The Immunological Activity of some of the Chymotryptic Peptides of Sperm-Whale Myoglobin

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1. Sperm-whale apomyoglobin was digested with chymotrypsin in a dialysis sac. The ultrafiltrate contained incompletely hydrolysed fragments which partially inhibited the precipitation of metmyoglobin and apomyoglobin by some antisera produced against metmyoglobin. The inhibitory activity was stable to heating at 100° and depended on the peptide structure. 2. The fragments were fractionated according to molecular size and were purified by ion-exchange chromatography. Six pure peptides and two peptides which contained a minor impurity were isolated. Their amino acid compositions and *N*-terminal amino acid sequences were determined and their entire amino acid sequences deduced from the known amino acid sequence of sperm-whale myoglobin. 3. The peptides formed no detectable precipitates with the antisera. Five of the eight peptides partially inhibited the precipitation of apomyoglobin and/or metmyoglobin by one antiserum. Six of the peptides inhibited the precipitation of apomyoglobin by one or other of two antisera; at least two of these peptides inhibited both antisera. One peptide failed to inhibit the precipitation of either antigen by either antiserum. Two of the peptides possessed the same serological specificity. 4. The molar ratios of inhibitors to antigen for 50% of the maximum inhibition decreased as the molecular size of the inhibitor increased. With one antiserum and with apomyoglobin as the antigen, molar ratios 12 and 80 were obtained for peptides with molecular weights 2051 and 793 respectively. 5. The size and structure of an antigenic site is discussed in relation to the known steric configuration of myoglobin.

Many attempts have been made to define the structural features that constitute an antigenic site of globular proteins. One approach to this problem has been to isolate and characterize fragments of the whole molecule that retain the power to combine with a specific antiserum. The success of this approach probably depends to a large extent on using a degradative procedure which does not disrupt the structural integrity of the antigenic site but which breaks the bonds attaching it to the remainder of the molecule. Degradation with enzymes has been used with some success and fragments containing some of the antigenic sites of the whole molecule have been isolated from enzymic digests of bovine serum albumin (Porter, 1957), human serum albumin (Lapresle & Durieux, 1957; Lapresle & Webb, 1960; Press & Porter, 1962), tobacco-mosaic-virus protein (Anderer, 1963; Young, Benjamini, Shimizu, Leung & Feingold, 1963) and the β -chain of human haemoglobin (Askonas & Smyth, 1964). The results suggest that

globular proteins possess a number of antigenic sites which may be of appreciable size and whose serological specificities depend on the three-dimensional arrangement of distinct sequences of amino acids. As a result, the definition of the structural features of an antigenic site should be facilitated by a study of the antigenic determinants of a globular protein whose three-dimensional structure and amino acid sequence are known. Crystalline sperm-whale myoglobin (Kendrew *et al.* 1961) and human haemoglobin (Muirhead & Perutz, 1963; Schroeder, 1963) are the only globular proteins which fulfil the above requirements. As the structure of sperm-whale myoglobin is known in greatest detail it was chosen for investigation, even though it is a very poor antigen. An examination of a partial chymotryptic digest of sperm-whale apomyoglobin showed that it contained a variety of active fragments which combined with but which did not form a precipitate with a specific antiserum. The isolation and properties of some of

these active peptides are described in the present paper, a preliminary report having already appeared (Crumpton, 1964).

MATERIALS

Sperm-whale myoglobin. Crystalline metmyoglobin was isolated by the method of Kendrew & Parrish (1956) from two samples of skeletal muscle kindly supplied by Dr J. C. Kendrew, F.R.S., and Chr. Salvesen and Co. Ltd., G.P.O. Box 217, Leith, Scotland. No differences were detected between metmyoglobins prepared from the muscles of different sperm whales. The crystalline material was stored at 2° as a paste in a saturated solution of $(\text{NH}_4)_2\text{SO}_4$, pH 7.0. Metmyoglobin was converted into the ferro form by adding a twofold molar excess of solid sodium dithionite (Hopkin and Williams Ltd.) to a stirred solution of the protein in 0.4 M- K_2HPO_4 at 2° followed by dialysis against deionized water.

Metmyoglobin used for immunization, for the immunological tests, and for preparing apomyoglobin for use in the immunological tests, was purified according to Edmundson & Hirs (1962); the fraction corresponding to 'components IV + V' of these authors was selected. The product was stored in a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ and was converted into the ferri form by adding a slight stoichiometric excess of 0.5 M-potassium ferricyanide to a solution of the protein in 0.1 M-phosphate buffer, pH 7.0, at 2° followed by dialysis against deionized water.

Ultracentrifugal analysis at pH 7.7 and starch-gel electrophoresis at pH 8.5 and 6.0 of the purified metmyoglobin showed no evidence of inhomogeneity. Amino acid analyses of the crystalline and purified metmyoglobins were in close agreement and the amino acid composition of the purified material was identical with that determined by Edmundson & Hirs (1962) for 'components IV + V'.

Apomyoglobin. Apomyoglobin was prepared according to the method of Theorell & Åkeson (1955) with slight modifications. A 1% (w/v) solution of reduced myoglobin in water at 0° was added dropwise with vigorous stirring to 20 vol. of acetone-HCl [2 ml. of N-HCl/l. of acetone (AnalaR)] at -13° to -15°. The precipitate was collected by centrifuging for 5 min. at -15° and dissolved in water at 0° to give a concentration of about 20 mg./ml. Occasionally this solution was noticeably coloured, in which case it was treated a second time with acetone-HCl. The solution of apomyoglobin was dialysed at 2° for 16 hr. against 0.01 M-sodium bicarbonate, followed by deionized water. A small amount of insoluble denatured apomyoglobin formed during dialysis; this was removed by centrifuging and the supernatant was stored at 2°. Recoveries of apomyoglobin were between 90 and 95% and the apomyoglobin contained not more than 1% (w/v) of undissociated myoglobin as judged from the absorption at 410 m μ . Apomyoglobin was usually used within 7 days of its preparation and was never used after 1 month.

Antisera. Antibodies were prepared in rabbits by the injection of metmyoglobin incorporated in a water in oil-adjuvant mixture. Equal volumes of a 1% (w/v) solution of purified metmyoglobin and 'complete adjuvant' [1.5 ml. of Arlacel A: Honeywell and Stein Ltd., London, W.1; 8.5 ml. of Bayol 55 (Bayol F): Printar Industries Ltd.,

London, E.16; 5 mg. of heat-killed *Mycobacterium tuberculosis*] were emulsified with a syringe. A sample of normal serum was obtained from each animal before immunization. The emulsion (1 ml.) was injected once a week for a period of 4 weeks; two injections intramuscularly were followed by one each intraperitoneally and subcutaneously. The animals were bled fortnightly, 6-10 weeks after the last injection. They were then re-immunized by three injections on successive days of either 0.5 ml. of 1% solution of metmyoglobin or 0.5 ml. of alum-precipitated metmyoglobin (5 mg./ml.) prepared according to Porter (1955), and bled 1 week later. More antisera were obtained by repeating these injections at intervals of about 2 months. Sera were preserved with thiomersalate (0.01%) and were stored at -20°. Approximately half of the immunized rabbits produced usable amounts of antibody. Antisera with similar characteristics were pooled. Antiserum WF represented sera collected from 15 rabbits after injection of antigen in adjuvant and after re-immunization with one course of alum-precipitated metmyoglobin; antiserum WH represented sera collected at the same times from two rabbits.

Other materials. Three-times-crystallized α -chymotrypsin (batch no. CDI707-13) and two-times-crystallized trypsin (batch no. TRSF771) were purchased from Worthington Biochemical Corp. and 'crystalline bacterial proteinase' from Nagase and Co. Ltd., Osaka, Japan. Papain was the inactive Hg dimer of Porter (1959); the enzyme was activated by dissolving it in 0.005 M-2-mercaptoethanol (L. Light and Co. Ltd.; redistilled before use), 0.001 M-EDTA (disodium salt) (Hopkin and Williams Ltd.), Sephadex G-50 (medium), DEAE-Sephadex A-25 (medium) and CM-Sephadex C-25 (medium) were obtained from Pharmacia, Uppsala, Sweden. Each material was dry-screened and the fraction which passed through sieve B.S.100 but was retained by sieve B.S.200 was used. Di-isopropyl phosphorofluoridate was purchased from Boots Pure Drug Co. Ltd.; 1 g. was added to 3.8 ml. of anhydrous propan-2-ol (AnalaR) to give an approx. 1 M solution. The dialysis tubing used for the preparation of enzymic digests was 8/32 in. Visking tubing and that used for the dialysis of peptides was 18/32 in. Visking tubing which had been heated in a hot-air oven at 90° for 4 days. L-Glutamic acid was obtained from Roche Products Ltd.

METHODS

Determination of protein concentration. Concentrations of metmyoglobin and apomyoglobin solutions in either water or 0.9% NaCl were determined by relating the extinction at 280 m μ measured with a cell of 1 cm. light-path to the specific extinction coefficient at the same wavelength. The solutions of metmyoglobin and apomyoglobin (about 20 mg./ml.) used for the determination of specific extinction coefficients had been exhaustively dialysed against deionized water. Their concentrations were determined by weighing samples (1.00 ml.) which had been dried to constant weight at 95° *in vacuo*. The specific extinction coefficients ($E_{1\text{cm}}^{1\%}$) at 280 m μ for solutions of metmyoglobin and apomyoglobin at neutral pH were 18.0 and 9.30 and for solutions in 0.1 N-NaOH were 17.9 and 9.2 respectively.

Detection of peptides. Peptides separated by fractionation on columns were detected in the eluate fractions by measurement of extinctions at 280 and 215 $m\mu$. Extinctions at 215 $m\mu$ were measured with a slit width of 0.8 mm. and a 1 cm. cell, and were corrected for any background absorption due to the solvent used for elution.

Determination of ammonium bicarbonate concentration. The amount of NH_4HCO_3 present in solutions of peptides was determined by relating the conductivity of the solution measured at 20° with a Phillips Conductivity Measuring Bridge PR9500 with cell no. PR9512/01 to the conductivities of solutions of NH_4HCO_3 of known concentration.

Ultracentrifugal analysis. Solutions (about 10 mg./ml.) of metmyoglobin in 0.15 M-KCl-7.8 mM-potassium phosphate, pH 7.7, were examined at 24° in a Spinco model E analytical ultracentrifuge; sedimentation was continued for 4 hr. at 59 780 rev./min.

Electrophoresis. Electrophoresis in starch gel in borate buffer, pH 8.5 (Smithies, 1955), and in 0.025 M-phosphate buffer, pH 6.0, was carried out as described by Smithies (1959); electrophoresis was continued for 17 hr. at 2.5 v/cm. High-voltage electrophoresis was carried out with the apparatus described by Katz, Dreyer & Anfinsen (1959) with 'white spirit 115' (Esso Petroleum Co. Ltd.) as coolant and pyridine-acetic acid-water, pH 6.5 (25:1:475, by vol.), and pH 3.7 (1:10:289, by vol.), as buffers. Peptide solutions were applied to Whatman no. 3MM paper as 2 cm. bands (0.01-0.05 $\mu\text{mole/cm.}$) and electrophoresis was continued for 45 min. at 80 v/cm. The peptides were located by dipping the dried paper in a solution of ninhydrin (1 g.) in ethanol-acetic acid-sym.-collidine (15:5:2, by vol.) and heating at 90° for 5 min. Electrophoresis on 16 cm.-long strips of cellulose acetate (Oxoid) was carried out in tris-boric acid buffer, pH 8.9 (Fazekas de St Groth, Webster & Datyner, 1963), for 4 hr. at 11 v/cm. Peptide solutions were applied as 2 cm. bands (0.01 $\mu\text{mole/cm.}$) and the strips were stained with Procion Brilliant Blue RS (Imperial Chemical Industries Ltd.) as described by Fazekas de St Groth *et al.* (1963).

Amino acid analysis. Absorption spectra were measured with a Unicam SP.700 recording spectrophotometer and a 1 cm. cell. Amino acid compositions were determined according to Crumpton & Wilkinson (1963) with 55 and 8 cm. columns for the separation of the neutral and acidic, and the basic amino acids respectively (Stark & Smyth, 1963; Dr S. Moore, The Rockefeller Institute, personal communication). As the Standard Calibration Amino Acid Mixture, type I (Spinco part no. 120-220), gave a low value for glutamic acid (A. B. Edmundson, personal communication), a freshly prepared solution of glutamic acid was used (2.00 ml. of 0.25 $\mu\text{mole/ml.}$). To a known volume of peptide solution containing 0.5-1 μmole was added 0.500 ml. of 2 μmoles of L-norleucine/ml. The mixture was dried and then hydrolysed with 1.0 ml. of constant-boiling HCl *in vacuo* for 24 hr. in a bath of boiling toluene. The hydrolysates were dried and redissolved in 6.50 ml. of citrate-HCl buffer, pH 2.2; 2.00 ml. samples were used for analysis. The results were calculated as amounts (μmoles) of amino acids present in the volume of peptide solution hydrolysed and were corrected for 100% recovery of the norleucine which was added (1 μmole). To compensate for destruction during hydrolysis the amounts of serine and threonine were corrected to zero time of hydrolysis. The amount (μmoles) of peptide

hydrolysed was taken to be equal to the average of the amounts of those amino acids which were present to the smallest extent; amino acids present in trace amounts only were ignored. The concentration ($\mu\text{moles/ml.}$) of each peptide solution was calculated from the volume of solution which was hydrolysed and the amount of peptide that this volume contained.

N-Terminal amino acid sequence. The phenyl isothiocyanate method of Edman (1950) was used to determine the N-terminal amino acid sequences of the peptides. The peptide (0.5-1 μmole) was treated with phenyl isothiocyanate (Hopkin and Williams Ltd.; redistilled before use) according to the method of Eriksson & Sjöquist (1960); cyclization and extraction of the N-terminal amino acid derivative were carried out according to Margoliash (1962). The extract was taken to dryness and redissolved in methanol to give a concentration of either 1 or 2.5 $\mu\text{moles/ml.}$ The yield and purity of the N-terminal amino acid derivative were determined from its absorption spectrum (230-310 $m\mu$) as described by Fraenkel-Conrat, Harris & Levy (1955). Phenylthiohydantoin amino acids were identified by thin-layer chromatography according to the method of Brenner, Niederwieser & Pataki (1961). MN-Silica-gel G (Macherey, Nagel and Co., 516 Düren, Germany) suspended in a 0.5% (w/v) solution of soluble starch (Hopkin and Williams Ltd.) was used as the supporting medium with the following solvent systems: chloroform-methanol (95:5 and 90:10, v/v) and, for the differentiation of the derivatives of aspartic acid and glutamic acid, 1,4-dioxan-water (4:1, v/v). Samples of known phenylthiohydantoin amino acids were included on each chromatogram. Chromatograms were sprayed with a fresh mixture of equal volumes of a solution of iodine (0.01 M-iodine in 0.5 M-KI) and 0.5 M-sodium azide; the amino acid derivatives appeared as white spots on a brown background.

The residual peptide was redissolved in 1.0 ml. of water; a sample (0.25 ml.) of this solution was used to determine its amino acid composition. The remainder of the residual peptide was treated again with phenyl isothiocyanate to identify the next amino acid.

Quantitative precipitin test. Increasing amounts of antigen dissolved in 0.20 ml. of 0.9% NaCl were added to 0.50 ml. of undiluted antiserum. Control tubes contained either 0.9% NaCl in place of the antigen solution or normal serum instead of the antiserum. The contents of the tubes were mixed, incubated at 37° for 1 hr. and then at 2° for 18 hr. The precipitates were washed three times with 0.7 ml. of 0.9% NaCl at 2°, were redissolved in 1.20 ml. of 0.1 N-NaOH and the extinctions measured at 280 $m\mu$ with a cell of 1 cm. light-path. Excess of antigen or antibody, present in the supernatants obtained after separation of the antigen-antibody precipitate, was detected by adding antigen to one-half of the supernatant and antiserum to the other half. These tubes were mixed, incubated at 37° for 1 hr., stored at 2° overnight, centrifuged and the presence of a precipitate was recorded. If no excess of antigen was detected, then the amount of antigen in the antigen-antibody precipitate was assumed to be the same as that added. The amount of antibody in the antigen-antibody precipitate was determined from the extinction at 280 $m\mu$ after subtracting the extinction due to the antigen. The specific extinction coefficient ($E_{1\text{cm}}^{1\%}$) at 280 $m\mu$ for rabbit γ -globulin in 0.1 N-NaOH was assumed to be 14.0 (Porter, 1957).

Quantitative inhibition of precipitation. As the enzymic digest gave no detectable precipitate with the antisera, the following method of testing for inhibitory activity was adopted. Increasing amounts of inhibitor diluted to 0.20 ml. with 0.9% NaCl were incubated for 2 hr. at 20° with 0.50 ml. of undiluted antiserum. An amount of metmyoglobin or apomyoglobin equal to that which gave maximal precipitation in the quantitative precipitin test was then added. The mixture was incubated at 37° for 1 hr. and at 2° overnight and the quantity of precipitate was estimated as previously described. The decrease in the extinction at 280 m μ due to the inhibitor was expressed as a percentage of the extinction obtained in the absence of inhibitor, determined in duplicate at the same time. Extinctions were usually reproducible within 2%.

Preparation and fractionation of peptides. Solutions (10 mg./ml.) of apomyoglobin for digestion with chymotrypsin were dialysed against 0.1 M-tris-HCl buffer, pH 8.4. A small amount of insoluble material formed during dialysis; this was removed by centrifuging before the addition of chymotrypsin (1% of the weight of apomyoglobin). Enzymic digestion was carried out in a dialysis sac under a positive pressure of 45 cm. Hg for 20 hr. at 0–2°. Di-isopropyl phosphorofluoridate (100 moles/mole of chymotrypsin) was added to the ultrafiltrate, which was concentrated by rotary evaporation *in vacuo* at 0–2°. The concentrated ultrafiltrate (about 20 ml.) was eluted from a column (145 cm. $\times$ 9.5 cm.²) of Sephadex G-50 with 0.025 M-NH₄HCO₃, pH 8.0; 5 ml. fractions were collected. The extinctions at 280 and 215 m μ of the eluate fractions were measured. Fractions corresponding to each peak were pooled, concentrated by rotary evaporation *in vacuo* at 0–2° to about 25 ml., dialysed in heated tubing for 4 hr. against 500 ml. of deionized water at 2°, concentrated to about 6 ml. and clarified by centrifuging. The pH values of the recovered solutions were between 7.8 and 8.2 and their conductivities were not greater than that of 0.06 M-NH₄HCO₃.

Peptides present in the above solutions were fractionated on DEAE-Sephadex. Samples (5 ml.) of the solutions were added to columns (40 cm. $\times$ 1.1 cm.²) of DEAE-Sephadex A-25 equilibrated with 0.01 M-NH₄HCO₃, pH 8.0. The columns were washed with 0.01 M-NH₄HCO₃, pH 8.0, and the adsorbed material was eluted with a gradient to 0.15 M-NH₄HCO₃, pH 8.0; a 150 ml. mixing chamber was used and 5 ml. fractions were collected. In those cases where all the 215 m μ -absorbing material added to the column was not eluted by the above procedure, the column was eluted further with a gradient to 0.5 M-NH₄HCO₃, pH 8.0. The extinctions at 280 and 215 m μ of the eluate fractions were measured and the fractions corresponding to each peak were pooled and concentrated as previously described.

Peptides which were not adsorbed by DEAE-Sephadex were fractionated on CM-Sephadex. Samples (5 ml.) of the concentrated solutions were adjusted with N-HCl to pH 6.5 and added to columns (40 cm. $\times$ 1.1 cm.²) of CM-Sephadex C-25 equilibrated with 0.01 M-phosphate buffer–0.01 M-NaCl, pH 6.5. Columns were eluted with either 0.01 M-phosphate buffer–0.2 M-NaCl, pH 6.5, or with 0.01 M-phosphate buffer–0.15 M-NaCl, pH 6.5, followed by a gradient to 0.01 M-phosphate buffer–0.4 M-NaCl, pH 6.5; a 150 ml. mixing chamber was used. The eluted peptides were detected and recovered as described above.

RESULTS

Antisera WF and WH

Antisera WF and WH were used to follow the purification and for the characterization of the inhibitory peptides present in a chymotryptic digest of apomyoglobin.

The precipitin curves given by antiserum WF with metmyoglobin and apomyoglobin, and the molar ratios of antibody to antigen in the precipitates, are shown in Fig. 1. It can be seen that 19.5 μ g. of metmyoglobin and 22 μ g. of apomyoglobin caused maximal precipitation and that the molar ratios of antibody to metmyoglobin and to apomyoglobin in the precipitate formed in a gross excess of antibody were 3.2 and 2.9 respectively. With antiserum WH maximal amounts of precipitate were given by 17 μ g. of metmyoglobin and 21 μ g. of apomyoglobin and the molar ratios of antibody to metmyoglobin and to apomyoglobin in gross antibody excess were 2.7 and 3.1 respectively.

No free antigen or antibody was detected in the supernatants corresponding to maximal precipitation of apomyoglobin by either antiserum. However, the supernatants corresponding to maximal precipitation of metmyoglobin gave detectable precipitates with apomyoglobin. The pre-immunization sera gave no precipitate with either antigen.

Preparation and purification of inhibitors

Native metmyoglobin was not hydrolysed by trypsin or chymotrypsin but apomyoglobin was

Fig. 1. Precipitin curves of apomyoglobin (O) and metmyoglobin (Δ) with antiserum WF and the molar ratio of antibody to antigen in the precipitates formed with apomyoglobin (●) and metmyoglobin (▲).

readily digested. As a result apomyoglobin was chosen as the substrate for enzymic degradation.

The probability of obtaining inhibitory fragments was considered to be greater if apomyoglobin was digested under conditions which would favour the selection of incompletely hydrolysed peptides. As a result enzymic digestions were carried out in dialysis sacs under a positive pressure since in this way diffusible fragments would be separated from the indiffusible enzyme as they were formed. Since it seemed probable that inhibitory activity would depend on a peptide retaining the three-dimensional structure that it possessed in the native molecule, all procedures were carried out at 0–2° and between pH 6.5 and 8.5, and drying from the frozen state was avoided.

Apomyoglobin was incubated in dialysis sacs with chymotrypsin, trypsin, papain and bacterial proteinase at 2°, at pH values between 7.0 and 8.5 and at different ratios of enzyme to substrate. The ultrafiltrates were tested for inhibitory activity with several antisera. It was found that chymotrypsin (1% of the weight of apomyoglobin) at pH 8.0–8.5 gave diffusible fragments which inhibited the precipitation of metmyoglobin and apomyoglobin by antisera WF and WH. Less inhibitory activity was detected with other antisera and with some sera no inhibition was detected. In general, antisera collected after immunization with antigen in adjuvant were inhibited to a greater degree than those collected after further injections of antigen. Apomyoglobin digested with the bacterial proteinase gave no detectable inhibition but digestion with trypsin and papain gave active ultrafiltrates; papain also produced a diffusible fragment which formed a precipitate with the antisera. In the work described below antisera WF and WH were used to follow the purification of the inhibitory peptides produced when apomyoglobin was digested with chymotrypsin.

Inhibitory activity of chymotryptic digest. Apomyoglobin (160 mg.) was digested with chymotrypsin as described in the Methods section. The ultrafiltrate contained about 100 mg. of peptides, which were separated from about 5 mg. of undigested apomyoglobin by elution from a column of Sephadex G-50. Fractions collected after the elution of apomyoglobin were pooled and concentrated to 8.0 ml., which contained approximately 80 mg. of peptides dissolved in 0.06 M-ammonium bicarbonate, pH 8.1. Controls, represented by (a) solvent incubated with chymotrypsin and (b) apomyoglobin with chymotrypsin previously inactivated by treatment with di-isopropyl phosphorofluoridate at 37°, were treated in an identical manner.

The recovered solutions gave no precipitates with antiserum WF or WH; the pH values of the

mixtures were between 7.5 and 7.8. Neither of the control solutions inhibited the precipitation of metmyoglobin or apomyoglobin, but, as shown in Fig. 2, the digest inhibited the precipitation of both antigens by antiserum WH. The effect on the inhibitory activity of heating at 100° for 30 min., and of incubation at 37° for 3 hr. with chymotrypsin or the bacterial proteinase (5% of the weight of the peptides) and the subsequent addition of di-isopropyl phosphorofluoridate (0.005 ml.) is also shown in Fig. 2. It can be seen that heating caused no decrease in activity, that incubation with chymotrypsin reduced the activity and that treatment with the bacterial proteinase almost completely destroyed it. A sample of the digest which had been incubated at 37° without enzyme and treated with di-isopropyl phosphorofluoridate showed no decrease in activity (Fig. 2). Closely similar results were obtained with antiserum WF; 0.20 ml. of the digest inhibited the precipitation of metmyoglobin and apomyoglobin by 15 and 29% respectively. Treatment with chymotrypsin, however, destroyed almost all the activity determined with metmyoglobin, whereas the reduction in activity determined with apomyoglobin resembled that obtained with antiserum WH.

Fig. 2. Inhibition of precipitation of antiserum WH by a chymotryptic digest of apomyoglobin (○), and by the digest which had been heated at 100° (△), incubated with chymotrypsin (□), incubated with bacterial proteinase (●) and incubated without enzyme (▲). Inhibition of precipitation (a) of metmyoglobin and (b) of apomyoglobin. Experimental details are given in the text.

Fig. 3. Elution diagram of the ultrafiltrate from a chymotryptic digest of 70 μ moles of apomyoglobin on a column of Sephadex G-50 in 0.025 M- NH_4HCO_3 , pH 8.0. (a) $E_{1\text{cm.}}$ at 280 $\text{m}\mu$ (○) and at 215 $\text{m}\mu$ (Δ). (b) Inhibition of precipitation of apomyoglobin on addition of sample (0.20 ml.) to 0.50 ml. of antiserum WF.

It was concluded that the chymotryptic digest of apomyoglobin contained incompletely hydrolysed active fragments whose inhibitory activity was dependent on the peptide structure.

Purification of inhibitors. The elution diagram of the ultrafiltrate from a digest of apomyoglobin (1.2 g.) on a column of Sephadex G-50 and the inhibitory activities of the eluate fractions determined with antiserum WF are shown in Fig. 3. Undigested apomyoglobin was followed by six 215 $\text{m}\mu$ -absorbing peaks (named A to F), the first four of which had inhibitory activity; the majority of the 280 $\text{m}\mu$ -absorbing peptides were located in peaks D and F. Antiserum WH gave a similar distribution of inhibitory activity except that peak C had a greater activity and peak D a much lower activity relative to peaks A and B than those measured with WF. Fractions corresponding to each peak were pooled and concentrated; the yields of fractions A, B, C and D were about 55, 40, 215 and 165 mg. respectively. Samples (0.10 ml.) of A, B, C and D gave inhibitions of 17, 15, 29 and 25% respectively, determined with apomyoglobin and antiserum WF. A pool (0.10 ml.) of equal volumes of A, B, C and D gave 24% inhibition whereas 28%

Fig. 4. Chromatography of fraction A on a column of DEAE-Sephadex with gradient elution from 0.01 to 0.15 M- NH_4HCO_3 , pH 8.0. ○, $E_{1\text{cm.}}$ at 215 $\text{m}\mu$; ●, inhibition of precipitation of apomyoglobin on addition of sample (0.20 ml.) to 0.50 ml. of antiserum WF.

Fig. 5. Chromatography of fraction B on a DEAE-Sephadex column in 0.01 M- NH_4HCO_3 , pH 8.0, followed at 270 ml. of eluate by gradient elution to 0.15 M- NH_4HCO_3 , pH 8.0.

inhibition would have been expected if the activities due to 0.025 ml. of each fraction were additive. This result may indicate that each fraction contained at least one active peptide which represented an independent antigenic site. However, it is also possible that the fractions contained peptides representing the same or part of the same site.

The elution diagrams of fractions A, B, C and D on columns of DEAE-Sephadex are shown in Figs.

Fig. 6. Elution diagram of fraction *C* on DEAE-Sephadex. The column was washed with 0.01 M-NH₄HCO₃, pH 8.0, followed at 150 ml. of eluate by gradient elution to 0.15 M-NH₄HCO₃, pH 8.0.

Fig. 7. Elution diagram of fraction *D* on DEAE-Sephadex. The column was washed with 0.01 M-NH₄HCO₃, pH 8.0, eluted from 120 ml. of eluate with a gradient to 0.15 M-NH₄HCO₃, pH 8.0, and from 600 ml. of eluate with a gradient to 0.5 M-NH₄HCO₃, pH 8.0. ○, *E*_{280 mμ}; ●, inhibition of precipitation of apomyoglobin on addition of sample (0.20 ml.) to 0.50 ml. of antiserum WF.

4, 5, 6 and 7 respectively. In *B* and *C* the eluate fractions were not tested for inhibitory activity owing to lack of antiserum. Fraction *A* (Fig. 4) gave two sharp 215 mμ-absorbing peaks (*A2* and *A4*), both of which were active; the activity of *A2* relative to its 215 mμ absorption was greater than that of *A4*. Fraction *B* (Fig. 5) gave one major peak (*B1*) and a number of minor peaks, one of which (*B2*) could not be differentiated from fraction *A4* by electrophoresis. It was concluded that peak *B* eluted from Sephadex G-50 contained some of

Table 1. *Yields and amino acid compositions of peptides isolated from a chymotryptic digest of 70 μmoles of apomyoglobin*
Experimental details are given in the text. Tryptophan was determined from the ultraviolet-absorption spectrum of the peptide.

Peptide	Amino acid content (moles/mole of peptide)																	No. of residues	Concn. of peptide soln. † (μmoles/ml.)	Yield ‡ (μmoles)
	Lys	His	Arg	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Met	Ile	Leu	Tyr	Phe	Trp			
A2	1.74	1.29	0.79	2.01	0.27	0.32	2.31	—	1.94	3.19	1.92	0.11	1.68	1.98	0.11	0.71	—	(19)*	0.71	2.2
A4	1.01	1.02	—	1.97	0.06	0.09	2.06	—	2.00	2.96	1.99	0.03	0.98	1.04	—	0.03	—	15	2.78	7.2
B1	2.77	0.96	—	1.02	1.02	1.06	1.04	—	1.01	1.05	2.00	—	—	1.97	—	—	—	14	3.24	12.2
C1a	3.96	2.01	—	—	—	—	1.93	1.00	1.00	1.01	—	—	—	2.00	—	—	—	13	2.30	6.7
C2	0.08	0.08	—	0.04	0.98	0.04	0.15	—	0.99	2.00	0.12	—	0.99	2.09	—	0.04	—	7	3.56	12.4
D1b	2.00	0.02	0.99	1.05	0.02	0.02	0.05	—	0.05	1.98	0.02	—	1.02	0.02	0.93	—	—	8	2.28	5.9
D2	1.07	0.06	0.04	0.08	—	0.05	1.99	—	1.93	0.08	—	—	1.02	0.96	0.11	—	—	7	3.67	9.9
D3	—	—	—	—	—	0.99	1.94	—	1.03	—	1.00	—	—	1.00	—	—	1.01	7	3.73	10.4

* See Table 2.
† Calculated from the amino acid analysis of a known volume of the peptide solution.
‡ Total amount of peptide recovered.

peptide A4. Fraction B2, but not B1, inhibited the precipitation of apomyoglobin. The activity of B2 was, however, insufficient to account for that of fraction B. Fraction C (Fig. 6) gave four 215 m μ -absorbing peaks (C1–C4), of which only C2 appeared to be pure. The majority of the inhibitory activity of fraction C was associated with fractions C3 and C4; these fractions have not been purified further. The first peak (C1) was purified by fractionation on CM-Sephadex; the column was washed with 0.01 M-phosphate buffer–0.15 M-sodium chloride, pH 6.5, and eluted with a gradient to 0.01 M-phosphate buffer–0.4 M-sodium chloride, pH 6.5. One major peak (C1a) was obtained. Fraction D (Fig. 7) gave three major 215 and 280 m μ -absorbing peaks (D1–D3), of which D1 was eluted at the front and D2 only had inhibitory activity. The first peak (D1) was purified by fractionation on CM-Sephadex. The column was eluted with 0.01 M-phosphate buffer–0.2 M-sodium chloride, pH 6.5; three peaks were distinguished, of which the second (D1b) represented the major component.

The yields of the purified peptides are given in Table 1.

Properties of peptides

Different preparations of the same peptide possessed closely similar physical, chemical and immunological properties.

Electrophoresis. Examination of A4, B1, C1a, D1b, D2 and D3 by high-voltage electrophoresis at pH 6.5 and 3.7 failed to reveal the presence in each of more than one peptide. Some tailing of A4 occurred; electrophoresis of this peptide on cellulose acetate at pH 8.9 gave one band only. High-voltage electrophoresis of A2 and C2 revealed the presence in each of a minor impurity.

Amino acid analysis. The absorption spectrum (220–320 m μ) of each peptide was determined at neutral pH and in 0.1 N-sodium hydroxide. The results indicated the presence of tyrosine in D1b

and D2, phenylalanine in A2 and tryptophan in D3. The amino acid compositions of the peptides are given in Table 1. It can be seen that peptides other than A2 and C2 were essentially pure. Peptide A2 would appear to be contaminated with another which contains a relatively high concentration of lysine.

N-Terminal amino acid sequence. The sequence of the first two N-terminal amino acids was determined for each peptide. The results were in complete agreement with the amino acid sequences given in Table 2. In the majority of cases yields of between 90 and 100% of the phenylthiohydantoin-amino acids were obtained. However, the arginine derivative from peptide D1b was obtained in only 40% yield and in this case arginine was shown to be N-terminal by amino acid analysis of the residual peptide. Chromatographic analysis of the derivative of the N-terminal amino acid of A2 revealed the presence of the phenylthiohydantoin of lysine as well as that of alanine; the alanine derivative represented the major component. It was concluded that A2 is impure and that the peptide with the sequence given in Table 2 is contaminated with a minor amount of another peptide which has lysine as its N-terminal amino acid.

Amino acid sequence of peptides. The amino acid sequences of the peptides were determined by comparing their compositions and N-terminal amino acid sequences with the sequence of myoglobin (A. B. Edmundson, personal communication). The sequences and the molecular weights of the peptides are presented in Table 2. An examination of the sequences of the peptides obtained after the complete digestion of apomyoglobin with chymotrypsin (A. B. Edmundson, personal communication) indicated that peptides A2, B1, C1a and D2 represented partial digestion products (Table 2).

Immunological activity. The pH values of the solutions of peptides A2, A4, B1, C2, D2 and D3 were between 7.8 and 8.2. The conductivities of the

Table 2. *Amino acid sequences of peptides isolated from a chymotryptic digest of apomyoglobin*

Peptide	Amino acid sequence	Mol. wt. †
A2	Ala-Lys-Val-Glu-Ala-Asp-Val-Ala-Gly-His-Gly-Glu-Asp-Ile-Leu*-Ile-Arg-Leu-Phe	2051
A4	Ala-Lys-Val-Glu-Ala-Asp-Val-Ala-Gly-His-Gly-Glu-Asp-Ile-Leu	1522
B1	Lys-Ala-Ser-Glu-Asp-Leu*-Lys-Lys-His-Gly-Val-Thr-Val-Leu	1523
C1a	Lys-Lys-Lys-Gly-His-His-Glu-Ala-Glu-Leu*-Lys-Pro-Leu	1513
C2	Thr-Ala-Leu-Gly-Ala-Ile-Leu	657
D1b	Arg-Lys-Asp-Ile-Ala-Ala-Lys-Tyr	963
D2	Lys-Glu-Leu-Gly-Tyr*-[Glu(NH ₂),Gly]	793
D3	Val-Leu-Ser-Glu-Gly-Glu-Trp	818

* Bonds hydrolysed by chymotrypsin (A. B. Edmundson, personal communication).

† Calculated from the amino acid composition of the peptide.

solutions were less than that of 0.3 M-ammonium bicarbonate, pH 8.2, 0.20 ml. of which failed to cause any detectable reduction in the amount of precipitate formed by apomyoglobin or metmyoglobin with 0.50 ml. of antiserum WF or WH.

No peptide gave detectable amounts of precipitate with either antiserum. The capacity of each peptide to inhibit the precipitation of apomyoglobin was determined by adding increasing volumes of peptide solution, up to a maximum of 0.20 ml., to 0.50 ml. of antiserum. In some cases the inhibition of precipitation of metmyoglobin by antiserum WF was also measured. The results of the titration of the inhibitory activity of peptide D2 are shown in Fig. 8. The maximum inhibitions caused by the peptides are given in Table 3 and the molar ratios of the inhibitors to antigen at half of the maximum inhibition which were calculated from the titration curves are presented in Table 4.

As was expected, different peptides caused different maximal inhibitions. Some peptides, such as D3, failed to give any detectable inhibition, even though as much as 0.75 μ mole of the peptide was added to the antiserum. Peptide D1b gave a low value (5%) for the maximum inhibition. This value was obtained on more than one occasion and was greater than the experimental error of the method (equivalent to about 2% inhibition). It was concluded that this peptide was active. As can be seen from Table 3, the inhibitory activities of the peptides depended on the antigen and

antiserum used to measure the activity. In those instances where measurements were made, peptides which inhibited the precipitation of apomyoglobin also inhibited the precipitation of metmyoglobin. On the other hand, one peptide (B1) inhibited the precipitation of the latter antigen but not the former. Also, whereas antiserum WH was inhibited by peptides C1a and C2, no inhibition was detected with antiserum WF; a much lower concentration of peptide D2 was also required to give maximal inhibition with antiserum WF than with WH. These results are in agreement with the relative activities, measured with WF and WH, of peaks C and D eluted from Sephadex G-50.

Different active peptides may represent different antigenic sites, or the same or parts of the same site. If two peptides represent the same site then a mixture, containing an amount of each peptide

Table 3. Maximum inhibition of precipitation of antigen-antibody complex caused by the peptides isolated from a chymotryptic digest of apomyoglobin

Peptide	Maximum inhibition (%) of precipitation of antigen-antibody complex		
	Antiserum WF		Antiserum WH
	With apomyoglobin	With metmyoglobin	With apomyoglobin
A2	12		
A4	12	8	9
B1	0	8	0
C1a	0		9
C2	0		> 7
D1b	5		
D2	15	7	> 6
D3	0	0	0

Fig. 8. Inhibition of precipitation of apomyoglobin with antiserum WF by peptide D2 (○), and by peptide D2 which had been incubated with chymotrypsin (△), incubated with bacterial proteinase (□) and incubated without enzyme (●). Experimental details are given in the text.

Table 4. Molar ratios of inhibitors to antigen

Inhibitor	Inhibitor:antigen molar ratio for 50% of maximum inhibition		
	Antiserum WF		Antiserum WH
	With apomyoglobin	With metmyoglobin	With apomyoglobin
A2	12		
A4	50	60	65
B1	—	145	—
C1a	—		150
C2	—		> 290
D1b	70		
D2	80	70	> 300

greater than that necessary for maximum inhibition, should give the same inhibition as that caused by either peptide alone. The amino acid sequence of peptide *A2* overlapped that of *A4* and both peptides gave the same maximum inhibition (12%). A mixture of amounts of *A2* and *A4* (0.07 and 0.28 μ mole respectively), which individually gave maximal inhibition, caused an inhibition of 12% only. It was concluded that these peptides represent the same antigenic site. On the other hand, if two peptides represent different antigenic sites then the inhibitory activity of a mixture should be equal to the sum of the activities of the individual peptides. To an amount (0.28 μ mole) of *A4* which caused maximal inhibition (12%) was added an amount (0.37 μ mole) of *D2* which gave an inhibition (13%) close to the maximum value of 15%. This mixture caused an inhibition of 18% whereas 25% would have been expected if the activities of the peptides had been additive. The activity of the mixture was, however, significantly greater than that of *A4*, but it was only slightly greater than the maximal inhibition given by *D2*. This result may indicate that *D2* and *A4* represent different antigenic sites but further experiments will be necessary to prove this. In view, however, of the relative positions occupied by these peptides in the Kendrew model of the myoglobin molecule (see the Discussion section) it is considered unlikely that they do not represent different antigenic sites.

If the activity of the peptides is due to their peptide structure, then digestion of the peptide with a non-specific protease should destroy the activity. Each of the active peptides was incubated at 37° for 2 hr. with 5% of their weight of the bacterial proteinase; di-isopropyl phosphorofluoridate (0.005 ml.) was then added. In each case at least 90% of the inhibitory activity was destroyed. Controls, represented by peptide treated in an identical manner, except that no enzyme was added, showed no decrease in activity. The results obtained with peptide *D2* are shown in Fig. 8. Peptide *D2* is hydrolysed by chymotrypsin (A. B. Edmundson, personal communication). This peptide was incubated at 37° for 2 hr. with 5% of its weight of chymotrypsin, was treated with di-isopropyl phosphorofluoridate (0.005 ml.) and the activity of the digest measured. The results (Fig. 8) indicated that more than half of the activity was destroyed. High-voltage electrophoresis of the digest at pH 3.7 and 6.5 showed the presence of material with the same mobility as that of the undigested peptide together with another peptide of different mobility. It is not known whether the residual activity of the digest was due to the presence of some undigested material or to the pentapeptide and/or dipeptide which were formed.

DISCUSSION

The results suggest that digestion of apomyoglobin with chymotrypsin under conditions which favoured the selection of partial digestion products gave rise to peptides which combined with antibodies to metmyoglobin. The number of active peptides and their capacities to inhibit the precipitation of antigen depended on the antiserum and antigen used in the inhibition test. Neither the whole digest nor any of the purified peptides inhibited completely the precipitation of antigen. On the contrary, the greatest amount of inhibition given by a purified peptide was 15% only (Table 3). However, the antisera used represented pools of serum collected from a number of different animals. As a result, in all probability they contained mixtures of antibodies directed against different antigenic sites, and different pools contained different ratios of antibodies directed against particular sites (cf. Press & Porter, 1962; Mage & Kabat, 1963a). Indeed, antiserum WH contained antibodies which combined with peptides *C1a* and *C2* but antibodies with these specificities were apparently not present in antiserum WF. Also, the maximum amount of precipitate produced by WH with apomyoglobin relative to that produced by metmyoglobin was greater than the ratio of the maximal amounts of precipitate formed by WF with apomyoglobin and metmyoglobin. The difference in behaviour of apomyoglobin compared with metmyoglobin in the quantitative precipitin test is believed to be due to the change in molecular shape which occurs when the haem group is removed from metmyoglobin (M. J. Crumpton & A. Polson, unpublished work). Examination of a number of different antisera suggested that antibodies produced early in immunization were inhibited to a greater degree than those produced in the same animal after a longer period of immunization. It is not known whether this was due to a change in the avidities of the antibodies (Grey, 1964), or to a change in the relative amounts of antibodies with different specificities.

The chymotryptic digest appeared to contain at least four peptides with different serological specificities. On the other hand, the maximum number of molecules of antibody bound by one molecule of antigen in the presence of a gross excess of antibody was close to three. The latter figure is sometimes referred to as the number of antigenic sites. In all probability this figure is not a measure of the total number of sites but it may represent the maximum number of molecules of antibody which can combine with and fit into the available space around a molecule of antigen.

Some of the peptides present in the digest were inactive. A peptide may be inactive because its

spatial configuration is different from that which it possessed when it was part of the whole molecule. Alternatively, the antiserum used may lack antibodies directed to this portion of the whole molecule. The survival of the original configuration will depend on the number of amino acid residues in the peptide and on whether the structure depended on the formation of non-covalent bonds with other parts of the whole molecule. As the activity of the initial digest was unaffected by heating at 100° for 30 min. it seems likely that the steric structures of the peptides were determined by their sequences and were recovered on cooling. It is, however, possible that the configurations of the peptides were changing continuously and that combination with specific antibody occurred when the peptide assumed its native form. Examination of the Kendrew model of the myoglobin molecule showed that the active heptapeptide *D2* represents the *C*-terminus, the last four amino acids of which are non-helical, that the inactive heptapeptide *D3* represents the *N*-terminus, which is helical, and that the active heptapeptide *C2* and the active octapeptide *D1b* are included in helical regions. The reason why *C2* is active is not understood; this peptide would be expected to be inactive if, as has been suggested (Doty & Lundberg, 1956), an octapeptide is the smallest peptide able to form a stable helical configuration.

Peptide *B1* inhibited the precipitation of metmyoglobin but was inactive when apomyoglobin was used as the antigen in the inhibition test. Examination of the Kendrew model of myoglobin showed that the histidine residue of this peptide is hydrogen-bonded to the molecule of water which is co-ordinated to the iron atom of the haem group. If this non-covalent bond stabilizes the configuration of this region of the metmyoglobin molecule, then it seems possible that this portion of the polypeptide chain has a different configuration in apomyoglobin (cf. Breslow, 1964). In this case the antibody with which *B1* combined may not react with apomyoglobin.

In general, the molar ratio of inhibitor to antigen at 50% of the maximum inhibition decreased as the molecular size of the inhibitor increased. For example, the molar ratio, determined with antiserum WF and apomyoglobin, decreased from 80 for a peptide (*D2*) of molecular weight 793 to 12 for peptide *A2* which had a molecular weight 2051. Increase in inhibitory power with increase in molecular size has also been shown for a series of oligosaccharides of increasing chain length from pneumococci type 3 polysaccharide (Mage & Kabat, 1963b). The above values were 10- to 100-fold as great as that for a peptide of molecular weight 7000 from human serum albumin (Press & Porter, 1962) but were much smaller than the

values (1000–3000) for haptens (Beiser, Burke & Tanenbaum, 1960) and for peptides of low molecular weight from silk fibroin (Cebra, 1961). The results obtained with the peptides from myoglobin appear to support the suggestion (Press & Porter, 1962) that the smaller, less inhibitory peptides form only part of the antigenic site which, consequently, may be of appreciable size. However, although this interpretation may be correct in some instances, the following evidence suggests that it is not true in every case. Peptide *A2* has a greater molecular size and a greater inhibitory power than *A4*; both peptides appear to possess the same serological specificity. If the above interpretation was correct, then *A2* should represent more of the antigenic site than *A4*. It is, however, unlikely that the extra four amino acids of *A2* form part of the antigenic site since examination of the Kendrew model of myoglobin showed that they are masked in the whole molecule. These results suggest that an antigenic site may have a relatively small size and that the larger the peptide the more closely the spatial configuration of the site corresponds to that in the whole molecule.

At present, owing to the limited number of inactive and active peptides available, it is not possible to judge whether the inhibitors possess a common structural feature which would account for antibodies being formed preferentially against these regions of the myoglobin molecule. It may, however, be significant that *A2* and *A4*, *B1*, *C1a* and *C2* are adjacent to or include corners of the folded polypeptide chain. With regard to the elucidation of the structure of an antigenic site the heptapeptide *D2* is potentially interesting. Digestion of peptide *D2* with trypsin should give the *C*-terminal hexapeptide of myoglobin and digestion with chymotrypsin should give a pentapeptide and the *C*-terminal dipeptide. A comparison of the inhibitory activities of these peptides would be of interest. However, these experiments must await the production of another antiserum with which peptide *D2* will combine.

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