Immunochemical Studies of Myoglobin with Synthetic Peptides†

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ABSTRACT: The heptapeptide, Lys-Glu-Leu-Gly-Tyr-Gln-Gly, representing the C terminus of sperm-whale myoglobin, and the hexa-, penta-, and tetrapeptide homologs of this heptapeptide, having sequences Glu-Leu-Gly-Tyr-Gln-Gly, Leu-Gly-Tyr-Gln-Gly, and Gly-Tyr-Gln-Gly, respectively, were synthesized by the solid-phase technique. An immunosorbent, prepared by coupling the heptapeptide to bromoacetylcellulose, was used to isolate the corresponding antibodies from rabbit anti-myoglobin sera. All adsorbed antibodies were eluted with the heptapeptide from this immunosorbent; the hexapeptide displaced only a small population of antibodies and no antibodies were eluted with the penta- or tetrapeptides. Using an immunosorbent prepared by coupling myoglobin to bromoacetylcellulose, all the peptides were capable of eluting at least a small amount of antibody from the immunosorbent but, even in this case, the major portion of antibody was eluted with the heptapeptide. These results demonstrate the important contribution of the N-terminal lysine residue of the heptapeptide in its binding with antibodies to this determinant. Disc electrophoresis of the antibodies eluted from the haptapeptide immunosorbent revealed that they possessed restricted electrophoretic heterogeneity. These antibodies were ultracentrifugally heterogeneous, consisting of two components. In addition, the tetradecapeptide, representing residues 56–69 of the helical region of myoglobin was synthesized by the solid-phase technique and the corresponding antibodies were isolated from the myoglobin immunosorbent.

Painstaking efforts have been made to prepare and isolate "homogeneous" populations of antibody in an attempt to elucidate the chemical basis of antigenicity and of the structure and specificity of antibodies. Much work has been done in this regard with anti-hapten antibodies (Kreiter and Pressman, 1964; Mamet-Bratley, 1966; Freedman and Painter, 1971) and with anti-polysaccharide antibodies (Kaplan and Kabat, 1966; Haber, 1970). Moreover, in recent years, a number of investigations have been conducted with a view to establishing the chemical nature of antigenic determinants of proteins. Thus, in the immunochemical study of tobacco mosaic virus protein (Young *et al.*, 1967) an antigenic determinant of this protein consisting of ten residues was synthesized and its reaction with antibodies to the whole protein was investigated; Arnon and Sela (1969) studied the determinants of lysozyme and have isolated antibodies to the "loop" peptide; antigenic determinants of staphylococcal nuclease and the corresponding antibodies were isolated (Omenn *et al.*, 1970).

Some years ago Crumpton and Wilkinson (1965) digested sperm-whale apomyoglobin with chymotrypsin and isolated several antigenically active peptides. In a previous paper the present authors (Givas *et al.*, 1968) reported briefly the chemical synthesis of one of these peptides, *i.e.*, the C-terminal heptapeptide, consisting of residues 147–153, and the isolation of the corresponding monospecific antibodies. In the present article the syntheses of the C-terminal heptapeptide of myoglobin and of its lower homologs, *i.e.*, the hexa-, penta-, and tetrapeptides, are reported in detail and a description is given of the isolation of the corresponding antibody populations and of attempts to delineate the immunodominant region of this antigenic determinant. Moreover, the synthesis of the tetradecapeptide, representing residues 56–69 of myoglobin (Edmundson, 1965), and its antigenic activity are described.

Materials and Methods

Solid-Phase Synthesis of Peptides. Chloromethylated polystyrene–2% divinylbenzene resin (containing 1.5 mequiv of Cl/g) was obtained from Cyclo Chemical Corp., Los Angeles, Calif. Boc- and Z-protected amino acids[1,2] were purchased from Schwarz BioResearch Inc., Orangeburg, N. Y., and their purity was checked by thin-layer chromatography. All reagents and solvents were of the highest grade commercially available.

Peptides were synthesized according to the procedure of Baxter *et al.* (1969) which incorporates minor variations of the original procedure of Merrifield (1964). Boc-amino acids were used for the synthesis of peptides except when lysine occurred as the N-terminal residue in which case bis-Z-lysine was coupled. Boc-amino acids with protected side chains were *O*-benzyltyrosine, γ-Benzylglutamic acid, β-benzylaspartic acid, *im*-benzylhistidine, *O*-benzylserine, *O*-benzylthreonine, and N^{ϵ}-Z-lysine. All coupling reactions were mediated with dicyclohexylcarbodiimide except that involving the carboxyl group of glutamine which was coupled directly as the *p*-nitrophenyl ester.

Synthesis of the C-Terminal Heptapeptide and Homologs. The C-terminal heptapeptide, Lys-Glu-Leu-Gly-Tyr-Gln-Gly, and its homologs, Glu-Leu-Gly-Tyr-Gln-Gly, Leu-Gly-Tyr-Gln-Gly, and Gly-Tyr-Gln-Gly, were synthesized as follows: Boc-glycine-resin (7 g containing 0.20 mmole/g of resin) was introduced into the synthesis vessel and subjected

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[1] Abbreviations used in this paper are: Boc, *tert*-butyloxycarbonyl; Z, benzyloxycarbonyl; PBS, phosphate-buffered saline (pH 7.1); BrAcC-H, bromoacetylcellulose-heptapeptide immunosorbent; BrAcC-Mb, bromoacetylcellulose-myoglobin immunosorbent; RγG, rabbit γ-globulin.

[2] All amino acids used in this study were of the L configuration.

to three cycles of deprotection, neutralization, and coupling. After coupling the fourth residue (glycine) a portion of the tetrapeptide-resin was removed from the reaction vessel. This was repeated after the fifth and sixth residues were coupled. Each protected peptide-resin was stored *in vacuo* over P_2O_5 prior to subsequent cleavage.

Synthesis of the Tetradecapeptide (Lys-Ala-Ser-Glu-Asp-Leu-Lys-Lys-His-Gly-Val-Thr-Val-Leu). For the synthesis of the protected tetradecapeptide, 5 g of Boc-leucine-resin (0.25 mmole/g of resin) was used at the start. Thirteen cycles of deprotection, neutralization, and coupling were carried out. At the conclusion of the synthesis the protected peptide-resin was removed from the vessel, collected on a filter, washed with methylene chloride and methanol, and dried *in vacuo* over P_2O_5. The weight was 6.52 g.

After cleavage from the resin (*vide infra*), the *im*-benzyl-protecting group was removed by reduction with sodium in liquid ammonia (Sifferd and du Vignaud, 1935) as follows. About 300 ml of liquid ammonia was dried over sodium. It was then redistilled under anhydrous conditions and collected at −70°. The *im*-benzyl-tetradecapeptide (150 mg) was then dissolved in the dry, distilled ammonia solution with vigorous stirring. The peptide-ammonia solution upon boiling (−30°), was then titrated with sodium[3] with the exclusion of moisture, until permanent light blue color remained. After a few seconds the reaction was quenched by adding a few drops of dry glacial acetic acid. The ammonia was removed by connecting the flask to a water aspirator with two soda-lime drying tubes in the line to avoid moisture. When the flask was dry, the crude deprotected peptide was dissolved in acetic acid and freeze-dried. The weight of the crude tetradecapeptide was 100 mg.

Cleavage of the Peptides from the Resin. The following general procedure was followed to remove the individual peptides from each peptide-resin. The protected peptide-resin was suspended in a mixture of trifluoroacetic acid–methylene chloride (1:1, v/v) containing 50 mmoles of anisole for each mmole of sensitive amino acid, *e.g.*, tyrosine (Stewart and Young, 1969). A slow stream of anhydrous HBr was bubbled into the solution for 70 min with the exclusion of moisture. The HBr was first scrubbed by passing it through a solution of resorcinol in trifluoroacetic acid. The free peptide was filtered and the resin washed several times with trifluoroacetic acid. The combined filtrates were evaporated at 10° on a rotary evaporator under reduced pressure.

The oily residue was triturated with ether and the precipitated peptide filtered and redissolved in a small volume of trifluoroacetic acid. It was reprecipitated with ether and filtered. The peptide was then dissolved in a suitable solvent, such as water or acetic acid, and freeze-dried.

Purification of the Peptides. The C-terminal peptides were purified by chromatography on a 1.1 × 40 cm column of DEAE-Sephadex, A-25, equilibrated with 0.01 M NH_4HCO_3. Elution was accomplished with a gradient of 0.01–0.15 M NH_4HCO_3. To form the gradient a nine-chamber Büchler Varigrad was used. The 0.01 M NH_4HCO_3 (160 ml) was introduced into the first chamber and 160 ml of the 0.15 M NH_4HCO_3 was introduced into each of the remaining eight chambers. The effluent was continuously monitored at 220 and 280 nm with a Gilford Model 2000 multiple sample absorbance recorder equipped with a Beckman DU monochromator; in this way the solvent absorption was continuously corrected for.

Thin-Layer Chromatography. The peptides were analyzed by thin-layer chromatography on silica gel H mounted on glass plates (20 × 20 cm). The solvent systems used were 75% phenol as well as 1-butanol–acetic acid–water (4:1:5, v/v, upper phase). The plates were sprayed with ninhydrin and the tetradecapeptide was sprayed with Pauly reagent as well.

Amino Acid Analysis. Samples of the products to be analyzed were hydrolyzed in 6 N HCl *in vacuo* at 110° for 20 hr. Analyses were performed on a Beckman amino acid analyzer, Model 120B, according to Moore *et al.* (1958).

Preparation of Antisera. Antisera to myoglobin[4] were obtained by intradermal injections, into the lower abdominal region at multiple sites of albino rabbits, of a total of 1 ml of a suspension consisting of 0.5 ml of 2% myoglobin dissolved in saline mixed with 0.5 ml of complete Freund's adjuvant. The first series of injections was followed 2 weeks later by a second series of injections totalling 1 ml and consisting of 0.5 ml of 4% myoglobin solution mixed with 0.5 ml of adjuvant. Following this the animals were injected intradermally at monthly intervals with a solution consisting of 1 ml of equal volumes of 4% myoglobin and adjuvant. The animals were bled 10 days after each injection. All sera were stored at −20° and were used individually.

Preparation of Immunosorbents. Cellulose (Whatman) was bromoacetylated according to Robbins *et al.* (1967). The bromoacetylcellulose (0.3 g dry weight) was suspended in 6 ml of 0.15 M phosphate–citrate buffer (pH 4.0) and 10 mg of heptapeptide, dissolved in 1 ml of buffer, was added. The suspension was stirred overnight at room temperature, centrifuged, and resuspended in 8 ml of 0.1 M $NaHCO_3$ (pH 8.9). It was then allowed to stand at 4° for 24 hr with occasional gentle stirring. After contrifugation it was resuspended in 10 ml of 0.1 M $NaHCO_3$ containing a few drops of ethanolamine (pH 9) and allowed to stand overnight at 4°. The suspension was then centrifuged, washed several times with PBS, and resuspended in 8 M urea and allowed to stand overnight at 4°. It was then washed well with PBS. To simulate the conditions for elution, it was incubated at 37° for 1 hr in 0.15 M glycine-HCl buffer (pH 2.5). The BrAcC-H immunosorbent thus prepared was centrifuged, washed well with PBS, and stored at 4° until ready for use.

For the preparation of BrAcC-Mb immunosorbent myoglobin (100 mg) was adsorbed to 0.5 g of bromoacetylcellulose in 0.1 M acetate buffer (pH 4.0) and the suspension was treated in all respects as described above.

Isolation of Antibodies. Antibodies were isolated from the immunosorbents using a batchwise method. Rabbit anti-myoglobin sera were incubated with the immunosorbents for 1 hr at 37° and overnight at 4°. The suspension was centrifuged at 20,000*g* and washed with PBS until the OD_{280} was 0.01. The immunosorbents were then treated with 10 μmole/ml of peptide dissolved in PBS followed by treatment with 0.15 M glycine-HCl (pH 2.5 and 2.0). In some cases the immunosorbents were eluted directly with glycine-HCl (pH 2.5 and 2.0). Each eluent solution was incubated with the immunosobent for 1 hr at 37°. The suspension was cen-

[3] This was accomplished with the use of a "sodium stick." Liquified sodium was drawn into a 1-ml pipet and fitted *via* a rubber stopper into the reaction vessel. The pipet was greased for easy movement. The titration was performed by a series of very quick immersions of the tip of the sodium stick into the ammonia solution.

[4] The myoglobin was prepared according to Hardman *et al.* (1966) and further purified on IRC-50 by the procedure of Edmundson and Hirs (1962).

TABLE I: Analysis of C-terminal Peptides.

Peptide	Sequence	Molar Ratio of Amino Acids					R_F[a]	
		Gly	Glu	Tyr	Leu	Lys	A	B
Tetrapeptide	Gly-Tyr-Gln-Gly	2.00	1.00	0.80			0.15	0.28
Pentapeptide	Leu-Gly-Tyr-Gln-Gly	2.00	0.99	0.91	1.02		0.23	0.44
Hexapeptide	Glu-Leu-Gly-Tyr-Gln-Gly	2.00	2.09	0.86	1.01		0.14	0.27
Heptapeptide	Lys-Glu-Leu-Gly-Tyr-Gln-Gly	2.00	2.00	0.86	1.00	1.09	0.13	0.23

[a] Solvent systems: (A) 1-butanol–acetic acid–water (4:1:5, v/v); system B, 75% phenol.

trifuged at 20,000*g* and the supernatant eluate passed through a Millipore filter (0.45 μ porosity). After incubation with each eluent solution, the immunosorbent was washed thoroughly with PBS (in the case of the peptides) or with glycine-HCl, until the OD_{280} was 0.01.

The purified antibody solutions were dialyzed against 0.1 M NH_4HCO_3 when eluted with peptide, followed by exhaustive dialysis against PBS. Otherwise, the eluates were dialyzed directly against PBS. The OD at 280 nm of the eluates was then recorded. The eluates were concentrated by negative pressure dialysis. The peptide could be recovered from the NH_4HCO_3 dialysate by evaporating off the solvent and bulk of the salt *in vacuo* at 40°, followed by gel filtration on Sephadex G-10.

Disc Electrophoresis. The eluted antibodies were analyzed by electrophoresis in 7% polyacrylamide gel using Tris-glycine buffer (pH 8.3) with a Büchler instrument (Fort Lee, N. J.) by passage of a 5-mA current/tube for 45 min. The gels were stained overnight with Amido Black and destained electrolytically at 6 mA/tube.

Radioiodination of Myoglobin. Myoglobin was radioiodinated with ^{125}I according to the procedure of Yagi *et al.* (1963) with minor modifications. To 3 mCi of ^{125}I was added 0.1 ml of KI (8×10^{-4} M in PBS, pH 7.5), 0.2 ml of Chloramine-T (0.1 M), and 2 mg of myoglobin dissolved in 0.2 ml of PBS (pH 7.5). The mixture was allowed to react for 5–6 min at room temperature when 0.1 ml of 0.3 M Na_2SO_3 was added. Free ^{125}I was removed from iodinated myoglobin by passing the reaction mixture through a Dowex 1-X8 column (1×15 cm) equilibrated with PBS (pH 6.8). The activity of the myoglobin was about 10^8 cpm/mg.

Radioimmunodiffusion. Radioimmunodiffusion was performed in 1.2% Noble agar (Difco). The eluted antibodies were mixed with normal RγG and were allowed to diffuse against sheep anti-rabbit serum for 24 hr at room temperature. RγG alone was also diffused against the sheep antiserum as a control. After diffusion the excess sheep antiserum was removed by washing the slides in PBS for several hours. Radioiodinated myoglobin was then applied to the same holes as the sheep antiserum and allowed to diffuse for 24 hr at room temperature. The slides were then washed exhaustively in PBS until the radioactivity of the washings approached that of background level. They were then washed in distilled water. The slides were thoroughly dried and wrapped in a thin sheet of Saran wrap (Dow Chemical Co.). Kodak No Screen X-Ray film was fixed over the slides for 3 days in a light-tight container. They were then stained with Carbofuscein dye.

Ultracentrifugation. The antibody eluted from the BrAc-C-H immunosorbent with glycine-HCl (pH 2.5) was concentrated by negative pressure dialysis and analyzed in a Spinco Model E ultracentrifuge in a synthetic boundary cell at 20° and 59,780 rpm. The solvent was PBS. Photographs were taken every 4 min after attaining full speed.

Results

Purification of the C-Terminal Peptides. The elution patterns of the C-terminal peptides chromatographed on DEAE-Sephadex are shown in Figure 1. As can be seen, as each residue was added to the growing peptide chain the complexity of the pattern obtained was increased, indicating the importance of complete coupling at each step. In this study no effort was made to monitor the degree of completion of each coupling reaction. In each elution pattern, in addition to the major component representing the desired product, at least one of the minor peaks represents a contaminant deficient in tyrosine, as indicated by its failure to absorb at 280 nm.

The results of the amino acid analyses and of thin-layer chromatography of the four C-terminal peptides are summarized in Table I. Each of these peptides revealed only one spot by thin-layer chromatography, which, when considered with the results of amino acid analyses, was interpreted as indicating a high degree of purity. The lower molar ratio for tyrosine obtained by amino acid analysis can be explained

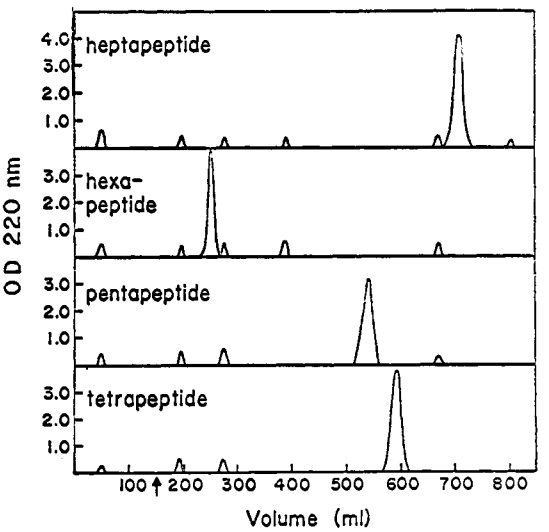

FIGURE 1: Elution pattern of the C-terminal peptides. A column (1.1×40 cm) of DEAE-Sephadex was equilibrated with 0.01 M NH_4HCO_3. Elution was accomplished with a gradient from 0.01 to 0.15 M NH_4HCO_3 (arrow indicates start of gradient).

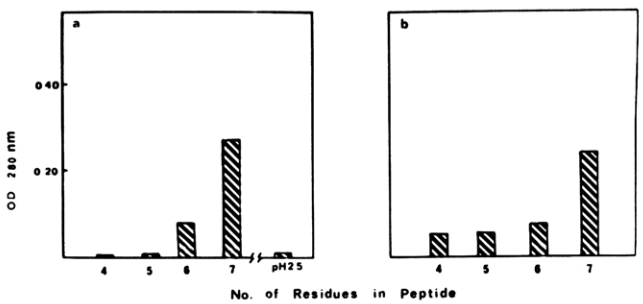

FIGURE 2: Sequential elution of antibody with peptides. The C-terminal tetra-, penta-, hexa-, and heptapeptides were used individually with each immunosorbent in order of increasing chain length. (a) Elution of antibody from BrAcC-H; (b) elution of antibody from BrAcC-Mb.

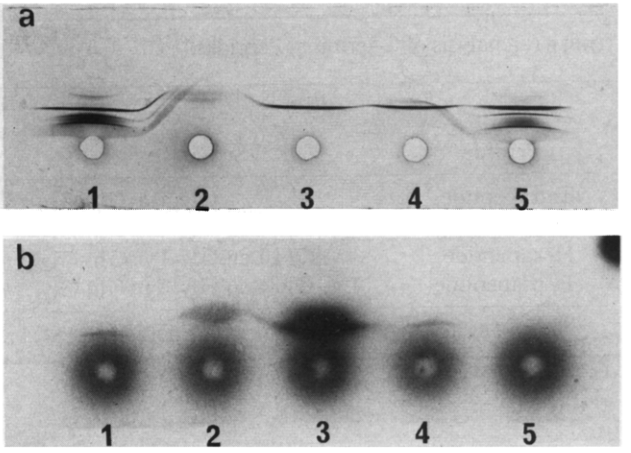

FIGURE 3: Radioimmunodiffusion of purified antibody. Samples were mixed with RγG and diffused against sheep anti-rabbit serum, followed by diffusion against [^{125}I]myoglobin. (a) Immunodiffusion pattern in agar gel; (b) radioautographic bands on X-ray film. (1) eluate from BrAcC-Mb at pH 2.5; (2) eluate from BrAcC-Mb at pH 2.0; (3) tetradecapeptide eluate from BrAcC-Mb; (4) heptapeptide eluate from BrAcC-H; (5) RγG control.

by the fact that this residue is somewhat acid sensitive and is partially destroyed under the conditions of hydrolysis employed here.

Thin-layer chromatography of the crude tetradecapeptide revealed one major spot and four minor contaminants with both solvent systems (the R_F value of the major component in 75% phenol was 0.42). The plate chromatographed in 1-butanol–acetic acid–water (4:1:5, v/v) was sprayed first with Pauly reagent and subsequent spraying with ninhydrin did not change the pattern. No spot could be detected corresponding to *im*-benzyl-tetradecapeptide indicating that the *im*-benzyl group had been completely removed. The tetradecapeptide was used without further purification to elute antibody from the myoglobin immunosorbent.

Elution of Antibodies from BrAcC-H and BrAcC-Mb Immunosorbents. BrAcC-H. Rabbit anti-myoglobin antiserum (12 ml) was incubated with BrAcC-H and eluted sequentially with a total of 40 μmoles each of tetra-, penta-, hexa-, and heptapeptides (in that order), and finally with glycine-HCl (pH 2.5). The results are shown in Figure 2a. The tetra- and pentapeptides, at the concentration used here (*i.e.*, 10 μmoles/ml), were ineffective in eluting material from the heptapeptide immunosorbent. A small amount of material was removed with the hexapeptide and the major portion of antibody was eluted with the heptapeptide. Subsequent treatment with glycine-HCl (pH 2.5) revealed no antibody in the eluate. Subsequent elution with glycine-HCl (pH 2.0) also revealed no protein in the eluate, ruling out the possibility of high-affinity antibody being still attached to the immunosorbent. None of the eluates gave precipitin bands on immunodiffusion against myoglobin. However, after incubation with BrAcC-H, the absorbed serum still showed a band indicating the presence of residual antibodies to other determinants of myoglobin. When antibodies bound to BrAcC-H were treated directly with glycine-HCl (pH 2.5), all the antibody appeared to be released since subsequent treatment at pH 2.0 did not result in further elution of protein. No precipitin bands were formed when the acid eluates from BrAcC-H were diffused against myoglobin.

BrAcC-Mb. This immunosorbent was incubated with rabbit antimyoglobin antiserum (12 ml) and eluted sequentially in the same way as BrAcC-H. From the results shown in Figure 2b it is evident that all the peptides had some ability to elute antibodies. However, again, the heptapeptide appeared to be the most effective in releasing antibodies from the immunosorbent. None of the eluates, nor the absorbed antiserum formed precipitin bands on immunodiffusion against myoglobin.

In some cases the antibody was eluted directly with glycine-HCl instead of with peptides. Elution of BrAcC-Mb with glycine-HCl (pH 2.5) resulted in the release of about 70–80% of the total anti-myoglobin antibodies and the remaining 20–30% could be eluted when the pH was lowered to 2.0. Both eluates gave precipitin bands on immunodiffusion *vs.* myoglobin.

Anti-myoglobin antiserum (25 ml) was incubated with BrAcC-Mb and eluted with the tetradecapeptide (total of 40 μmoles). After exhaustive dialysis the OD_{280} of the 2.0-ml concentrate was 0.41. The eluate did not give a precipitin band on immunodiffusion with myoglobin.

Radioimmunodiffusion. The results of the radioimmunodiffusion are shown in Figure 3. All of the eluates tested reacted specifically with radioiodinated myoglobin, as revealed by the presence of bands on the X-ray film (Figure 3b). However, although the control (RγG) showed, as would be expected, the formation of a precipitin band (Figure 3a), no corresponding radioactive band was visible on the film.

Disc Electrophoresis. The results of the electrophoresis in polyacrylamide gel are shown in Figure 4. In comparison with the pattern obtained with whole serum as well as with that of the antibodies eluted from BrAcC-Mb at pH 2.5 (Figure 4a), the heptapeptide eluate from BrAcC-H (Figure 4b) revealed a more limited heterogeneity covering a very narrow range of the electrophoretic mobilities of γ-globulins. In addition, a band corresponding to that of IgM globulins was visible, as well as a trace of albumin.

Ultracentrifugal Analysis. The antibody eluted from BrAcC-H was ultracentrifugally heterogeneous containing two components. The corrected s_{20} values of the slower and faster moving components were 5.8 and 16.5 S, respectively, corresponding to those of IgG and IgM antibodies.

Discussion

In the present study an attempt was made to arrive at a more precise definition of the antigenic determinant contained in the C-terminal heptapeptide of sperm-whale myoglobin. The C-terminal tetra- and pentapeptides were ineffective in removing antibody bound to the BrAcC-H immunosorbent

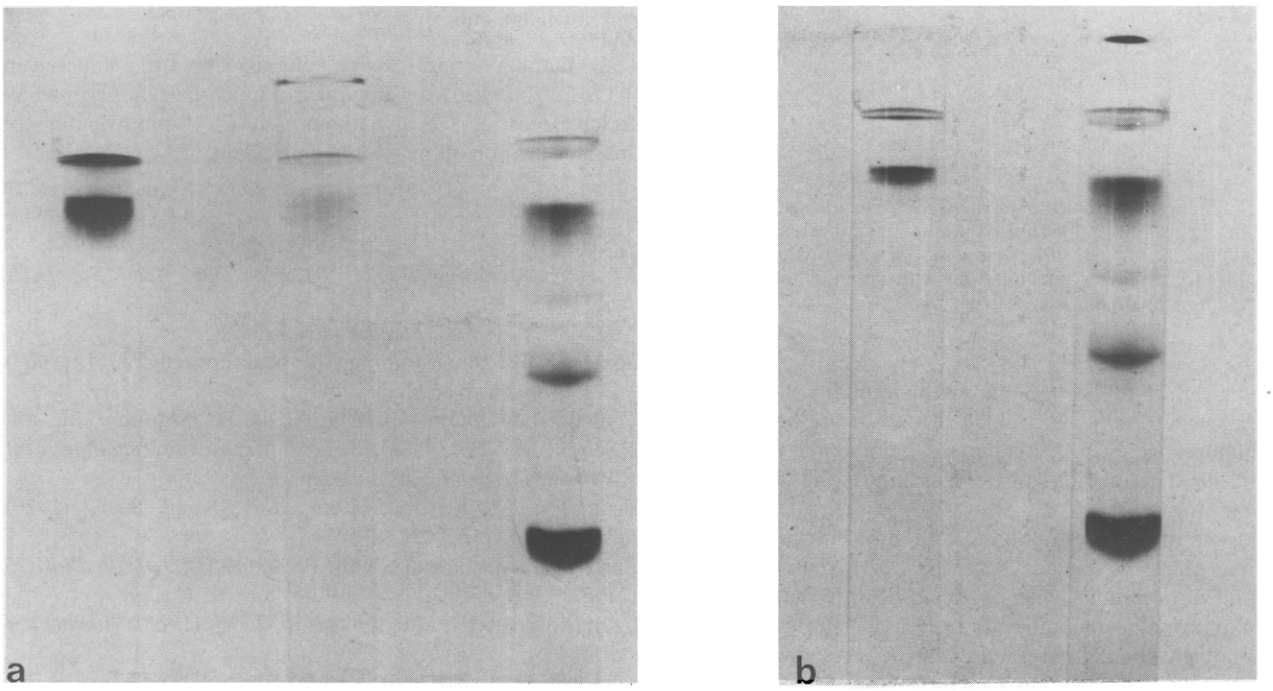

FIGURE 4: Disc electrophoretic patterns of purified antibody. (a) Acid eluates from BrAcC-Mb; left, pH 2.5 eluate; center, pH 2.0 eluate; right, whole anti-myoglobin serum. (b) Left, heptapeptide eluate from BrAcC-H; right whole anti-myoglobin serum.

indicating that the residues up to and including the pentapeptide, Leu-Gly-Tyr-Gln-Gly, did not possess sufficient binding energy to remove antibodies bound to this immunosorbent. However, the addition of the sixth residue, glutamic acid, provided the necessary binding energy for the release of at least a small portion of antibodies. Furthermore, with the addition of the final residue, lysine, the cumulative binding energy sufficed to release the bound antibodies, as evidenced by the fact that no antibody could subsequently be eluted at pH 2.5 or at pH 2.0. In these experiments, no attempt was made to establish if the antibodies eluted with the hexa- and heptapeptides represented different populations of antibodies whose antibody combining sites differed from each other, or if they represented the same population of antibodies, a fraction of which could be removed by the hexapeptide at the particular concentration used.

It seems evident that BrAcC-H was not effective in binding all the antibodies directed against the C-terminal heptapeptide since the tetra- and pentapeptides, which did not elute antibodies off BrAcC-H, were capable of eluting a small portion of the corresponding antibodies bound to BrAcC-Mb. It is possible that steric factors may have been responsible for the inability of some antibodies to bind to BrAcC-H. However, the results obtained with both immunosorbents indicate that the N-terminal lysine residue of the heptapeptide did indeed play a major role in the binding between this peptide and the corresponding antibodies. Regrettably, because of the small quantity of antibodies available in each antiserum, no quantitative precipitin tests were performed on the original antisera to determine their antibody content, nor was the amount of antibody adsorbed to the immunosorbents quantitatively measured.

These results contrast somewhat with those reported by Crumpton *et al.* (1970) who showed that the hexa- and heptapeptides possessed the same activity, as measured by inhibition of precipitation. This discrepancy may be ascribed to the different methods used to measure binding activity or to the different antisera used in the two studies. It should also be noted that these authors used whole serum to measure binding, rather than purified antibodies, and it is conceivable that the serum may have contained peptidases, which may have altered the heptapeptide reducing thus its activity to that of the hexapeptide. Such peptidases have been found to interfere in inhibition studies with peptides (Schechter *et al.*, 1966). These workers (Crumpton *et al.*, 1970) also established that the tetra- and pentapeptides possessed a small amount of inhibitory activity, these findings being supported by the results of this study that these smaller peptides were capable of eluting antibodies from BrAcC-Mb.

Nitration of tyrosines-146 and -151 (Figure 5) had been found to cause complete loss of the binding activity of a C-terminal peptide consisting of residues 132–153 (Atassi, 1968), indicating that one or both of these tyrosine residues were present in an antigenically reactive region of myoglobin. These results are contrasted by the finding that replacement of tyrosine-151 with phenylalanine or *p*-methoxyphenylalanine in C-terminal hepta- and hexapeptides (Crumpton *et al.*, 1970) did not alter the activity of these peptides. It has also been reported that a peptide consisting of residues 139–146, N terminal to peptide 147–153, possessed a slight inhibitory activity (Crumpton and Wilkinson, 1965). The results presented in this paper point to the importance of lysine-147 in binding. Considering all these results, it may be suggested that tyrosine-146 forms a part of the antigenic site and that it is the C-terminal portion of peptide 139–146 and the N-terminal portion of peptide 147–153, which together constitute the antigenic determinant.

Although lysine-147 plays an important role in binding to antibody, this residue may not necessarily be the immunodominant group (Luderitz *et al.*, 1966) of the C-terminal heptapeptide, *i.e.*, it may not contribute the highest proportion of the binding energy (Schlossman and Levine, 1967). The actual binding energy contributed by lysine-147 may be relatively small; however, without the additional increment

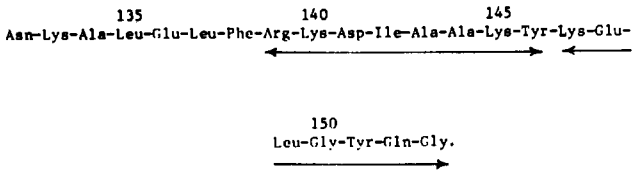

FIGURE 5: Amino acid sequence of the C terminus of sperm-whale myoglobin. Peptide 139–146 inhibited the precipitation of anti-metmyoglobin antibodies with apomyoglobin to the extent of 5%. Peptide 147–153 inhibited precipitation with apomyoglobin up to 15% (Crumpton and Wilkinson, 1956).

provided by this residue the cumulative binding energies of the other residues may be insufficient to elute antibody from an immunosorbent. The actual immunodominant group of this antigenic determinant can be determined only by quantitative binding studies.

The results of the electrophoretic and ultracentrifugal analyses show that the eluate from BrAcC-H represented a population of antibodies with restricted heterogeneity. Thus, it would appear that the antibody response, even to a single, small, relatively simple, naturally occurring protein antigenic determinant, is a complex one. Nor is the lack of homogeneity of antibodies directed to the C-terminal heptapeptide of myoglobin limited to rabbit antisera. Purified horse anti-heptapeptide antibodies were shown, by electrophoresis, to contain three components (Centeno *et al.*, 1968). The apparent discrepancy between the present results indicating a limited heterogeneity of purified antibodies directed to the C-terminal heptapeptide of myoglobin and the previous findings (Givas *et al.*, 1968) suggesting the isolation of homogeneous monospecific antibodies may be due to the fact that in the earlier study the purified antibodies were dialyzed against water prior to electrophoresis; this procedure would be expected to remove the euglobulin fraction.

The elutability of antibodies from BrAcC-H and from BrAcC-Mb with the C-terminal peptides, as well as with the tetradecapeptide from BrAcC-Mb, is considered as evidence that the corresponding antibodies were indeed directed against a single antigenic determinant in each case. Their specificity was shown by the fact that they combined with radioiodinated myoglobin as demonstrated by the band produced on the X-ray film.

The antibodies directed against the tetradecapeptide are an interesting subject for further study since this essentially nonhelical free peptide (Crumpton and Small, 1967) combines with antibodies directed against this region which in the native myoglobin molecule is helical (Kendrew *et al.*, 1961). This behavior contrasts with that of the conformation-dependent antibodies directed against the "loop" peptide of lysozyme (Maron *et al.*, 1971).

In summary, it has been shown by sequential elution of antibodies, from appropriate immunosorbents with a series of synthetic peptides of increasing chain lengths and related to the C-terminal heptapeptide of myoglobin, that the N-terminal lysine residue plays an important role in the binding of myoglobin with the antibodies directed to the C terminus of myoglobin. The isolated antibodies possessed limited electrophoretic and ultracentrifugal heterogeneity. Moreover the synthetic tetradecapeptide, corresponding to a helical region in myoglobin (residues 56–69), was shown to possess antigenic activity.

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