Analysis of an evolutionarily conserved antigenic site on mammalian cytochrome *c* using synthetic peptides

(*N*-acetylation/antigenic design/immunodominant)

RONALD JEMMERSON, PHILLIP R. MORROW, NORMAN R. KLINMAN, AND YVONNE PATERSON*

Department of Immunology, Scripps Clinic and Research Foundation, 10666 North Torrey Pines Road, La Jolla, CA 92037

Communicated by E. Margoliash, November 16, 1984

ABSTRACT Two synthetic peptides inclusive of the NH₂-terminal *N*-acetyl-Gly-Asp-Val-Glu tetrapeptide of mammalian cytochrome *c* (cyt *c*) were used in this study to address the question of whether mammals can respond immunologically to an evolutionarily conserved region of a protein. These peptides were assessed for their capacity (*i*) to act as immunogens for the production of anti-self cyt *c* antisera and (*ii*) to bind rabbit anti-rodent cyt *c* antibody. The findings from these studies indicate the existence of an immunogenic determinant in an evolutionarily conserved region of cyt *c* that contains residues 1–4. This determinant can induce anti-self cyt *c* antibodies whether presented as a peptide on a carrier protein or in the context of the intact molecule as polymerized mammalian cyt *c*.

A commonly accepted view of the antigenicity of a protein is that the predominant epitopes correspond to those regions where the immunizing protein differs in amino acid sequence from the homologous protein of the immunized animal (1). This concept followed directly from the phenomenon of immunological tolerance. If B cells having specificities for structures on self proteins are aborted or rendered tolerant, then only those B cells having specificities for foreign antigenic determinants should remain in a state that can be activated (2). This is obviously an oversimplification in that T cells and macrophage processing probably have a large effect on which parts of a protein will be antigenic in a particular host. Nonetheless, data obtained from recent studies, most notably with cytochrome *c* (cyt *c*), continue to support the hypothesis (3–6). Thus, for example, three different groups (4–6) found that the antigenic determinants on horse cyt *c*, as recognized by rabbits, correspond to three regions of the molecule—namely, around residues 44, 60, and 89/92, where horse and rabbit cyt *c* differ in amino acid sequence.

In apparent conflict with the hypothesis was the observation that antibodies could be elicited in rabbits against their own cyt *c* if the molecule was appropriately presented to the immune system by polymerization with glutaraldehyde or by coupling to bovine IgG (7), conditions that may circumvent T-cell-mediated tolerance. The antibodies produced against the self antigen appeared to bind in the same region as the antibodies that were previously produced against foreign cyt *c* and, hence, were apparently directed to evolutionarily variable regions (8).

Recent evidence from many laboratories has indicated that synthetic peptides that represent regions within proteins could be used as representative immunogens for those regions (9–13). We constructed two synthetic peptides corresponding to an evolutionarily conserved region that includes the NH₂-terminal tetrapeptide of mammalian cyt *c* to use as probes to identify potential autoantigenic sites within con-

served regions of cyt *c*. Both peptides were assessed for their capacity to act as immunogens for the production of anti-self cyt *c* antisera and to bind rabbit anti-rodent cyt *c* antibody. The findings from these studies indicate the existence of an immunogenic determinant in an evolutionarily conserved region of cyt *c* that contains residues 1–4. This determinant can induce anti-self cyt *c* antibodies whether presented as a peptide on a carrier protein or in the context of the intact molecule as polymerized mammalian cyt *c*.

MATERIALS AND METHODS

Native Protein Antigens. Mouse cyt *c* was a gift from Emanuel Margoliash (Northwestern University, Evanston, IL). All other cyt *c* used in this study, including rat cyt *c*, which is identical in sequence to mouse cyt *c* (14), was purchased from Sigma.

Cyt *c* is only weakly immunogenic in monomeric form (15); thus, for immunization it was used either polymerized with 0.1% glutaraldehyde by the method of Reichlin *et al.* (15) or conjugated to the immunogenic carrier protein bovine IgG. Two procedures were used for generating immune responses in rabbits. In one case, a primary immunization of glutaraldehyde-polymerized rat cyt *c* (100 µg) in complete Freund's adjuvant was followed in 3 wk with the same antigen in incomplete Freund's adjuvant. In the other procedure, cyt *c* (100 µg) coupled to bovine IgG with glutaraldehyde (16) was used for the primary immunization followed in 2–3 wk by polymerized cyt *c*.

In antibody assays, the proteins were used as simple solutions of their crystalline monomeric form.

Design and Preparation of Peptide Antigens. Two peptides that correspond to the following segments of rabbit cyt *c*, residues *N*-acetyl-(1–9)-Tyr, with sequence CH₃CO-Gly-Asp-Val-Glu-Lys-Gly-Lys-Lys-Ile-Tyr-OH (peptide A) and residues *N*-acetyl-(1–4)-Gly-(97–100), with sequence CH₃CO-Gly-Asp-Val-Glu-Gly-Tyr-Leu-Lys-Lys-OH (peptide B), were synthesized by Peninsula Laboratories (San Carlos, CA). The composition of the peptides was confirmed by amino acid analysis: peptide A (Gly, 2.04; Asp, 0.96; Val, 0.82; Glu, 0.85; Lys, 3.15; Ile, 0.68; Tyr, 0.78), peptide B (Gly, 2.06; Asp, 0.93; Val, 0.81; Glu, 0.87; Tyr, 1.09; Leu, 1.06; Lys, 2.19). The rationale for the construction of these peptides was as follows: the residues on an antigen encompassed by an antibody-combining site probably depend on (*i*) the size of the site, (*ii*) which residues of the antigen are immunodominant, and (*iii*) the spatial orientation of the antibody relative to the antigen. On examination of the three-dimensional structure of the horse cyt *c* (17), it was deduced that the presentation of the NH₂-terminal residues 1–4 of cyt *c* to the antibody-combining site might involve residues 5–9 or 97–100 depending on the orientation of cyt *c*. Therefore,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: cyt *c*, cytochrome *c*.

*To whom reprint requests should be addressed.

we attempted to design peptides that would mimic these two different surface presentations of the potential antigenic site at the NH₂ terminus. Two analogues of these peptides in which a cysteinyl residue was incorporated between the *N*-acetyl end group and the first residue, glycine, were also synthesized for us by Peninsula Laboratories for studies on structural recognition.

To monitor the conjugation of the peptides to carrier proteins for assays and immunization of animals, we used ¹²⁵I-labeled peptides in analytical trials. The amino acid sequence of cyt *c* contained in the peptide *N*-acetyl-(1-4)-Gly-(97-100) included a tyrosine residue to which the ¹²⁵I could be covalently attached. Since the first nine residues of cyt *c* do not include tyrosine, it was added to the carboxyl terminal end of peptide 1-9. The NH₂-terminal end of each peptide was blocked with an acetyl group because *N*-acetylation occurs naturally in vertebrate cyt *c* as a post-translational modification (18).

A peptide that represents the common segment in the two peptides described above—i.e., *N*-acetyl-(1-4)—was synthesized by us on a polyamide resin (Chemical Dynamics, South Plainfield, NJ) with a cleavage-resistant sarcosyl-ethylene diamine spacer arm using *N*- α -fluorenylmethoxycarbonyl amino acid derivatives, tert-butyl side-chain protecting groups for the side-chain carboxylic groups, and standard coupling procedures (19, 20). The *N*- α -fluorenylmethyl-oxycarbonyl group was cleaved by 20% piperidine between couplings and the NH₂ terminus of the peptide was *N*-acetylated before side-chain deprotection using 10% acetic anhydride and 10% diisopropylethylamine in dimethylformamide. Side-chain protecting groups were cleaved by 2 M HBr in acetic acid/trifluoroacetic acid (1:1) with the fully deprotected peptide remaining attached by its COOH terminus to the resin. The composition of the completed peptide was confirmed by amino acid analysis (Gly, 0.95; Asp, 1.10; Val, 0.89; Glu, 1.14).

Subsequent RIA of this peptide was performed with the peptide still covalently attached to the polyamide resin by the procedure of Smith *et al.* (21). RIAs of the antisera on the polyamide resin alone and on a peptide corresponding to region 41-46 in rodent cyt *c*, which was attached to the resin, were also performed to assess binding that is not antigen specific. Binding of nonspecific antibodies to the peptides was measured with preimmune rabbit immunoglobulin (15 μ g/ml) prepared from the antisera of control rabbits by ammonium sulfate fractionation.

Samples of dry peptide/polyamide (1 mg) were swollen and washed 3 times with 0.1 M borate buffer (pH 8.5) containing Tween 20 (0.2%) and NaCl (1 M). Affinity-purified rabbit antibodies from hyperimmune antisera were diluted with this buffer containing 0.5% bovine serum albumin to an antibody concentration of 15 μ g/ml. Antibody binding was detected with ¹²⁵I-labeled goat anti-rabbit IgG.

In addition to the synthetic peptide antigens described above the three major cyanogen bromide-cleaved fragments of mouse cyt *c*—namely, residues 1-65, 66-80, and 81-104—were prepared from the native protein as described (22).

Coupling the Peptide to Carriers. The synthetic peptides used were coupled to polypeptide carriers. For assay purposes, the carriers were bovine serum albumin (Sigma) and poly-L-lysine (*M_r*, $\approx$ 260,000; Sigma) and for immunization purposes bovine IgG (Pentex, FII, Miles) was the carrier protein.

To couple the peptides to carriers using 0.1% glutaraldehyde, carrier (5 mg) and peptide (1 mg) were dissolved in 1 ml of 0.1 M ammonium acetate (pH 7.0). Glutaraldehyde was added slowly with stirring to a final concentration of 0.1% and was allowed to react for 2 hr at room temperature. The reactants and products were separated by dialysis; in some experiments, further purification by chromatography with

Sephadex G-100 was necessary. Using ¹²⁵I-labeled peptides as a tracer, we determined that the efficiency of coupling for both peptides was 20%–30% and 5–10 molecules of each peptide were coupled to each carrier molecule.

New Zealand Red rabbits were primed and given booster injections with 375 μ g of the peptide-protein conjugate by injection into positions along the back intradermally and in the hind footpads. The primary dose was an emulsification in complete Freund's adjuvant, and the secondary dose was given in incomplete Freund's adjuvant; animals were bled 2–4 wk later.

Solid-Phase Antibody Binding Assays. Antisera were examined for antigenic specificity using ELISA (23) and solid-phase RIA (24). Antisera were incubated on antigen-coated 96-well plates at various dilutions. The plates were developed using goat anti-rabbit IgG, which was either conjugated to horseradish peroxidase (ELISA) or labeled with ¹²⁵I (RIA). To determine whether antisera contained different antibody populations specific for each peptide antigen, antisera were preincubated on a plate coated with one peptide, and unbound material was then assayed for binding to the other peptide by the procedure described above. In some experiments, the antibodies were purified prior to assay by affinity chromatography on rodent cyt *c* covalently coupled to cyanogen bromide-activated Sepharose 4B (25).

Antibody titers were determined using either an RIA or ELISA. The principles of these assays are identical except for the detecting signals. The antigens used to assess antibody binding were rat cyt *c* and the two peptides representative of the NH₂ terminus of cyt *c* coupled to carrier polypeptides. To measure nonspecific binding, the carrier polypeptide alone and bovine serum albumin were used. As an index of the reactivity of each antiserum, the ratio of the binding of antibodies to each peptide over its binding to cyt *c* was calculated as follows:

% reactivity =

$$\frac{\text{cpm bound to peptide on carrier} - \text{cpm bound to carrier}}{\text{cpm bound to cyt } c - \text{cpm bound to bovine serum albumin}}$$

The values used in this calculation were taken at serum dilutions at which antibody bound was not saturating.

RESULTS

The Immunogenicity of a Peptide Including Residues 1-4 of Mammalian cyt *c*. Serum from a New Zealand Red rabbit immunized with peptide *N*-acetyl-(1-4)-Gly-(97-100) coupled to bovine IgG was tested by both ELISA and RIA for binding to both the homologous peptide and the peptide *N*-acetyl-(1-9)-Tyr, each coupled to polylysine, as well as to intact mammalian cyt *c*. The results of such an assay using affinity-purified antibodies on the peptides are shown in Fig. 1. It can be seen that this antiserum contained antibodies that bound to the homologous peptide *N*-acetyl-(1-4)-Gly-(97-100) and to the peptide *N*-acetyl-(1-9)-Tyr. The antisera induced by peptide *N*-acetyl-(1-4)-Gly-(97-100) also included antibodies that bound to intact rodent cyt *c* (Fig. 2). Thus, it appears that immunization of rabbits with a peptide including the first four residues of mammalian cyt *c* can give rise to antibodies that bind to a region of the molecule evolutionarily conserved throughout mammalian species (26) and heretofore believed to be immunologically silent.

To provide further evidence for this specificity, solid-phase RIAs were carried out with antibodies affinity purified on mouse cyt *c*. A variety of species variant cytochromes and synthetic peptides coupled to polylysine were used as

FIG. 1. RIA of affinity-purified antibodies to *N*-acetyl-(1-4)-Gly-(97-100) coupled to bovine IgG on *N*-acetyl-(1-4)-Gly-(97-100)-polylysine ($\square$), *N*-acetyl-(1-9)-Tyr-polylysine ($\circ$), or polylysine ($\bullet$).

antigens (Fig. 3). The antibodies cross-reacted with a panel of cyt *c* from various mammalian sources, all of which are *N*-acetylated and are homologous for residues 1-4. There is little reactivity to two cyt *c* from the yeasts *Saccharomyces* and *Candida* (Fig. 3a), which are not acetylated and have little homology in this region (26). The reactivity of the antibody to tuna cyt *c*, in which alanine replaces glutamic acid at residue 4 in the molecule, is significantly lower than to the other vertebrate cytochromes; however, the avian cytochromes, which also have one amino acid substitution in this region (isoleucine replaces valine at position 3), show complete reactivity to antibodies to *N*-acetyl-(1-4)-Gly-(97-100).

The binding of the antibodies to *N*-acetyl-(1-9)-Tyr and *N*-acetyl-(1-4)-Gly-(97-100) could be blocked by incorporating a cysteinyl residue in the peptide between the *N*-acetyl end

FIG. 2. ELISA of rabbit antisera to *N*-acetyl-(1-4)-Gly-(97-100) coupled to bovine IgG on mouse cyt *c* (Δ) or bovine serum albumin ($\bullet$).

group and glycine, the first residue (Fig. 3b). In addition, preadsorption by either peptide removed the population of antibodies available for binding to the other peptide (Fig. 3b). This indicates that the specificity of these antibodies resides in the common four residues of these peptides—i.e., *N*-acetyl-(1-4). That these antibodies can in fact bind to a peptide of this length was confirmed by synthesizing this peptide on a polyamide resin by the method described in *Materials and Methods*, which enables the peptide to be assayed while still attached to the solid-phase resin (21). Fig. 3c shows antibody specifically bound to the peptide *N*-acetyl-(1-4) attached to resin compared to nonspecific binding to the resin alone. Nonspecific binding to control peptide representing region 41-46 in cyt *c* showed similar levels to the polyamide control (results not shown). Nonspecific rabbit antibodies showed no significant binding to either the resin or the resin-attached peptides.

FIG. 3. RIA of affinity-purified rabbit anti-*N*-acetyl-(1-4)-Gly-(97-100) on different panels of antigens. Values shown were obtained from dilutions of antibody preparations that gave less than saturated binding. (a) Eukaryotic cyt *c*. (b) Synthetic peptides coupled to polylysine. A, *N*-acetyl-(1-9)-Tyr-polylysine; B, *N*-acetyl-(1-4)-Gly-(97-100)-polylysine; Cys A, *N*-acetyl-Cys-(1-9)-Tyr-polylysine; Cys B, *N*-acetyl-Cys-(1-4)-Gly-(97-100)-polylysine; blank, polylysine. (c) Synthetic peptide *N*-acetyl-(1-4) covalently attached to a polyamide resin through its COOH terminus.

Table 1. Reactivity with NH₂-terminal peptides of four rabbit antisera to polymerized mouse cyt *c*

Antiserum	% reactivity with peptides	
	<i>N</i> -acetyl-(1-9)-Tyr	<i>N</i> -acetyl-(1-4)-Gly-(97-100)
1	91	95
2	72	67
3	69	49
4	8	5

Antiserum 1: First immunization and booster injection was glutaraldehyde-polymerized mouse cyt *c*. Antisera 2 and 3: First immunization was glutaraldehyde-polymerized conjugate of bovine IgG and mouse cyt *c*; booster injection was glutaraldehyde-polymerized mouse cyt *c*. Antiserum 4: Obtained from Urbanski and Margoliash (3); soluble fraction from glutaraldehyde-polymerized mouse cyt *c* used for immunization.

That antibodies to *N*-acetyl-(1-4)-Gly-(97-100) were specific for the first four residues of the peptide was further demonstrated by removing all reactivity to cyt *c* and peptides *N*-acetyl-(1-4)-Gly-(97-100) and *N*-acetyl-(1-9)-Tyr by preadsorption on *N*-acetyl-(1-4) covalently bound to the polyamide resin. Adsorption of the antibodies by polyamide resin alone or control peptide 41-46 had no effect on the reactivity of the antibodies to these antigens.

It should be noted that a rabbit immunized with peptide *N*-acetyl-(1-9)-Tyr coupled to bovine IgG produced antibodies that were strongly reactive with peptide *N*-acetyl-(1-9)-Tyr but were weakly reactive with the peptide *N*-acetyl-(1-4)-Gly-(97-100) and not reactive at all with intact mouse cyt *c* (results not shown).

Antibodies to Polymerized Rodent cyt *c* Are Reactive with Residues 1-4. The above findings demonstrate not only that a peptide displaying a self antigenic determinant could be immunogenic, but also that such a peptide could be an excellent probe for detecting the presence within an antiserum of

an antibody population to a given region of a protein. We therefore examined several antisera generated in rabbits by immunization with various forms of rodent cyt *c* to determine whether antibodies to the region of 1-4 were present but heretofore undetected. We generated antisera from three rabbits: one by immunization with polymerized rat cyt *c* and two by a primary immunization with rat cyt *c* coupled to bovine IgG followed by immunization with polymerized cyt *c*. The fourth antiserum was obtained from E. Margoliash and was one of the antisera previously tested by Urbanski and Margoliash for reactivity to regions of cyt *c* (3). All four antisera contained reactivity to both peptides and the reactivities to the peptides of antisera 1, 2, and 3 (raised in this laboratory) are considerably greater than that of antiserum 4 (prepared by Urbanski and Margoliash) (Table 1). This is possibly due to differences in the immunization procedures used in the two studies. Although polymerized cyt *c* was used both by us and by Urbanski and Margoliash, a much larger quantity of immunogen (5 mg) was used for each immunization in the latter study (3). In addition, the degree of aggregation of cyt *c* used in our work was much greater; indeed, our immunogen contained particulate material, whereas the other workers used only soluble complexes for immunization. It is likely that polymerization causes alterations in the polypeptide that could result in changes within potentially immunogenic determinants. In addition, New Zealand Red rabbits were used in our study, whereas New Zealand White rabbits were used by Urbanski and Margoliash.

The recognition of the NH₂ terminus by the antisera raised in our laboratories was further delineated by assessing binding to various antigens. The results obtained for all three antisera were similar and are exemplified in Fig. 4, which represents the analyses of antiserum 1 (Table 1). It can be seen that these antibodies recognize all tested vertebrate cytochromes, which are identical at the NH₂ terminus but do not recognize yeast cytochromes, which have considerably different NH₂ termini (26) (Fig. 4a). Furthermore, the anti-

FIG. 4. RIA of rabbit antiserum (antiserum 1 in Table 1) to polymerized rat cyt *c* on different panels of antigens. Values were obtained from dilutions of antibody preparations that gave less than saturated binding. (a) Eukaryotic cyt *c*. (b) Antigens coupled to polylysine. Mouse cyt *c*, CNBr-cleaved fragments from mouse cyt *c* 1-65, 66-80, 81-104, and polylysine control (blank). (c) Synthetic peptides coupled to polylysine. A, *N*-acetyl-(1-9)-Tyr-polylysine; B, *N*-acetyl-(1-4)-Gly-(97-100)-polylysine; Cys A, *N*-acetyl-Cys-(1-9)-polylysine; Cys B, *N*-acetyl-Cys-(1-4)-Gly-(97-100)-polylysine; blank, polylysine.

genicity is predominantly located in the region of residues 1–65 shown by the binding of the antibodies to cyanogen bromide-cleaved fragments of cyt *c*, each of which are coupled to polylysine to ensure that the peptides bind equally well to the plates (Fig. 4b). Finally, the reactivity can be isolated to residues 1–4 because preadsorption of the antibody on either peptide removes the reactivity of the anti-cyt *c* antibodies to the other peptide (Fig. 4c), and the inclusion of a cysteinyl residue between the *N*-acetyl end group and residue 1 in both *N*-acetyl-(1–9)-Tyr and *N*-acetyl-(1–4)-Gly-(97–100) blocks binding (Fig. 4c).

DISCUSSION

In this study, we have used synthetic peptides representative of the NH₂-terminal sequence of mammalian cyt *c* to ask directly whether mammals can respond to an evolutionarily conserved region of a protein. The results indicate that one such peptide can elicit antibodies in rabbits that recognize not only the immunizing peptide but two other peptides that contain the same four NH₂-terminal residues as well as native rabbit cyt *c*. Furthermore, these peptides were used to show that antisera raised in rabbits immunized with rodent cyt *c* include antibodies against this evolutionarily conserved region. The levels of this population of antibodies vary among different antisera and may depend on the strain of rabbit used and the degree of aggregation of cyt *c* used for immunization.

The NH₂-terminal region of mammalian cyt *c* represents not only a highly conserved region of a mammalian protein but also a region that heretofore had been thought to be non-immunogenic. It should be noted, however, that reactivity to this region of cyt *c* may arise from circumstances that are consistent with the hypothesis that conserved regions of proteins are not, in general, immunogenic (8). The immunogenicity of mouse cyt *c* has been shown to involve residues 44, 62, and 89 (3). The NH₂ terminus of the cytochrome molecule is in topographic proximity to one of these immunodominant residues—in fact, residue 1 is ≈6 Å from residue 89. What we may be detecting, therefore, with synthetic peptide *N*-acetyl-(1–4) are antibodies to a topographic determinant on the intact protein that includes both the NH₂-terminal region and residue 89. Such a situation is thought to occur with myoglobin where surface residues, which are remote in sequence from the short contiguous sequences originally proposed by Atassi as representing the total antigenicity of the molecule (27), have been clearly identified as being involved in the antigenic sites of this protein (28). If this is the case, the primary response to this determinant on polymerized cyt *c* may, in fact, be stimulated by the evolutionarily variant residue 89.

It is also possible that the chemical reaction of polymerization required for increasing immune responses to cyt *c* may cause disturbances in polypeptide folding, which then trigger an antibody response. The NH₂-terminal segment of cyt *c* may be more susceptible to this kind of conformational alteration than other regions of the molecule, possibly allowing for breaking of tolerance at the B-cell level. Alternatively, tolerance at the T-cell level could be broken through conformational alteration of T-cell determinants at other sites on cyt *c*, in which case the relative immunogenicity of the first few residues may be a consequence of their presentation as an exposed terminus that is available for “end on” recognition by the immunoglobulin receptor on B cells. Such recognition has previously been found to be important in antibody binding for carbohydrates (29). Evidence for this view is provided by the observation that none of the antibodies that recognize the NH₂ terminus of cyt *c* binds to the peptides ex-

tended by an NH₂-terminal cysteinyl residue. Such an addition would be expected to markedly affect end on recognition but to interfere less with recognition of determinants within the peptide sequence.

The studies presented here not only confirm the work of many others in demonstrating the utility of synthetic peptides for the study of the immunogenicity of defined regions of proteins but also extend this approach to the analysis of potential reactivities to self determinants.

It is clear that further studies using synthetic peptides constructed of regions that are conserved in contrast to those that are not conserved both as immunogens and as assay probes for antibodies against intact molecules will be highly useful in elucidating heretofore undetected antigenic regions in proteins.

This work was supported by Grants AI 19499 and AI 15797 from the National Institutes of Health. R.J. was the recipient of a Damon Runyon/Walter Winchell Fellowship for Cancer Research. P.R.M. is the recipient of a fellowship from the Arthritis Foundation.

1. Reichlin, M. (1975) *Adv. Immunol.* **20**, 71–123.
2. Burnet, F. M. (1975) *Aust. J. Sci.* **20**, 67–69.
3. Urbanski, G. J. & Margoliash, E. (1977) *J. Immunol.* **118**, 1170–1180.
4. Jemmerson, R. & Margoliash, E. (1979) *J. Biol. Chem.* **254**, 12706–12716.
5. Eng, J. & Reichlin, M. (1979) *Mol. Immunol.* **16**, 225–230.
6. Berman, P. W. & Harbury, H. A. (1980) *J. Biol. Chem.* **255**, 6133–6137.
7. Nisonoff, A., Margoliash, E. & Reichlin, M. (1967) *Science* **155**, 1273–1275.
8. Jemmerson, R. & Margoliash, E. (1979) *Nature (London)* **282**, 468–471.
9. Arnon, R. (1980) *Annu. Rev. Microbiol.* **34**, 593–618.
10. Walter, G., Scheidtmann, K.-H., Carbone, A., Laudano, A. P. & Doolittle, R. F. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 5197–5200.
11. Audibert, F., Jolivet, M., Chedid, L., Alouf, J. E., Boquet, P., Rivaille, P. & Siffert, O. (1981) *Nature (London)* **289**, 593–594.
12. Baron, M. H. & Baltimore, D. (1982) *J. Virol.* **43**, 969–978.
13. Lerner, R. (1982) *Nature (London)* **299**, 592–596.
14. Carlson, S. S., Mross, G. A., Wilson, A. C., Mead, R. T., Wolin, L. D., Bowers, S. F., Foley, N. T., Muijsers, A. O. & Margoliash, E. (1977) *Biochemistry* **16**, 1437–1442.
15. Reichlin, M., Nisonoff, A. & Margoliash, E. (1970) *J. Biol. Chem.* **245**, 947–954.
16. Reichlin, M. (1980) *Methods Enzymol.* **70**, 159–165.
17. Dickerson, R. E. (1972) *Sci. Am.* **226** (4), 58–72.
18. Margoliash, E. (1972) *Harvey Lect.* **66**, 177–247.
19. Arshady, R., Atherton, E., Clive, D. L. J. & Sheppard, R. C. (1981) *J. Chem. Soc. Perkin Trans. 1*, 529–537.
20. Atherton, E., Logan, C. J. & Sheppard, R. C. (1981) *J. Chem. Soc. Perkin Trans. 1*, 538–546.
21. Smith, J. A., Hurrell, J. G. & Leach, S. J. (1977) *Immunochimistry* **14**, 565–568.
22. Corradin, G. & Harbury, H. A. (1970) *Biochim. Biophys. Acta* **221**, 489–496.
23. Engvall, E. (1980) *Methods Enzymol.* **70**, 419–439.
24. Pierce, S. K. & Klinman, N. K. (1977) *J. Exp. Med.* **146**, 509–519.
25. Jemmerson, R. & Margoliash, E. (1980) *Methods Enzymol.* **70**, 244–262.
26. Borden, D. & Margoliash, E. (1976) in *Handbook of Biochemistry and Molecular Biology*, ed. Fasman, G. D. (The Chemical Rubber Co., Cleveland, OH), Vol. 3, pp. 268–279.
27. Atassi, M. Z. (1975) *Immunochemistry* **12**, 423–438.
28. Benjamin, D. C., Berzofsky, J. A., East, I. J., Gurd, F. R. N., Hannum, C., Leach, S. J., Margoliash, E., Michael, J. G., Miller, A., Prager, E. M., Reichlin, M., Sercarz, E. E., Smith-Gill, S. J., Todd, P. E. & Wilson, A. C. (1984) *Annu. Rev. Immunol.* **2**, 67–101.
29. Kabat, E. A. (1966) *J. Immunol.* **97**, 1–11.