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# MONOCLONAL AND ANTI-IDIOTYPIC ANTIBODIES: PROBES FOR RECEPTOR STRUCTURE AND FUNCTION

# MONOCLONAL AND ANTI-IDIOTYPIC ANTIBODIES: PROBES FOR RECEPTOR STRUCTURE AND FUNCTION

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# Contents of Volumes 1, 2, and 3

## Volume 1: Membranes, Detergents, and Receptor Solubilization



## Volume 2: Receptor Purification Procedures

## Volume 3: Molecular and Chemical Characterization of Membrane Receptors

# Preface

The recent rapid advances in our knowledge of receptor structure and function emanate to a considerable extent from the application of new technologies, such as monoclonal antibody production, to this area. Our understanding of the molecular nature of receptors has been considerably advanced by hybridoma technology and monoclonal antibodies. This new technology is uniquely suited for receptor biochemistry, a field that has been severely hampered, in part, by extremely small amounts of receptor protein in most tissues. Monoclonal antibodies have provided a means of receptor purification and structural characterization as well as information on the evolution of receptor proteins and the structural homology of receptor subunits, findings confirmed by receptor sequencing and gene cloning.

The discovery that anti-idiotypic antibodies with specificity for receptor proteins can be generated by immunizing animals with hormones or neurotransmitters has even further expanded the immunological approaches in receptor biochemistry.

In **Monoclonal and Anti-Idiotypic Antibodies: Probes for Receptor Structure and Function,** Volume 4 of this series, we have assembled a collection of studies by pioneers in the use of monoclonal and anti-idiotypic antibodies for receptor characterization. While not a symposium proceedings, this volume evolved out of a symposium by the same title presented by the American Society for Pharmacology and Experimental Therapeutics at the 67th Annual Meeting of the Federation of American Societies for Experimental Biology.

Chapter 1 serves as an excellent introduction to the utility of monoclonal antibodies as probes of protein conformation, even at the level of single functional antigenic determinants on a well-characterized protein, myoglobin. These data illustrate the tremendous degree of specificity which can be achieved with monoclonal antibodies. Chapters 2 to 6 describe the application of hybridoma technology and monoclonal antibodies to the purification and characterization of nicotinic acetylcholine receptors, insulin and related growth factor receptors, thyrotropin receptors, and neurotransmitter receptors of the autonomic nervous system. As illustrated in these chapters, monoclonal antibodies have had a variety of uses in receptor studies including mapping of antigenic determinants on receptors and their subunits, purification of receptors, characterization of structural homology between related receptor proteins, determination of specificities of autoimmune sera in certain disease states such as myasthenia gravis and Graves' disease, and study of the evolution of receptor subtypes.

Chapter 7 provides an overview to the field of anti-idiotypic antibodies and the concept that anti-idiotypes may serve as internal images of antigens. As illustration of this concept, Chapters 8 and 9 describe the production of anti-idiotypic antibodies to $\beta$-adrenergic and nicotinic acetylcholine specific ligands which interact with

β-adrenergic receptor and the nicotinic acetylcholine receptors, respectively. In the final chapter, identification of reovirus type 3 hemagglutinin receptors is described using monoclonal anti-idiotypic antibodies to reovirus type 3 hemagglutinin.

We believe that the reader will find the articles in this volume to be an extremely detailed review of the rapidly expanding field of receptor immunology. These chapters describe the current state of the art application of monoclonal and anti-idiotypic antibodies to the understanding of a variety of receptor systems. In addition, the techniques and approaches presented in this volume will be useful to investigators undertaking the immunological characterization of other integral membrane proteins.

**J. Craig Venter**
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1

# Monoclonal Antibodies as Probes of Antigenic Structure

Jay A. Berzofsky

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## INTRODUCTION

The use of monoclonal antibodies [Köhler and Milstein, 1975] as probes of the antigenic structure and conformation of proteins is one aspect of our overall research effort to understand the biochemical and genetic regulation of the immune response to a well-characterized natural protein antigen, sperm whale myoglobin. We have studied the genetic regulation of the immune response [Berzofsky, 1978, 1980; Berzofsky et al, 1979; Richman et al, 1980; Berzofsky and Richman, 1981; Kohno and Berzofsky, 1982a–c], the sites recognized by T lymphocytes [Berkower et al, 1982], and the sites recognized by monoclonal antibodies [Berzofsky et al, 1980, 1982]. This chapter shall be confined to only the last of these subjects.

Myoglobin is not a cell surface receptor, the subject of most of this volume, but it has been so well characterized in terms of primary amino acid sequence [Edmundson, 1965; Herrera and Lehmann, 1974; Bogardt et al, 1980], three-dimensional structure [Kendrew et al, 1960; Takano, 1977], and function [Rossi-Fanelli et al, 1964; Friend and Gurd, 1979], that it serves as a useful model antigen. It is hoped that the principles learned from studying this model system will be useful in similar studies of other functional proteins, such as cell surface receptors, which are not as well characterized.

Sperm whale myoglobin consists of a single polypeptide chain of 153 amino acid residues, with no disulfide bonds, and with an iron protoporphyrin, or heme, prosthetic group which binds oxygen. The heme can also serve as a reporter group for spectroscopic probes of myoglobin conformation, as will be seen later.

As is apparent from Figure 1, myoglobin has a considerable amount of alpha-helical structure. When one removes the heme to make apomyoglobin, the protein conformation changes but much structure remains. When one cleaves at the two methionine residues, 55 and 131, with cyanogen bromide, to produce three large fragments, most of the alpha-helical structure is lost [Hurrell et al, 1977]. We have used both fragments such as these, and myoglobins from different species which have known amino acid substitutions, to analyze sites recognized by monoclonal antibodies.

### Monoclonal Antibodies to Myoglobin

In collaboration with Dr. John Minna of the National Cancer Institute, we raised a series of monoclonal antibodies to sperm whale

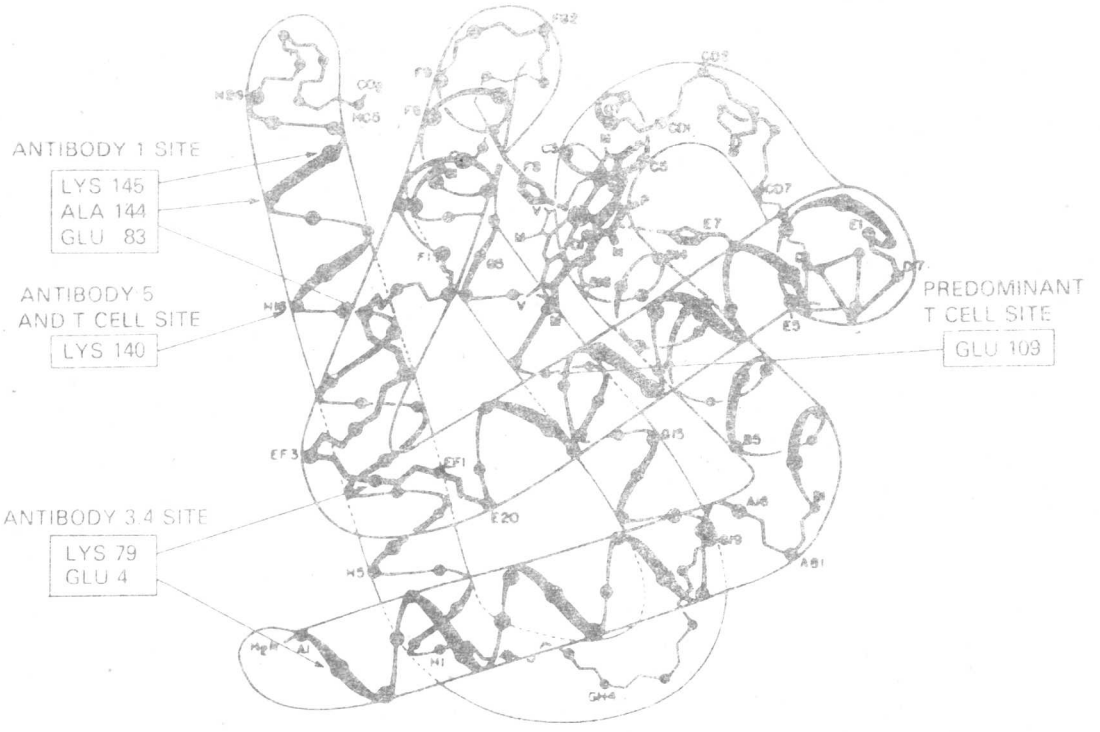


**Fig. 1.** *Line drawing representing the three-dimensional structure of the polypeptide alpha-carbon backbone of native sperm whale myoglobin, modified from Dickerson [1964] with permission. The alpha helices are labeled A through H from the amino terminal to the carboxyl terminal. Side chains are omitted except for two histidine rings (F8 and E7) involved with the heme. The methionines at positions 55 (D5) and 131 (H7) are the sites of cyanogen bromide cleavage to yield the three peptides discussed, 1-55, 56-131, and 132-153. Sites recognized by monoclonal antibodies [Berzofsky et al, 1982], as described in the text, and sites recognized by T cells [Berkower et al, 1982,1983] are indicated. Note that the immunodominant T cell site (recognized by the majority of myoglobin specific T cells of high responder B10.D2 and B10.S mice) does not correspond to any known antibody-defined determinant. However, a minor T cell site coincides with the determinant recognized by one of the monoclonal antibodies.*

myoglobin. Mice of strain A.SW, which were genetic high responders to myoglobin, were hyperimmunized by injection intraperitoneally with 200 $\mu$g myoglobin in complete Freund's adjuvant followed by from three to ten boosts with myoglobin in saline. Spleen cells were fused with a drug-marked nonsecretor plasmacytoma cell line, NS-1, using polyethylene glycol to induce fusion [Köhler and Milstein, 1975; Galfre et al, 1977], and the resulting hybridomas were cloned by limiting dilution in microtiter wells. The clones were screened for production of antimyoglobin antibodies in the culture supernatant by both solid phase and solution radioimmunoassays [Berzofsky et al, 1980]. Six clones were selected for growing in larger quantity for further study.

These were studied for binding to myoglobin and fragments in a solution radioimmunoassay using $^{3}$H-labeled myoglobin and precipitation of the immunoglobulin plus bound antigen by polyethylene glycol, leaving the free antigen in solution [Berzofsky et al, 1980]. The binding titration fit a linear Scatchard plot [Scatchard, 1949] (Fig. 2), as would be expected for a homogeneous monoclonal antibody with only a single affinity. In

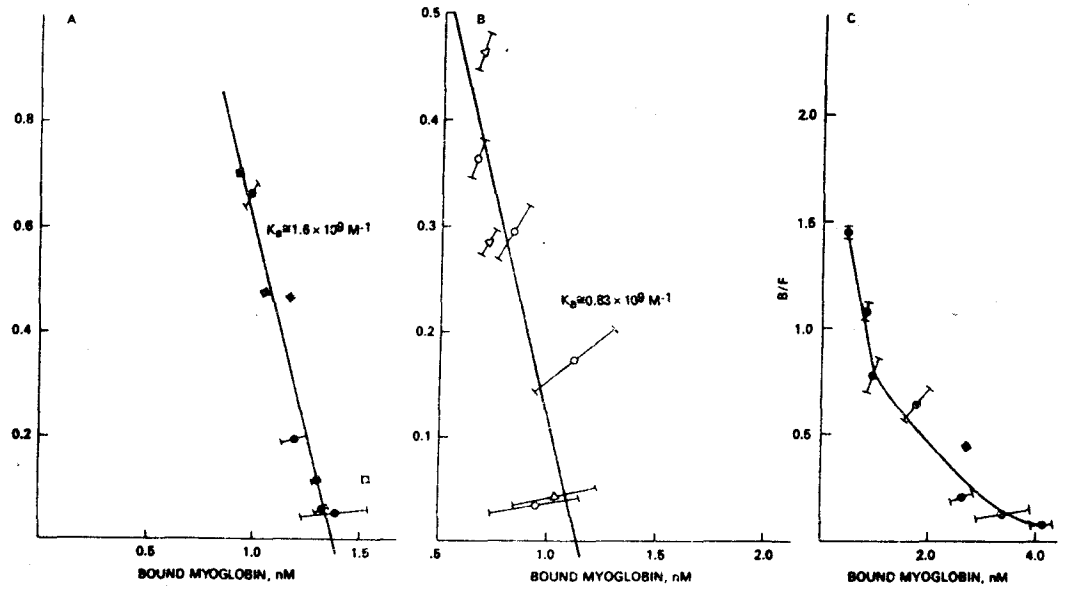


**Fig. 2.** *Scatchard analysis of hybridoma monoclonal antibodies specific for sperm whale myoglobin. A) Clone 5, 1:50 dilution of culture supernatant. B) Clone 6, 1:50 dilution of culture supernatant. Increasing concentrations of [$^3$H]sperm whale myoglobin were added to a constant concentration of monoclonal antibody. After equilibrium was achieved, at pH 7.6 and 4°C, polyethylene glycol (MW 6,000, final concentration 10% W:W) was added to precipitate all the immunoglobulin and bound myoglobin, leaving free myoglobin in the supernatant. The bound/free [$^3$H]myoglobin ratio is plotted versus the concentration of bound myoglobin. C) Sixth bleed immune serum from mouse 1329, whose spleen was used for the HAL fusion, diluted 1:1,500 in order to achieve a concentration of specific antibodies comparable to that in the diluted hybridoma supernatants. Curvature of the plot in C indicates heterogeneity of antibody affinities (reproduced from Berzofsky et al [1980], with permission of the publisher).*

contrast, the serum antibodies from the mice whose spleens were used for fusion gave curved Scatchard plots indicative of the heterogeneity of affinity. Interestingly, the cells producing the one "monoclonal" antibody that gave a biphasic Scatchard plot ("clone 3") were recloned and shown to consist of two clones with distinct affinities and fine specificities (clone 3.4 and 3.5).

The affinities of these six antibodies, by Scatchard analysis, were all high—between 2 × $10^8$ and 2 × $10^9$ $M^{-1}$ (Table I). The preponderance of high-affinity antibodies may be due to the preferential detection of these in the assay method used to screen hybridoma clones.

Despite the high affinity, none of the six monoclonal antibodies could be demonstrated to bind significantly to any of the three cyanogen bromide cleavage fragments, 1-55, 56-131, or 132-153, which together span the whole sequence of myoglobin [Berzofsky et al, 1980, 1982]. Since it was unlikely that all

**TABLE I. Summary of Monoclonal Antimyoglobin Antibodies**

| Clone | Detailed designation | Ig subclass | Affinity for sperm whale myoglobin $M^{-1} \times 10^{-9}$ |
|---|---|---|---|
| 1 | HAL19-201$A_{10}$ | $IgG_1K$ | 1.9 |
| 2 | HAL32-201$B_{11}$ | $IgG_{2a}K$ | 2.2 |
| 3.4 | HAL38-200$E_6$ Subclone $ID_5$ | $IgG_1K$ | 0.2 |
| 4 | HAL39-201C3 | $IgG_1K$ | 0.71 |
| 5 | HAL43-201$E_{11}$ | $IgG_1K$ | 1.6 |
| 6 | HAM1-201$F_3$ | $IgG_1K$ | 0.83 |

of the antibodies bound exactly at the two methionine residues at which cyanogen bromide cleaves, the loss of binding on cleavage presumably reflected the loss of native conformation on cleavage. Thus, these six antibodies all appear to be conformation-specific. As will be seen below, the conformation specificity of at least some of these antibodies is probably due to recognition of an antigenic site (determinant or epitope) which consists of a cluster of amino acid residues which are close together on the surface of the native myoglobin, but which are far enough apart in the primary sequence that they are separated when the native conformation is lost as the protein is denatured or cleaved into fragments. In fact, they may be separated onto different fragments. We call such sites topographic antigenic determinants.

Because of the lack of reactivity with fragments, we could not use smaller and smaller fragments to define the sites bound by these antibodies. Instead, we had to use a series of myoglobins from different species with known amino acid sequences and very similar native three-dimensional structures to correlate changes in structure with changes in binding affinity. An important assumption implicit in this approach is that amino acid substitutions perturb the structure and local conformation in their immediate surface environment, but do not significantly alter the overall tertiary structure of the protein, so as to affect antibody binding to a distant site. The evidence to justify this assumption is that the tertiary structures of those myoglobins studied by x-ray crystallography are nearly identical [Bradshaw et al, 1969; Takano, 1977; Scouloudi, 1978; Scouloudi and Baker, 1978]. Furthermore, as will be seen below, substitutions which severely affect the binding of one monoclonal antibody have no effect on the binding of a second monoclonal antibody, and vice versa. Thus, neither set of substitutions sufficiently alters the global structure of the protein to affect the binding of all the antibodies. Using this approach, in collaboration with Professor Frank R.N. Gurd of Indiana University, we have identified residues involved in the binding of three of the antibodies, but cannot yet unambiguously identify sites of the other three with the myoglobin variants available.

**Monoclonal antibody 5.** Solution-competitive binding radioimmunoassays were performed using the polyethylene glycol precipitation method mentioned above, with a tracer of native sperm whale myoglobin which had been preferentially labeled at the aminoterminal alpha-amino group by [$^3$H]propionylation. [Berzofsky et al, 1982]. An example of the competition curves for one of the monoclonal antibodies (clone 5) is shown in Figure 3. Note that for a given monoclonal antibody, the effect of the radiolabeling modification of the amino terminal on the affinity for the tracer is constant, so that one can validly compare relative affinities of different competitors even if absolute affinities are affected. The concentrations of competitor required to give 50% inhibition, taken as an estimate of the relative affinities [Berzofsky and Schechter, 1981], are compared with amino acid sequence changes (Table II).

The myoglobins studied for binding to clone 5 antibody breakdown sharply into two dis-

**TABLE II. Inhibition of Monoclonal Antibody 5 Binding by Myoglobin Variants***

| Inhibitor myoglobin | Residue No. 140 | 144 | 145 | Concentration for 50% inhibition (nM) |
|---|---|---|---|---|
| Sperm whale | Lys | Ala | Lys | 17 |
| Dwarf sperm whale | Lys | Ala | Lys | 26 |
| Dall porpoise | Lys | Thr | Lys | 10 |
| Goosebeaked whale | Lys | Ala | Lys | 32 |
| Human | Lys | Ser | Asn | 30 |
| Minke whale | Lys | Ala | Lys | 21 |
| Killer whale | Lys | Ala | Lys | 55 |
| California sea lion | Asn | Ala | Lys | > 20,000 |
| Dog | Asn | Ala | Lys | > 40,000 |
| Horse | Asn | Ala | Lys | 6,400 |
| Beef | Asn | Glu | Lys | > 40,000 |
| Sheep | Asn | Ala | Gln | > 40,000 |

*Modified from Berzofsky et al [1982] with permission.

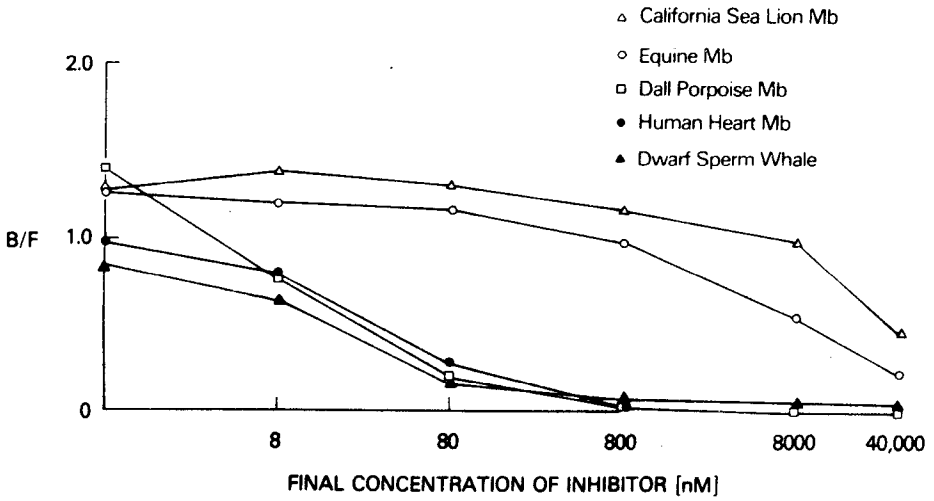


**Fig. 3.** *Competitive inhibition by various myoglobins of clone 5 monoclonal antibody binding to $^{3}$H-sperm whale myoglobin. Final concentration of labeled sperm whale myoglobin, 7 nM, and of clone 5 monoclonal antibody, approximately 5 nM in binding sites. B/F, bound/free; Mb, myoglobin. The assay was as described in Figure 2 and in the text, except that the labeled tracer sperm whale myoglobin and monoclonal antibody concentrations were held constant, and the unlabeled competitor myoglobin concentration was varied as indicated on the abscissa (reproduced from Berzofsky et al [1982], with permission).*

tinct subsets, which differ by several orders of magnitude in affinity for this antibody (Table II). This pattern correlates uniquely with one amino acid substitution at position 140. All myoglobins which have Lys 140, as in sperm whale, have a high affinity for this antibody, whereas all myoglobins with Asn 140 have a markedly lower affinity (Table II) [Berzofsky et al, 1982]. No other amino acid substitution shows any correlation with affinity. Thus, in the case of monoclonal antibody 5, we can make a unique assignment of Lys 140 as critical for antibody 5 binding. The most likely interpretation of this result is that Lys 140 is actually a contact residue in the antigenic site recognized by antibody 5. However, it is possible that Lys 140 → Asn 140 perturbs a nearby antigenic determinant without being in it. As discussed above, we believe it unlikely that the Lys 140 → Asn substitution acts at a distance on an antigenic site on the far side of the molecule. Therefore, these results at least localize the site recognized by monoclonal antibody 5 to the region surrounding Lys 140. Of course, other residues as well, which we cannot identify by this approach, must also be involved in the binding of this antibody, because antibody 5 is conformation-specific. Note also Table II that substitutions at Ala 144 and Lys 145, such as in Dall porpoise and human myoglobins, do not affect the binding of this antibody. Observations such as these place limits on the extent of this antigenic site, and suggest that effects are indeed localized, since substitutions at these sites significantly affect binding of monoclonal antibody 1, to be discussed below.

All three of these residues are close together in the H-helix of myoglobin (Fig. 1). Their spatial relationship is even more apparent if we rotate the left side of the molecule (as seen in Fig. 1) 90° toward us, and examine a computer-generated space-filling model from this "left view" prepared by Richard Feldmann,

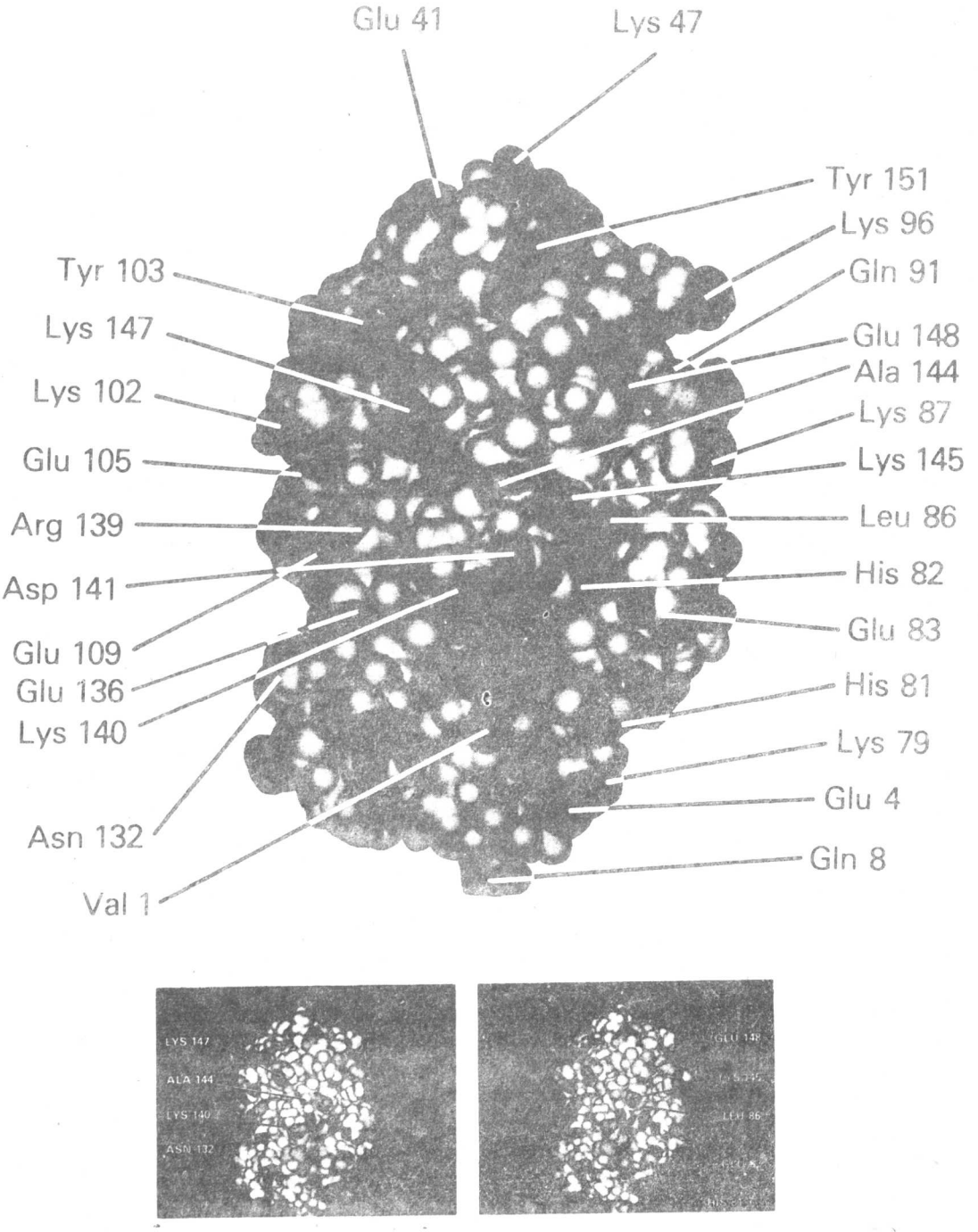
Glu 41
Lys 47
Tyr 151
Lys 96
Gln 91
Tyr 103
Lys 147
Glu 148
Ala 144
Lys 102
Lys 87
Glu 105
Lys 145
Arg 139
Leu 86
Asp 141
His 82
Glu 83
Glu 109
Glu 136
His 81
Lys 140
Lys 79
Glu 4
Asn 132
Gln 8
Val 1
LYS 147
ALA 144
LYS 140
ASN 132
GLU 148
LEU 86

Fig. 4.

DCRT, NIH [Feldmann et al, 1978] using the x-ray crystallographic coordinates of Takano [1977] (Fig. 4).

**Monoclonal antibody 1.** The analysis of a second monoclonal antibody, that from clone 1, proved more complex. We could not make a unique assignment of any single residue which could account for all of the lower-affinity myoglobins. However, we were fortunate to find one informative pair of myoglobins which differ from each other at only one residue, position 83. Pilot whale myoglobin has Glu 83, like sperm whale myoglobin, and has a similar affinity (Table III and Berzofsky et al [1982]), whereas killer whale myoglobin, which differs from the pilot whale protein by the single substitution, Glu 83 → Asp, has a tenfold lower affinity for monoclonal antibody 1. The effect of this single substitution allows us to localize the antigenic site recognized by monoclonal antibody 1 to the region around residue 83. However, several low-affinity myoglobins have no substitution at position 83. This finding implies that substitutions at positions other than 83 must also, independently, be able to affect the affinity for antibody 1. To find these, we postulated that the other changes must be nearby on the myoglobin surface in order to fit into the same antibody-combining site. The only nearby residues (on the surface of the native molecule) which could account for all of the other low affinity myoglobins were positions 144 and 145 (Table III). In fact, beef and sheep myoglobins, with nonconservative substitutions at positions 144 and 145, respectively, have the lowest affinity of any myoglobins tested, even though they retain Glu 83.

Thus we have identified a *cluster* of three residues, Glu 83, Ala 144, and Lys 145, a substitution at any one of which could lead to reduced affinity for monoclonal antibody 1. These residues are close together on the surface of the native myoglobin (Fig. 4) (of necessity from the criteria used to identify them). However, they happen, fortuitously, to be separated by 61 residues in the primary sequence. Therefore, their proximity on the surface de-

**TABLE III. Inhibition of Monoclonal Antibody 1 Binding by Myoglobin Variants***

| Inhibitor myoglobin | Residue No. 83 | 140 | 144 | 145 | Concentration for 50% inhibition (nM) |
|---|---|---|---|---|---|
| Sperm whale | Glu | Lys | Ala | Lys | 10 |
| Dwarf sperm whale | Glu | Lys | Ala | Lys | 13 |
| Goosebeaked whale | Glu | Lys | Ala | Lys | 3 |
| Dog | Glu | Asn | Ala | Lys | 5 |
| Horse | Glu | Asn | Ala | Lys | 3 |
| Pilot whale | Glu | Lys | Ala | Lys | 24 |
| Killer whale | Asp | Lys | Ala | Lys | 240 |
| California sea lion | Asp | Asn | Ala | Lys | 160 |
| Human | Glu | Lys | Ser | Asn | 470 |
| Dall porpoise | Asp | Lys | Thr | Lys | 500 |
| Beef | Glu | Asn | Glu | Lys | > 40,000 |
| Sheep | Glu | Asn | Ala | Gln | > 40,000 |

*Modified from Berzofsky et al [1982] with permission.

**Fig. 4.** *The upper panel shows a left view of a computer-generated space-filling molecular model of sperm whale myoglobin, based on the Takano [1977] x-ray diffraction coordinates. This orientation is rotated 90° relative to that in Figure 1, which is arbitrarily designated the "front view." The computer method was described by Feldmann et al [1978]. The carboxyl oxygens are shaded darkest, followed by the heme and aromatic carbons, aliphatic side-chain carbons, noncarboxylic oxygens, primary amino groups, and finally other nitrogens. The backbone and side chains of nonaliphatic residues, except for functional groups, are shown in white. Note that the direction of the helices is not readily apparent on the surface, in contrast to the backbone drawing in Figure 1. The residues Glu 4 and Lys 79, and Glu 83, Ala 144, and Lys 145, are believed to be part of topographic antigenic determinants recognized by monoclonal antibodies to myoglobin, whereas Lys 140 is part of the site recognized by a third monoclonal antibody. The lower panel shows the same view as a stereo pair, which may be viewed in three dimensions using an inexpensive stereoviewer such as the "stereoscopes" sold by Abrams Instrument Corp., Lansing, MI, or Hubbard Scientific Co., Northbrook IL (modified from Berzofsky et al [1982], with permission).*