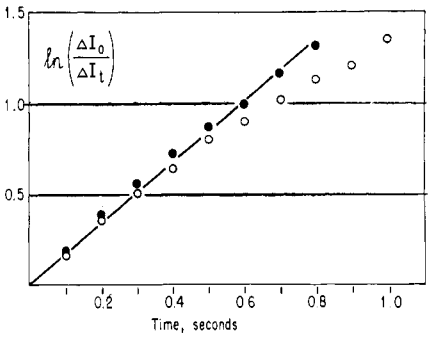

FIGURE 9: The pseudo-first-order kinetic plot of the reaction at 20° of human cytochrome *c* (3.3 μM) with a preparation of anti-human *c* Fab fragments which were combined with an excess of monkey *c* (●). The data for the reaction of purified anti-Ile antibodies with human *c* at this temperature is included for comparison (○). The time scale for this second set of data has been adjusted so that the two curves should coincide if the reactions have the same second-order rate constant. The buffer was 0.1 M phosphate (pH 7).

antibody with human cyt *c* were studied. In Figure 8 are shown the pseudo-first-order kinetic plots of the reaction at 30, 20, and 13° when *0.67* μM Ab (in binding sites) is mixed with *6.7* μM human cyt *c*. Overall, the reaction is reasonably homogeneous. The activation energy of the reaction as calculated from the reaction rates at these temperatures is 8.8 kcal/mole.

There was some concern that the properties of the site-specific antibodies might have been affected by the isolation procedure. To show that this was not the case the following experiment was carried out. To a solution of anti-human cyt *c* Fab fragments (0.2 mg/ml) sufficient monkey cyt *c* was added to be certain that all of the antibodies which would cross react with this protein were bound. The final molar ratio of Fab fragments to monkey cyt *c* was 1.6, considerably lower than the measured stoichiometry. This mixture was then reacted with human cyt *c* in the stopped-flow apparatus and the kinetics of the combination of the unbound Fab fragments with the human protein was examined. The concentration of human *c* after mixing in the flow apparatus was 3.3 μM. In Figure 9 the pseudo-first-order plot of this reaction is compared to that of the purified anti-Ile fraction at the same temperature, 20°. The rates are very similar. The kinetics of the total anti-human cyt *c* Fab saturated with monkey cyt *c* is the more homogeneous, however.

In their earlier studies on the reaction of antibodies with heme protein antigens, Noble *et al.* (1969) noted that the overall reaction of human cyt *c* with anti-human cyt *c* antibodies was slower than the reaction of those antibodies with monkey cyt *c*. On the basis of this they predicted that the rate of reaction of anti-Ile antibodies with human cytochrome *c* would be slower than the overall reaction rate of the total anti-human cyt *c* antibody population. We have confirmed this prediction. In Figure 10 are shown the pseudo-first-order plots of the reaction of human cytochrome *c* (3.3 μM) with anti-human cyt *c* Fab fragments and with specific anti-Ile antibodies. The reaction of the site-specific anti-Ile antibodies is both much slower and much more homogeneous than the reaction of the total anti-human cyt *c* antibody population.

Discussion

We have isolated three different antibody populations that react with their specific protein antigens with a one to one stoichiometry. One of these populations, the anti-oxidase antibodies, is isolated by virtue of an accident of excess synthesis, and the isolation procedure offers no information as to its site of binding on the cyt *c* molecule. The other two populations, anti-Val and anti-Ile are specific to single amino acid alterations in their protein antigens. The site of binding of these two populations to their antigen molecules is almost certainly in the region of the amino acid to which they are sensitive. They cannot be sensitive to a general change in the conformation of the antigen since this would be inconsistent with the observed binding stoichiometry of unity. It is also unlikely that the single binding site is some distance from the amino acid to which the antibodies are sensitive. Reichlin (1972) has shown that immunologically active amino acid alterations in the human hemoglobin molecule are always at the surface of the molecule in one of the regions in which the structure of human hemoglobin differs from the structure of the hemoglobin of the immunized organism. This should hardly be the case if an amino acid alteration could produce an immunologically active allosteric change in the antigen's structure at a distant site. In addition, as previously mentioned, Nisonoff *et al.* (1970) reported that their antibody fraction, which reacted with human but not with monkey cyt *c*, would react with only one other of the many cyt *c*'s tested, that of

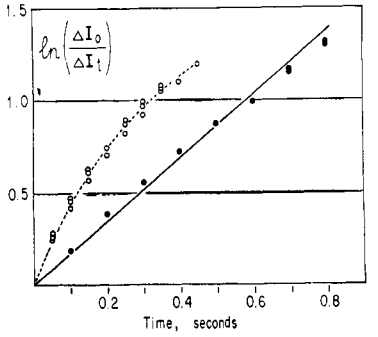

FIGURE 10: The kinetics of the reaction of anti-human cyt *c* Fab fragments (2.0 μM) with human cytochrome *c* (3.3 μM) at 20° (○) is compared to that for the reaction of monkey cyt *c* saturated anti-human cyt *c* Fab fragments with human cytochrome *c* (●). Because of the binding stoichiometry, human cytochrome *c* was present in large excess and the reactions were treated as pseudo-first order. The buffer was 0.1 M phosphate (pH 7).

the kangaroo. Kangaroo cyt *c* differs from human cyt *c* at 10 positions of its sequence, but it is the only one of the tested cyt *c*'s that has in common an isoleucine residue at position 58. Therefore, this antibody population is apparently specific to this particular substitution while being unaffected by numerous other modifications in the protein antigen. This is easily explained only by single site specificity toward a region of the antigen's surface that includes the amino acid to which the antibody population is specific. However, such indirect evidence, although compelling, is not proof; and some doubt will remain until a direct demonstration of the site of binding is achieved.

If their binding site is as we suspect, then these site-specific antibodies represent a portion of the organism's response to a single antigenic site. As previously mentioned, the anti-Val and anti-Ile antibodies which we isolate are those which do not bind at all to the modified antigen during our isolation procedure or at the concentrations used in our fluorescence quenching titrations. This requires that an enormous affinity difference result from the alteration of a single amino acid on the antigen. The amount of site-specific material which we isolate is considerably less than would be predicted from either the number of antibody binding sites on the antigen molecules or from the differences detected by complement fixation between the original and modified antigen molecules. There must be other antibody populations which are sensitive to the amino acid alteration but which still bind significantly to the modified antigen.

In general, site-specific antibodies differ in their properties from the total set of antibodies directed toward a protein antigen. Most notable is their increased homogeneity. When titrated with specific antigen, their fluorescence is quenched linearly with antigen addition. Furthermore, the reactions of the site-specific antibodies with their antigens are kinetically quite homogeneous. Such functional homogeneity in no way establishes molecular homogeneity in these fractions. This aspect of the problem is still under study. Antibodies to one antigenic site also differ from those directed toward a different antigenic site. The rate constants for the reactions of the anti-Ile fraction and the unpurified antioxidase antibodies with cyt *c* are 0.45×10^6 and 0.8×10^6 M^{-1} sec^{-1}, respectively. The rate constant for the reaction of anti-Val antibodies with HbS is 0.58×10^6 M^{-1} sec^{-1}. There is also evidence that the amount of fluorescence quenching resulting from the combination of antibody with antigen differs from one antigenic site to another. The fluorescence of anti-HbS Fab fragments is quenched 42% by HbS, while the fluorescence quenching of the anti-Val Fab fragments, corrected for the nonreactive material present, is only 29%. Anti-human cyt *c* Fab fragments are quenched 41% by human cyt *c*, but the quenching of the fluorescence of anti-Ile Fab fragments by human cyt *c*, when corrected for nonreactive material, is 55%. The fluorescence which results from the interaction of an antibody with its heme protein antigen depends on the number and location of the tryptophan residues on the antibody molecule as well as on the distance from the antigenic site to the heme. That various site-specific antibodies should differ from one another in these respects is not surprising; but this, along with the kinetic differences between these populations, serves to point out that the numerous antigen–antibody reactions that occur at the surface of an antigen molecule are chemically distinct.

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