Monoclonal Antibodies as Probes of Conformational Changes in Protein-engineered Cytochrome c*

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Determination of the nature of the antigen-antibody complex has always been the ultimate goal of three-dimensional epitope mapping studies. Various strategies for epitope mapping have been employed which include comparative binding studies with peptide fragments of antigens, binding studies with evolutionarily related proteins, chemical modifications of epitopes, and protection of epitopes from chemical modification or proteolysis by antibody shielding. In this study we report the use of protein engineering to modify residues in horse cytochrome c that are in or near the epitopes of four monoclonal antibodies specific for this protein. The results demonstrate not only that site-specific changes in the antigen binding site dramatically affect antibody binding, but, more importantly, that some of the site-specific changes cause local and long-range perturbations in structure that are detected by monoclonal antibody binding at other surfaces of the antigen. These findings emphasize the role of native conformation in the stabilization of the interaction between protein antigens and high affinity monoclonal antibodies. Furthermore, the results demonstrate that monoclonal antibodies are more sensitive probes of changes in conformation brought about by protein engineering than low resolution spectroscopic methods such as circular dichroism, where similar spectra are observed for all the analogues. These findings suggest a role for monoclonal antibodies in detecting conformational changes invoked by nonconservative amino acid substitutions or substitutions of evolutionarily conserved residues in protein-engineered or recombinant proteins.

Studies on the nature of the antigen-antibody complex have been limited by the methods available to identify the complete epitopes of protein antigens. Most studies have relied on model protein antigens of known primary and tertiary structure including myoglobin, cytochrome c, and lysozyme (Benjamin et al., 1984) and have identified immunodominant residues using panels of evolutionarily related proteins. Other methods have mapped epitopes using protection of residues in the epitope from chemical modification (Burnens et al., 1987) or from proteolysis (Jemmerson and Paterson, 1986a, 1986b) by antibody shielding. The use of synthetic peptides for mapping epitopes has not proved successful with monoclonal antibodies (mAbs)1 with strong conformational requirements (Benjamin et al., 1984; Berzofsky, 1985; Jemmerson and Paterson, 1986a, b).

A major breakthrough in our knowledge of antigen-antibody interactions has been provided by x-ray crystallographic studies on immune complexes. So far two structures of Fab fragments complexed with protein antigens have been solved. In the first structure the antigen was the model protein lysozyme (Amit et al., 1986), and in the second the influenza virus neuraminidase was the antigen (Coleman et al., 1987). In both structures the residues of the antigen contacting the antibody combining site were found to derive from discontinuous regions of the polypeptide chain which created an interface with the antibody extending over a quite large topographic surface area of the protein antigen (about 500 Å²). However, the two structures differed in the degree of conformational adaption required by both antigen and antibody to form the complexes. In the case of the lysozyme complex no changes in conformation between the lysozyme molecule bound to the antibody and the previously determined lysozyme structure were observed, suggesting that strong complimentarity occurred between the interacting sites. The structure of the neuraminidase complex, however, suggested that rearrangements may occur in both the antibody and the antigen on complex formation. No doubt our knowledge of protein-antibody interactions will be further extended by future x-ray crystallographic studies on the structures of immune complexes. Unfortunately, high quality crystals of antibody-antigen complexes for x-ray diffraction are very difficult to obtain. In addition, the analysis of the data obtained from such large structures can take years. The fact that the three-dimensional structure of murine IgGs has been well studied (Amzel and Poljak, 1979; Davis and Metzger, 1983) and that, of the antigens, lysozyme (Blake et al., 1965) and neuraminidase (Varqhe and Paterson, 1983) were known made the structural determinations in these cases less complicated. These limitations, therefore, necessitate additional lines of approach that can be applied more generally to determine the discontinuous regions of an antigen that interact with antibody and the conformational constraints of the interaction.

Using various methodologies we have mapped residues in the epitopes recognized by four mAbs to horse cytochrome c (Carbone and Paterson, 1985; Jemmerson and Paterson, 1986a; Cooper et al., 1987). Our studies with these mAbs have indicated a strong requirement for native conformation of the antigen for recognition in that all four antibodies fail to bind peptide fragments known to be a part of the epitopes (Jemmerson et al., 1987). The abbreviations used are: mAb, monoclonal antibody; Boc, tert-butoxycarbonyl; Hse, homoserine; PBS, phosphate-buffered saline, 0.01 M, pH 7.4.

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merson and Paterson, 1986a). Furthermore, disruption of the native structure in horse cytochrome c brought about by a site-directed chemical modification of Tyr<sup>104</sup> influenced recognition of one mAb whose epitope does not include this residue (Cooper et al., 1987). The four mAbs are of high affinity (K<sub>d</sub> ≈ 10<sup>9</sup>) and bind at two distinct areas of the cytochrome c molecule. The E8 mAb binds to a region around Lys<sup>96</sup> (Carbone and Paterson, 1985; Jemmerson and Paterson, 1986a). The three other mAbs bind on the opposite side of the molecule and have overlapping epitopes. mAbs C3 and C7 bind to residues His<sup>99</sup> and Pro<sup>104</sup>; and E3 binds to Thr<sup>97</sup> (Carbone and Paterson, 1985; Jemmerson and Paterson, 1986a; Cooper et al., 1987). Having identified these epitopes, we used molecular modeling to aid in identifying other possible epitopes seen by the mAbs and engineered 11 cytochrome c analogues using semisynthesis with two goals in mind. First, we have modified an evolutionarily conserved residue suspected to be a part of the E8 epitope (Glu<sup>95</sup>—Gln and Lys) to continue the epitope mapping studies. Second, we have chemically modified residues, removed residues, and broken peptide bonds between residues in or near the antibody epitopes to determine what effect these local changes have on antibody binding near the site of perturbation and if these local changes would cause long-range structural perturbations that affected antibody binding at distant surfaces.

The availability of mAbs of single specificity from hybridoma cell lines has resulted in the use of antibodies for many nonimmunological purposes which include the isolation and purification of cellular proteins (Schulman et al., 1978), the detection of the expression of recombinant gene products (St. John et al., 1986), and their use as catalysts of organic reactions (Tramontano et al., 1986). The studies described in this paper extend the general utility of mAbs to the field of conformational analysis of proteins. These studies have demonstrated that panels of mAbs with well defined epitopes on the antigen are effective probes of conformational changes invoked in a protein molecule by substitutions of evolutionarily conserved residues, or by the removal of peptide bonds and loop regions.

**EXPERIMENTAL PROCEDURES**

**Materials**

Cytochrome c (horse heart, type III), carboxypeptidase B (pancreatic, type I), and trypsin inhibitor (soya-bean type I) were obtained from Sigma; trypsin (1-1-1-trasylol-2-phenylethyl chloromethyl ketone (TPCK)-treated) from Worthington. Methyl acetylimidyl modification of lysine is shown in Fig. 1. The pK of this group is 12.7–12.8 (Wallace and Harris, 1984), and therefore acetylimidyl-lysine is positively charged at neutral pH. This modification, therefore, represents only a small group addition to lysine that retains native charge. The purity of the products was checked by ion-exchange chromatography on SP (sulfopropyl)-Sephadex C-25 as described (Wallace and Harris, 1984).

**Methods**

**Semisynthesis of Cytochrome c Analogues**

Acetimidylated Cytochrome c—Acetimidylation of cytochrome c was performed by method (IV) of Wallace and Harris (1984). The acetimidyl modification of lysine is shown in Fig. 1. The pK of this group is 12.7–12.8 (Wallace and Harris, 1984), and therefore acetylimidyl-lysine is positively charged at neutral pH. This modification, therefore, represents only a small group addition to lysine that retains native charge. The purity of the products was checked by ion-exchange chromatography on SP (sulfopropyl)-Sephadex C-25 as described (Wallace and Harris, 1984).

**Hse<sup>96</sup>—Cytochrome c** was treated with CNBr under conditions such that cleavage occurred only at Met<sup>96</sup> and not Met<sup>98</sup> (Corradin and Harbury, 1970). The two fragments (1-65 and 66-104) were condensed under neutral conditions to re-form the native protein (Wallace and Rose, 1985). In the dithionite-reduced complex the missing peptide bond, 65-66, is re-formed by aminolysis of the COOH-terminal homoserine lactone of 1-65 by the α-amino group 66-104 (Corradin and Harbury, 1974). For this reason, all of the semisynthetic analogues in groups I and II except acetimidylated cytochrome c have homoserine, not methionine, at position 65. This substitution does not affect the functional or physical properties of cytochrome c (Boswell et al., 1981).

(Acetimidylated 1-65)-(66-104) and (1-65)-(Acetimidylated 66-104).—Using the procedure described above, chimerae were prepared in which a native fragment was combined with an acetimidylated fragment, at either the NH<sub>2</sub>-terminal or COOH-terminal position (Wallace, 1984).

Gln<sup>96</sup> and Lys<sup>98</sup>—The semisynthetic substitutions at position 66 were prepared as described previously (Wallace and Corthesy, 1986). Briefly, acetimidylated-cytochrome c was treated with CNBr to yield the 1-65 and 66-104 fragments. The acetimidylated 66-104 peptide was truncated at the amino end by Edman degradation (Wallace and Offord, 1979) to remove the glutamic acid at position 66. Then N<sup>C</sup>-Boc-glutamine-<i>N</i>-hydroxy succinimide ester or N<sup>C</sup>-di-Boc-lysine-<i>N</i>-hydroxy succinimide ester were used for coupling to the acetimidylated fragment 67-104 (Wallace and Offord, 1979). Acetimidylated 1-65 and acetimidylated 66-104 (Gln<sup>96</sup> or Lys<sup>98</sup>) were condensed as described above for Hse<sup>96</sup>. The lysine protecting group was either left on (acetimidylated Gln<sup>96</sup>, acetimidylated Lys<sup>98</sup>) or removed (Lys<sup>98</sup>, Gln<sup>96</sup>) (Hunter and Ludwig, 1962).

1-38-39-104—Acetimidylated cytochrome c was digested with trypsin, and the tryptic peptides were separated by gel filtration on Sephadex G-50 (Harris and Offord, 1977). The tryptic peptides, N<sup>C</sup>-acetimidyl fragment 1-38 and N<sup>C</sup>-acetimidyl fragment 39-104 were each further purified by cation-exchange chromatography (Wallace and Proudfoot, 1987). The peptides were desalted by gel filtration on Sephadex G-25 in 1% acetic acid and were recovered from solution by freeze-drying. The noncovalent complex was prepared and purified as described by Wallace and Proudfoot (1987). The lysine side chain protection (acetimidylated group) was removed with concentrated ammonium acetate (Hunter and Ludwig, 1962).

1-37-38-104—N<sup>C</sup>-Acetimidyl fragment 1-37 was prepared by carboxypeptidase B digestion of N<sup>C</sup>-acetimidyl fragment 1-38 (Harris, 1978). The fragment was purified by cation-exchange chromatography as described above.

Elongation of the N<sup>C</sup>-acetimidyl fragment 39-104 was achieved by methods similar to those described for N<sup>C</sup>-acetimidyl fragment 81-104 (Wallace and Offord, 1979). The hydroxybenzotriazole ester of t-<i>b</i>-arginine was formed before coupling.

Products of coupling were purified after deprotection by cation-exchange chromatography. Confirmation of coupling was obtained by amino acid composition and end group determinations. Formation of the noncovalent complex (1-104) occurred upon mixing of the two fragments in equimolar amounts in dilute neutral phosphate buffers (Proudfoot et al., 1986). The lysines were deprotected as described above.

Fig. 1. Chemical modification of lysine residues using methyl acetylimidate. The acetimidylated group can be removed with concentrated ammonia/ammonium acetate buffer.

**Lysine**

\[
\text{NH}_2^+ \xrightarrow{H_2N-(CH_2)_4-CH} \xrightarrow{CH_3-C-\overset{\equiv}{O}CH_2} CH_3 \\
\text{pH} > 11.0 \quad \xrightarrow{C-\overset{\equiv}{NH}-(CH_2)_4-CH} \xrightarrow{\text{NH}_2^+}
\]

**Probes**

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COOH-terminal carboxyl group. Acetimidylated groups were then removed.

Circular Dichroism Spectroscopy

CD spectra were obtained with an Aviv Model 61DS solid-state CD instrument (Aviv Associates, Lakewood, NJ). The instrument is equipped with a 50 KHz photoelastic modulator and an end-on photomultiplier. The instrument was calibrated with d-10-camphor-sulfonic acid (Chen and Yang, 1977). The concentration of protein solutions was determined by absorbance using an extinction coefficient e222 = 1.95 ml mg⁻¹ cm⁻¹ for cytochrome c. The CD was measured in a 2.0-mm sample cell which was maintained at 25 °C with a thermostated cell holder. Five scans were averaged and were corrected for the base line. The corrected data set was multiplied by a constant to obtain the mean residue ellipticity, θ. Secondary structure was estimated by a nonlinear least-squares curve-fitting program (Chang et al., 1978).

Production of mAbs to Horse Cytochrome c

The production of anti-horse cytochrome c mAbs C3, C7, E3, and E8 was described by Carbone and Paterson (1986). The mAbs were purified from ascites fluid by affinity chromatography on horse cytochrome c coupled to CNBr-Sepharose (Carbone and Paterson, 1985).

Radioimmunoassay of Antibodies on Microtiter Plates

Microtiter plates were coated with 50 µl of antigen solution (0.01 mg/ml) per well and incubated for 12-16 h at 4 °C. The plates were then blocked with 5% horse serum in PBS for 1 h at room temperature and washed three times with 1% horse serum in PBS. The plates were then coated with 50 µl of affinity purified anti-horse cytochrome c mAbs (concentrations were 0.48-0.81 mg/ml, undiluted) at various dilutions (10⁻¹-10⁻⁴) in 1% horse serum and incubated at room temperature. After 4 h the plates were washed and a rabbit antibody directed against mouse IgG was added. Following a 2-h incubation period the plates were washed and ¹²⁵I-labeled, affinity-purified goat anti-rabbit IgG was added. After incubation overnight, the plates were washed with water, dried, and counted. Nonspecific binding (background) was determined for microtiter plates blocked with 5% horse serum that had not been coated with antigen.

Radioimmuneosssays were also performed at pH 5, 6, and 7 for the 1-38-39-104 cytochrome c analogue. For these assays, PBS was adjusted to the appropriate pH with 6 M HCl. The assays were performed as usual except that all reactions were carried out at the designated pH.

Radiolabeling

¹²⁵I labeling of affinity-purified goat anti-rabbit antibodies was performed according to standard procedures using chloramine T (Jemmerson and Margolish, 1981).

RESULTS

Semisynthesis of Cytochrome c Analogues

The 11 analogues that were prepared for this study by semisynthesis are shown in Table I. The group I analogues were prepared to determine if chemical modification of the lysines would have any effect on antibody binding. The group II analogues were prepared to determine if Glu(E8) was a part of the E8 mAb epitope. The substitutions represented a conservative change (Glu → Gln) and a nonconservative change (Glu → Lys). The group III analogues were prepared to determine the effect of structural modifications of antigen that involved no change to amino acid side chains, near antibody epitopes (C3, C7, E3) and distant from an antibody epitope (E8), on antibody binding.

Structural Analyses

Each of the cytochrome c analogues was analyzed by circular dichroism spectroscopy to confirm that the modifications had not caused denaturation of the antigen. Far UV CD measurements were made from 260 to 195 nm, and the ellipticities at 222 nm of each analogue are shown in Table I. The spectra of those analogues (acetimidylated 1-65)-(66-104), acetimidylated Lys(85), and (acetimidylated 1-65)-(66-104)-(C7) were measured at 25 °C in 2.0-mm cells and the buffer used for all analyses was phosphate-buffered saline (PBS), pH 7.4.

TABLE I

<table>
<thead>
<tr>
<th>Biological activities and mean residue ellipticities of chemically modified cytochrome c analogues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biological activity</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>Group I</td>
</tr>
<tr>
<td>(Acetimidylated 1-65)-(66-104)</td>
</tr>
<tr>
<td>(1-65) (Acetimidylated 66-104)</td>
</tr>
<tr>
<td>(Acetimidylated cytochrome c)</td>
</tr>
<tr>
<td>Group II</td>
</tr>
<tr>
<td>Hes&lt;sup&gt;−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acetimidylated Lys&lt;sup&gt;−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Acetimidylated Gln&lt;sup&gt;−&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group III</td>
</tr>
<tr>
<td>des-40-55</td>
</tr>
<tr>
<td>1-38-39-104</td>
</tr>
<tr>
<td>1-37-38-104</td>
</tr>
<tr>
<td>Native cytochrome c</td>
</tr>
</tbody>
</table>

* All analogues were tested in the depleted mitochondrial succinate assay system of Jacobs and Sanadi (1960). This method has been discussed by Wallace (1984).


Wallace and Harbury, 1970.

Wallace and Corthesy, 1986.


Proudfoot et al., 1986.
ondary structure of cytochrome c, as judged by circular dichroism. NMR spectroscopy has also shown that total acetimidylation of horse cytochrome c causes no gross structural changes (Boswell et al., 1983). The fact that these analogues have nearly native biological activities (Table I) and redox potentials (Wallace, 1984) also argues against any denaturation caused by this modification.

In all group II analogues in which there are substitutions at Glu<sup>66</sup>, the spectral data do indicate that there are disruptions in structure (Table I) in that they show a mean residue ellipticity at 222 nm of 62–81% of unmodified cytochrome c. This is really not surprising since Glu<sup>66</sup> is believed to be important to the conformational stabilization of the protein through salt bridge formation (Wallace and Corthesy, 1986).

Glu<sup>66</sup> is situated on the exposed surface of the 62–70 helix in cytochrome c; thus the structural perturbations detected by CD may mean that the helix has been partially disrupted in these analogues. The disruptions, however, do not appear to affect biological activity (Table I).

In the group III analogues, removing the loop that encloses the bottom edge of the heme group (des-40–55) apparently causes some small structural change, since the proportions of α-helix and 3<sub>10</sub> bend are not explicable on the basis of the loss of the loop alone (Takano et al., 1977) (Table I, Fig. 2). The loss of biological activity is believed to be principally due, however, to the change in redox potential that is caused by increased exposure of the heme group (Wallace and Proudfoot, 1987). The disruption of native structure in the loop that is caused by breaking a peptide bond between residues 38 and 39 (1–38.39–104) also similarly affects biological activity without manifesting significant changes in the CD spectrum (Table I). Greater changes accompany the absence of the 37–38 peptide bond in 1–37.38–104; yet, in this case, 55–70% of the biological activity is retained, implying a structure that is more nearly able to mimic that of the native protein (Proudfoot et al., 1986a). In general, therefore, there seems to be no simple correlation between change in molar ellipticity and deviation from the native conformation as manifested by functional characteristics.

From the spectral data (260–195 nm scans) of each of the analogues, the secondary structural content was estimated by the curve-fitting procedure of Chang et al., 1978. (Table II). All of the analogues appeared to have near native amounts of α-helix (79–49.2%; control = 36.5%), no predicted β-structure except for the acetimidylated Gln<sup>m</sup> analogue (9.6%), and near native amounts of both 3<sub>10</sub> bends (15.6–28.6%; control = 22.9%) and random structure (35.3–48.5%; control = 40.5%). From the structural data analyses, although there do appear to be modifications in the secondary structure of some of these analogues, none of the modifications have resulted in those major effects on the native structure that would be readily detectable by CD spectroscopy.

Radioimmunoassays

Group I Analogues (Acetimidylated Modifications of Lysines)—One of the first questions we addressed was whether mAbs could recognize the difference between Lys and acetimidylated Lys. For the C3 mAb, the acetimidylated modification of the NH<sub>2</sub>-terminal fragment (acetimidylated 1–65). (66–104) or COOH-terminal fragment (1–65) (acetimidylated 66–104) has no effect on binding, although the totally acetimidylated protein is not recognized as well as native cytochrome c (Fig. 3A).

In the case of the C7 mAb, none of the acetimidylated modifications had any effect on binding (Fig. 3B). These results indicate that modification of lysine side chains of cytochrome c has little or no effect on C3 and C7 binding and imply that either these side chains are not part of the epitopes for these monoclonal antibodies or that acetimidylated lysine side chains can also interact with these mAbs. The acetimidylated modification of 66–104, however, does appear to affect E3 binding (Fig. 3C and Table III). The (acetimidylated 1–65). (66–104) recognition appears normal, whereas (1–65). (acetimidylated 66–104) and acetimidylated cytochrome c 1–104 are both bound with less avidity (7 and 67 times more Ab needed for 50% binding level of the control, native cytochrome c). This implies that one or more lysines in the COOH-terminal fragment is a part of the epitope. The E8 mAb recognizes a Lys in the NH<sub>2</sub>-terminal fragment of the molecule (Lys<sup>66</sup>) yet the data indicate that the modification of Lys<sup>66</sup> (acetimidylated 1–65). (66–104), has no affect on binding, whereas the modification of lysine in the COOH-terminal fragment, (1–65). (acetimidylated 66–104) and acetimidylated cytochrome c 1–104, indicates that at least one lysine within the sequence 66–104 is also important for recognition. Another possible explanation for these results is that modifications of the lysines, per se, do not directly affect binding, but that indirect, slight structural changes due to the modification do.

Group II Analogues (Substitutions at Position 66)—Data from epitope mapping studies has suggested that residues in the epitope for E8 lie within the sequence 61–73 (Jemmerson and Paterson, 1986a). Therefore, we made substitutions at the surface residue Glu<sup>66</sup> to determine if this was a part of the E8 epitope. Because a modification at residue 66 is not a part of the epitopes of C3, C7, or E3, these mAbs could be used as probes of long-range structural changes caused by the modifications at position 66. In these analogues Glu<sup>66</sup> was substituted with a glutamine or a lysine. All of the group II analogues and the group I analogues, (acetimidylated 1–65). (66–104) and (1–65). (acetimidylated 66–104) have Hse instead of Met<sup>66</sup>. This substitution by itself appears to have no effect on recognition by any of the mAbs (see Table III). C3 recognized Gln<sup>66</sup> and Lys<sup>66</sup> as native, whereas the acetimidylated Gln<sup>66</sup> and acetimidylated Lys<sup>66</sup> (Fig. 4A, Table III) were not. In fact,
**FIG. 3.** Solid-phase radioimmunoassays for mAb C3 (panel A), C7 (panel B), E3 (panel C), and E8 (panel D) on the group I cytochrome c analogues. Cytochrome c, ●; (acetimidylated 1–65), ○; (1–65) (acetimidylated 66–104), □; acetimidylated cytochrome c, △; nonspecific binding (background), ▼.

**TABLE III**

Relative avidities of cytochrome c analogues

Relative avidity = antibody concentration for 50% binding of analogue/antibody concentration for 50% binding of control. Numbers represent the factor of antibody concentration needed to obtain 50% of the binding level of control (cytochrome c). Thus an analogue with the same avidity as cytochrome c has an avidity of 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>C3</th>
<th>C7</th>
<th>E3</th>
<th>E8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group I</td>
<td>(Acetimidylated 1–65) (66–104)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>(1–65) (acetimidylated 66–104)</td>
<td>1</td>
<td>2</td>
<td>7⁺</td>
</tr>
<tr>
<td></td>
<td>Acetimidylated cytochrome c</td>
<td>4⁺</td>
<td>1</td>
<td>67⁺</td>
</tr>
<tr>
<td>Group II</td>
<td>Hse⁶⁶</td>
<td>2</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Acetimidylated Lys⁶⁶</td>
<td>15⁺</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Lys⁶⁶</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Acetimidylated Gln⁶⁶</td>
<td>8⁺</td>
<td>2</td>
<td>10⁰⁺</td>
</tr>
<tr>
<td></td>
<td>Gln⁶⁶</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Group III</td>
<td>des-40–55</td>
<td>(&gt;5 × 10⁹)⁺</td>
<td>(&gt;2 × 10⁹)⁺</td>
<td>(&gt;3 × 10⁹)⁺</td>
</tr>
<tr>
<td></td>
<td>1–38:39–104</td>
<td>(&gt;5 × 10⁹)⁺</td>
<td>(&gt;2 × 10⁹)⁺</td>
<td>(&gt;3 × 10⁹)⁺</td>
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<td></td>
<td>1–37:38–104</td>
<td>(&gt;5 × 10⁹)⁺</td>
<td>(&gt;2 × 10⁹)⁺</td>
<td>(&gt;3 × 10⁹)⁺</td>
</tr>
</tbody>
</table>

⁺p < 0.1.
⁺⁺p < 0.01.
⁺⁺⁺p < 0.001.
⁺⁺⁺⁺p < 0.0001.
Not above background.
there is a general trend in loss of avidity by C3 for acetimidylated cytochrome c, acetimidylated Glu\textsuperscript{66}, and acetimidylated Lys\textsuperscript{66} (4, 8, and 15 times more Ab needed for 50% binding level as control, native cytochrome c), indicating that the charge changes at residue 66 in the acetimidylated derivatives appear to exacerbate the avidity changes.

All of the group II analogues were recognized as native by the C7 mAb, which indicated native structure at the C7 epitope (Fig. 4B, Table III). E3 also recognized the Glu\textsuperscript{66} and Lys\textsuperscript{66} analogues as native, but again indicated a sensitive lysine modification in the acetimidylated Glu\textsuperscript{66} and acetimidylated Lys\textsuperscript{66} analogues (compare Figs. 3C and 4C). This sensitivity has been shown to involve a lysine in the 66-104 area of the molecule (Fig. 3C).

All three mAb (C3, C7, E3) recognized the Glu\textsuperscript{66} and Lys\textsuperscript{66} derivatives as native. The only exceptions were that the acetimidyl-modified analogues bound with lower avidity by C3 and E3. These data simply confirm the findings shown in Fig. 3 that binding by both of these mAbs is influenced directly or indirectly by the acetimidylated modification. The E8 data shown in Fig. 4D, however, indicate something quite different. The Glu\textsuperscript{66} and Lys\textsuperscript{66} are bound with substantially less avidity than native cytochrome c (4 and 13 times as much E8 needed for 50% binding level of control, Fig. 4D, Table III). This diminished binding probably results from a direct change in the epitope, although a structural change, as suggested by the CD data, near the epitope cannot be totally ruled out. The general loss of binding of E8 to acetimidylated analogues is confirmed with the acetimidylated Lys\textsuperscript{66} and acetimidylated Glu\textsuperscript{66} analogues (Fig. 4D).

The group III analogue data demonstrate the effects on antibody binding by a loss of part of the epitope (des-40-55) or a structural change near an epitope (1-37:38-104 and 1-38:39-104). Three of the four mAbs have part of their epitopes missing in the des-40-55 analogue (C3-Pro\textsuperscript{44}, C7-Pro\textsuperscript{44}, E3-Thr\textsuperscript{77}), and these three mAbs have residues in their epitopes near the missing peptide bonds 37.38 and 38.39. Only the C7 mAb is able to recognize any of these analogues to any extent above background (Fig. 5, A-C). This indicates that all three mAbs are sensitive to local structural changes at or near their epitopes on cytochrome c and that the remaining residues in their epitopes are not sufficient to stabilize the interaction with antibody.

The group III modifications include changes near to (1-37.
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FIG. 5. Solid-phase radioimmunoassays for mAb C3 (panel A), C7 (panel B), E3 (panel C), and E8 (panel D) on the group III cytochrome c analogues. Cytochrome c, ●; des-40-55, ○; 1-38-39-104, □; 1-37-38-104, △; nonspecific binding (background), ▼.

38–104 and 1–38–39–104) and distant from (des-40–55) the residue Lys⁶⁶, which is part of the epitope on cytochrome c recognized by E8. We believe, however, that Lys⁶⁶ is on the edge of the epitope for E8 since blocking studies with the four mAbs (Carbone and Paterson, 1985) demonstrated that E8 could bind the cytochrome c molecule simultaneously with any one of the other three mAbs. In addition, the studies presented here indicate Glu⁶⁶ and at least one lysine in the carboxyl-terminal region (66–104) are also in the epitope for E8. All of the group III modifications result in a total abrogation of binding by E8. Thus, mAb E8 is able to recognize long range structural perturbations in cytochrome c that were caused by these site-directed changes. Furthermore, the long-range effects that are detected by E8 are probably subtle ones, since these analogues appear to have near-native CD spectra.

Antibody Binding at Lower pH

Many of the cytochrome c analogues have a lower pK for the alkaline transition than the native protein (Wallace, 1984; Wallace and Proudfoot, 1987), which results in a conformational change near the loop structure that covers the heme crevice. This is particularly relevant for (1–38–39–104) which has an alkaline transition in the physiological range in which the previous assays were performed, i.e. pH of 7.1 (Wallace and Proudfoot, 1987). Thus, radioimmunoassays were also performed at high ionic strength (PBS, 0.01 M phosphate, 0.15 M NaCl) and at lower pH (5, 6, and 7). Results of these assays on the 1–38–39–104 analogue were the same as those at pH 7.4 (data not shown). This confirms that any lack of binding by mAbs to these analogues is not simply due to the alkaline transition conformational change of these antigens.

DISCUSSION

The four mAbs used in this study are of high affinity (\(K_d = 10^9–10^{10}\) M⁻¹; Carbone and Paterson, 1985) and from previous studies appear to recognize only determinants with native conformation (Jemmerson and Paterson, 1986a; Cooper et al., 1987). In the studies presented here, we have shown that they also appear to be sensitive to small changes in antigen conformation that are brought about by site-directed changes. The fact that antibodies can recognize conformational changes is consistent with previous studies using other antigens. Reichlin and co-workers (Lando and Reichlin, 1982; Lando et al., 1982) demonstrated that serum antibodies to myoglobin contain two distinct populations, one that reacts with peptide fragments from the native molecule, and one that is specific for intact antigen. In later studies, Berzofsky (Berzofsky et al., 1982) produced high affinity mAbs to sperm
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... whale myoglobin which were specific for the intact antigen. However, although these and other studies (Friguet et al., 1984; Hollander and Katchalski-Katzir, 1986) have demonstrated the sensitivity of mAbs to rather large conformational changes such as those invoked by denaturing the antigen or cleaving it into fragments, the studies presented here indicate that mAbs may be very sensitive probes of structure and can recognize even subtle changes in conformation.

From previous epitope mapping studies, we have established that His\textsuperscript{65} and Pro\textsuperscript{44} are a part of the antigen epitope recognized by mAbs C3 and C7; and that Thr\textsuperscript{47} is a part of the antigen binding site of mAb E3 (Carbone and Paterson, 1985; Jemmerson and Paterson, 1986; and Cooper et al., 1987). From the studies presented here, the radioimmunoassay data for the acetimidyl-modified analogues indicates that lysine modification affects antibody binding for each of the mAbs to varying degrees. Both mAbs C3 and C7 are only slightly affected by this modification, with C3 binding diminished by total acetimidylated only (four times more antibody needed for 50% binding level of control, native cytochrome c, \(p < 0.1\) and C7 binding diminished only with the COOH-terminal acetimidylated analogue, (1-65) (acetimidylated 66-104) (two times as much C7 antibody needed for 50% binding level of control (not significant). Since the totally acetimidylated analogue was bound with the same avidity as native cytochrome c, this implies that C7 is unaffected by these lysine modifications. C3, on the other hand, was only affected by total acetimidylation and not affected by acetimidylation of either fragment. This demonstrates that it is probably not the lysine modification itself that causes the change in avidity, but a slight structural change such as a steric hindrance effect.

With the other two mAbs, E3 and E8, we see quite dramatic avidity changes for both (1-65) (acetimidylated 66-104) and acetimidylated cytochrome c (Table III). This implies that both mAbs have a previously unidentified lysine in the 66-104 fragment that is a part of their epitopes. Furthermore, the lysines are not the same because these mAbs have been shown to have nonoverlapping recognition sites (Carbone and Paterson, 1985). The fact that modification of Lys\textsuperscript{66} known to be a part of the E8 epitope, appear as native to E8, whereas a lysine in the COOH-terminal fragment does not, shows that interpretation of these results is not entirely straightforward. It is possible, however, that the interaction of Lys\textsuperscript{66} with mAb E8 is stabilized mainly by electrostatic forces which are not influenced by acetimidylation of modification whereas steric effects are important for the lysine in the 66-104 fragment of the molecule.

Although E8 did not, three mAbs, C3, C7, and E3, tolerated the Glu\textsuperscript{66} substitutions, indicating that the three overlapping epitopes distant from position 66 were in a native conformation, and had therefore not been disrupted by the site-directed modification. In the group III analogues, structural modifications near the epitopes totally abrogated binding of the C3 and E3 mAb, and greatly diminished the binding of the C7 mAb. In addition, E8 did not recognize these analogues, even though epitope is distant from the region of modification. The changes to cytochrome c structure in the group III analogues are in a loop region of cytochrome c which is stabilized by seven intraloop hydrogen bonds and 11 hydrogen bonds that link the loop to the remainder of the molecule (Proudfoot et al., 1986) (see Fig. 6A). Some of these hydrogen bonds may occur between residues in the loop region and residues in the epitopes of the mAbs in other regions of the molecule, e.g. His\textsuperscript{65}, which is hydrogen bonded to residue 44. Two of the group III analogues differed from native cytochrome c only in the absence of a peptide bond, 37-38 or 38-39 (see Fig. 6A). The inability of all four of the mAbs to recognize any of the group III analogues suggests that not only local conformational perturbations involving the loop region itself occur in the absence of either 40-55, or the hydrogen bond between residues 37 and 38 or 38 and 39, but that the hydrogen bond network between the loop and the remainder of the molecule is disrupted leading to long-range conformational perturbations. The lack of reactivity of these mAbs to peptide fragments and whole tryptic digests of cytochrome c (Jemmerson and Paterson, 1986a, b) is consistent with their inability to interact with the refolded intact polypeptide chain in the absence of one peptide bond. Residues in the epitopes of all four mAbs are shown in two different stereo views in Fig. 6, A and B.

mAb epitope mapping studies have identified Lys\textsuperscript{66} as a part of the antigen binding site for E8 (Carbone and Paterson, 1985; Jemmerson and Paterson, 1986a) using fine specificity analyses with natural mutants and the proteolysis of immune complexes. Furthermore, the proteolysis data indicated that the peptide 61-73 was retarded in its release from the antigen-antibody complex during tryptic digestion suggesting that some part of this peptide is bound to the mAb E8 (Jemmerson and Paterson, 1986a). The data presented in this study clearly indicate that residue 65 is not one of the other possible contact residues for E8 in the 61-73 peptide. In all of the 1-65-66-104 complexes residue 65 was a homoserine and in each case this modification was completely tolerated. Since this modification has been shown to cause a slight perturbation in structure around His\textsuperscript{66} (Boswell et al., 1981), this region must also be outside the epitope for E8. Glu\textsuperscript{66} is a surface residue within the 61-73 peptide that is within 10 A of Lys\textsuperscript{66} and thus could be a part of the epitope for E8 (these residues are displayed in Fig. 6B). From the E8 antibody binding data, it appears that this prediction was correct. Binding of Glu\textsuperscript{66} and Lys\textsuperscript{66} analogues by E8 was substantially reduced (4 and 13 times as much E8 was needed for 50% binding level of control (p < 0.1 and p < 0.02, respectively). These results indicate that the epitope recognized by the E8 mAb has been affected by these site-directed changes. This loss of binding is not caused by a general denaturation since the three other mAbs recognize the Gln\textsuperscript{66} and Lys\textsuperscript{66} analogues as native. These are the only site-directed changes that affect the binding of a single mAb and thus imply that this effect is caused by a direct change in the epitope. Whether or not the recognition changes are caused by substitution of the residue itself, or a structural change in the immediate vicinity of the substitution, remains to be established. The CD data indicate that there has been a loss of helical content in these analogues. However, while the helical contents for the Gln\textsuperscript{66} and Lys\textsuperscript{66} derivatives are almost identical, the binding data, compared to native cytochrome c, are very different (p < 0.1 versus p < 0.02). This suggests it is indeed a residue change that is causing loss of antibody binding. A more sensitive system to have measured these changes would have been an assay system in which the modified cytochrome c competes with native cytochrome c for binding to the mAb (Carbone and Paterson, 1985; Cooper et al., 1987). Unfortunately, because of the limited quantities of engineered analogues, we were not able to perform these sorts of analyses.

The emerging picture of the E8 combining site is shown in stereo view (Fig. 6B). The surface residue Glu\textsuperscript{66} occurs on a stretch of polypeptide chain between 60 and 66 and clearly falls into an area between the residues known to be a part of the E8 binding site. In addition, this position represents one of the six amino acid changes between horse and mouse cytochrome c and may therefore be immunogenic. Thus, in...
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FIG. 6. Stereo-computer graphics representations by J. Tainer (Scripps Clinic), constructed with the program GRAMPS (Connolly and Olsen, 1985) and GRANNY (O'Donnell and Olsen, 1981), showing the α-carbon backbone of horse cytochrome c and selected side chains. The horse cytochrome c coordinates used in this representation were derived by J. Sayre (Scripps Clinic) and J. Tainer from the tuna cytochrome c coordinates (Takano and Dickerson, 1981) using molecular dynamics calculations. A, in this view of the molecule the α-carbon backbone is dashed for the loop region 40-55 and the side chains of Arg38 indicates the region of the backbone where specific cleavages were made. B, this view indicates the proximity of residues 57, 62, and 85 to residues in the epitope for E8 (60 and 66).

In future studies, we will engineer substitutions at position 62. Defining all the residues that form the antigenic site will greatly enhance the utility of these mAbs. Once the complete epitopes are known, then their ability to measure structural perturbations along the surface of cytochrome c will be more straightforward. Furthermore, a complete definition of their epitopes would enhance their utility in structure and functional studies with cytochrome c and its surface interactions with its physiological partners in the electron transport chain.

These results have important implications for the role of structural complementarity in protein-protein interactions in general. Protein and DNA engineering are used increasingly in the manipulation of surface regions of pathogenic protein antigens to provide more efficacious recombinant vaccines, in applications to mapping the binding sites of interacting protein systems outside the immune system, and in structure-function studies involving modification of residues in the catalytic sites of enzymes. Our results indicate that site-directed changes in proteins brought about by site-directed mutagenesis or semisynthesis may not be directly responsible for observed changes in biological activity but may invoke their effects through structural perturbations in other regions of the molecule besides the site of modification. The ability to monitor the surface topography of a modified protein with mAbs has the potential to determine the level of structural change along the total surface of the antigen. This study clearly shows that the ability of mAbs to detect localized structural changes is certainly better than low resolution spectral techniques such as circular dichroism.

In conclusion, the use of site-directed chemical modification of horse cytochrome c has permitted the identification of a second amino acid residue in the epitope of a mAb (E8). Furthermore, the lysine modification studies have established that E3 and E8 have at least one lysine in the fragment 66-104 that is a part of their epitopes. Computer modeling studies along with other types of chemical modifications can eventually establish the boundaries of the interaction zone between these antibodies and cytochrome c. The use of protein-engineered cytochrome c analogues has provided site-specific changes not found in natural variants and thus has been useful not only in epitope mapping, but also has helped to demonstrate that conformational disruptions in the epitope dramatically disrupt recognition by high affinity mAbs. These data suggest that a high degree of complementarity between antigen and antibody is required for binding by these mAbs as was shown in the crystal structure analysis of the lysozyme antigen and a mAb raised against it (Amit et al., 1986) and does not appear to indicate conformational changes on complex formation as detected in the neuraminidase-antibody structure (Coleman et al., 1987). More importantly, our data illustrate the fact that site-directed changes in a protein cause local as well as long-distance perturbations in structure that may not be seen by low resolution spectral techniques such as circular dichroism, but are easily detected by binding studies with mAbs of this type.
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