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The *C*-Terminal Antigenic Site of Sperm-Whale Myoglobin: the Immunological Activities of Synthetic Peptides Related to the *C*-Terminus of Myoglobin

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Previous attempts to define the structure of the *C*-terminal antigenic site of sperm-whale myoglobin have relied on peptides obtained by degradation. Enzymic cleavage of the immunologically active *C*-terminal heptapeptide

-Lys-Glu-Leu-Gly-Tyr-Gln-Gly

suggested that the site was no larger than the *C*-terminal hexapeptide, and that it included the leucylglycyl sequence and the *C*-terminal glycine and/or glutamine residues (Crumpton, 1967). The more precise definition of this antigenic site required peptides that differed in both size and sequence from those obtained by enzymic cleavage. This communication describes the synthesis and immunological activities of the *C*-terminal hepta-, hexa-, penta- and tetra-peptides, and of the hepta-, hexa- and penta-peptides in which the tyrosine residue is replaced by phenylalanine or *p*-methoxyphenylalanine.

Experimental and results. The synthetic protected *C*-terminal pentapeptide was formed by coupling *o*-nitrophenylsulphenyl-L-leucylglycine to *O*-benzyl-L-tyrosyl-L-glutaminylglycine *p*-nitrobenzyl ester. Active esters were used in the synthesis of the above fragments, and *NN'*-dicyclohexylcarbodi-imide in the coupling reaction. The *N*-terminal residues of the heptapeptide were added stepwise to the *O*-benzyl-pentapeptide *p*-nitrobenzyl ester by using the *N*-hydroxysuccinimide esters of *o*-nitrophenylsulphenyl-γ-benzyl-L-glutamate and N^{α}-(*o*-nitrophenylsulphenyl)-N^{ϵ}-benzyloxycarbonyl-L-lysine respectively. *o*-Nitrophenylsulphenyl protecting groups were removed at each stage with ethereal HCl. The protected *C*-terminal tetrapeptide was prepared by coupling *N*-benzyloxycarbonylglycine *N*-hydroxysuccinimide ester to the *O*-benzyl-tripeptide *p*-nitrobenzyl ester. L-Phenylalanine and *p*-methoxy-L-phenylalanine analogues were obtained by incorporating these residues into the *C*-terminal tripeptide. In all syntheses the protecting groups were finally removed by hydrogenolysis and the free peptides were purified by elution from a column (150cm × 2.8cm) of Sephadex G-25 with 20mM-NH_4HCO_3, pH8.0. The heptapeptide and its *p*-methoxyphenylalanine analogue were further purified by elution from DEAE-Sephadex as for the natural heptapeptide (peptide *D*2; Crumpton & Wilkinson, 1965). The natural *C*-terminal hepta- and hexa-peptides were prepared as described by Crumpton (1967).

No impurities were detected in the peptides by either paper chromatography in butanol–acetic acid–water (4:1:5, by vol.) and butan-1-ol–pyridine–acetic acid–water (45:35:9:36, by vol.), or high-voltage electrophoresis at pH6.5; peptides were detected with ninhydrin and hypochlorite starch–iodide (Pan & Dutcher, 1956). Natural and synthetic peptides (hepta- or hexa-peptides) occupied identical positions after chromatography and electrophoresis, and the electrophoretic mobilities of the peptides agreed with those predicted from their sizes and compositions (Offord, 1966).

Amino acid analyses were performed by using an automatic analyser (Benson & Patterson, 1965). Peptides were hydrolysed with HCl as described by Crumpton & Wilkinson (1963). However, as *p*-methoxyphenylalanine is converted into tyrosine under these conditions, peptides containing *p*-methoxyphenylalanine were also hydrolysed with 1.8M-H_2SO_4 for 24h at 130°C (Law & du Vigneaud, 1960). Analysis of these hydrolysates gave a peak in a position identical with that of an authentic sample of *p*-methoxy-L-phenylalanine (97min after norleucine with a 50cm column and a buffer flow of 50ml/h). Peptides (0.1μmol in 1ml of 0.1M-NH_4HCO_3, pH7.5) were also hydrolysed with 50μg of aminopeptidase M (Pfleiderer & Celliers, 1963) for 24h at 23°C. The compositions of the above hydrolysates agreed closely with the predicated values and no impurities were detected.

Immunological activity was measured by competitive inhibition of precipitation (Crumpton & Wilkinson, 1965) by using apomyoglobin as the antigen and anti-(metmyoglobin) serum WD; the experimental error of the method was equivalent to ±1% inhibition. The inhibitory activities of the peptides are shown in Fig. 1. The activities of the synthetic and natural hepta- and hexa-peptides were not significantly different. In contrast, the synthetic pentapeptide was less active and the synthetic tetrapeptide was much less active than the hepta- and hexa-peptides. Further, the replacement of the tyrosine residue of the hepta-, hexa- and

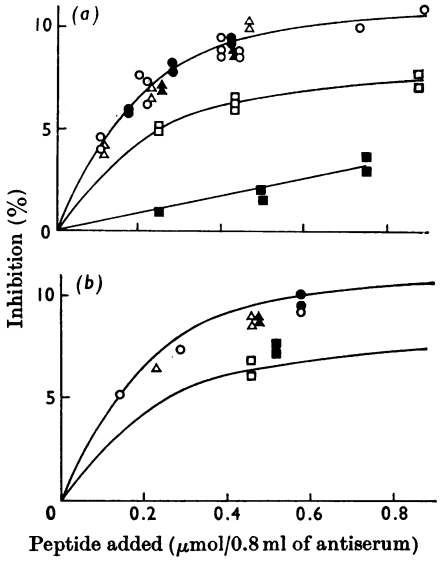

Fig. 1. Inhibition of precipitation of 18.2μg of sperm-whale apomyoglobin with 0.8ml of an antiserum to sperm-whale metmyoglobin (WD) by increasing amounts (dissolved in 0.2ml) of natural and synthetic *C*-terminal peptides of myoglobin, and of synthetic peptides in which the tyrosine residue was replaced by a phenylalanine or a *p*-methoxyphenylalanine residue. The washed precipitates were dissolved in 0.5ml of 0.1M-NaOH and the extinctions measured at 280nm in a 1cm cell. The decrease in extinction due to the inhibitor was expressed as a percentage of the extinction obtained in the absence of any inhibition. (*a*) Natural heptapeptide (○), synthetic heptapeptide (●), natural hexapeptide (△), synthetic hexapeptide (▲), synthetic pentapeptide (□) and synthetic tetrapeptide (■). (*b*) Phenylalanine analogues of the heptapeptide (○), hexapeptide (△) and pentapeptide (□), and *p*-methoxyphenylalanine analogues of the heptapeptide (●), hexapeptide (▲) and pentapeptide (■). The curves shown are taken from Fig. 1(*a*).

penta-peptides by phenylalanine or *p*-methoxyphenylalanine gave no significant change in activity relative to that of the respective tyrosine-containing peptide.

Discussion. The results revealed that the *N*-terminal glutamic acid and leucine residues of the hexapeptide form part of the *C*-terminal antigenic site detected by antiserum WD, and confirmed that the *N*-terminal lysine residue of the heptapeptide does not interact with the antibodies to this site. A comparison of the activities of the hepta-, hexa-, penta- and tetra-peptides suggested that the leucine residue represents the immunodominant group of the site (cf. Kabat, 1966). In this case, since the leucine is situated at a corner in myoglobin (Kendrew *et al.* 1961), it appeared that the antibody was not only formed preferentially against the corner area (Crumpton, 1968), but was also directed primarily against the corner residue. The slight activity of the tetrapeptide (cf. Crumpton, 1967) supported the previous suggestion that the *C*-terminal glycine and/or glutamine residues are included in the antigenic site (cf. Goodman, 1969), especially since the tyrosine residue probably does not interact with antibody. It is concluded that the *C*-terminal antigenic site of myoglobin is no smaller and may be no larger than the hexapeptide.

A comparison of the activities of the tyrosine, phenylalanine and *p*-methoxyphenylalanine analogues indicated that the tyrosine hydroxyl group does not interact with antibody, and that replacement of the hydrogen atom by a methyl group does not hinder peptide–antibody interaction. Further, since the tyrosine phenyl ring is not exposed on the surface of myoglobin (Kendrew *et al.* 1961), the tyrosine side chain probably does not interact with antibody. The tyrosine residue may, however, still represent an essential part of the site if it is required to orientate the hexapeptide so that interaction with antibody can occur. This interpretation is not inconsistent with the observation (Atassi, 1968) that nitration of the tyrosine residues of the *C*-terminal 22-peptide of myoglobin destroyed the inhibitory activity of the peptide. In this case it seems likely that, owing to lack of space around the *C*-terminal tyrosine in myoglobin (Kendrew *et al.* 1961), the addition of nitro groups would prevent the *C*-terminal hexapeptide from assuming a conformation complementary to that of the antibody site. In view of the proximity in myoglobin (Kendrew *et al.* 1961) of the tyrosine hydroxyl group and a methyl group of the antigenically important leucine residue, it is of note that the methyl group of the *p*-methoxyphenylalanine analogues did not decrease peptide–antibody interaction.

The similar inhibitory activities of the tyrosine and phenylalanine analogues are noteworthy from another point of view. It is known that horse myoglobin cross-reacts with anti-(sperm-whale myoglobin) sera (M. J. Crumpton, unpublished work), and that the *C*-terminus of horse myoglobin is identical with that of sperm-whale myoglobin except for the replacement of the tyrosine by a phenylalanine residue (Dautrevaux, Boulanger, Han, Moschetto & Biserte, 1967). The present results suggest that antibodies to the *C*-terminal antigenic site of sperm-whale myoglobin would react equally well with the *C*-terminus of horse myoglobin, provided that the sites have identical conformations in each myoglobin.

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Atassi, M. Z. (1968). *Biochemistry, Easton*, **7**, 3078.
Benson, J. V. & Patterson, J. A. (1965). *Analyt. Chem.* **37**, 1108.
Crumpton, M. J. (1967). *Nature, Lond.*, **215**, 17.
Crumpton, M. J. (1968). *Biochem. J.* **108**, 2P.
Crumpton, M. J. & Wilkinson, J. M. (1963). *Biochem. J.* **88**, 228.
Crumpton, M. J. & Wilkinson, J. M. (1965). *Biochem. J.* **94**, 545.
Dautrevaux, M., Boulanger, Y., Han, K. K., Moschetto, Y. & Biserte, G. (1967). *Bull. Soc. Chim. biol.* **49**, 1409.
Goodman, J. W. (1969). *Immunochemistry*, **6**, 139.
Kabat, E. A. (1966). *J. Immun.* **97**, 1.
Kendrew, J. C., Watson, H. C., Strandberg, B. E., Dickerson, R. E., Phillips, D. C. & Shore, V. C. (1961). *Nature, Lond.*, **190**, 666.
Law, H. D. & du Vigneaud, V. (1960). *J. Am. chem. Soc.* **82**, 4579.
Offord, R. E. (1966). *Nature, Lond.*, **211**, 591.
Pan, S. C. & Dutcher, J. D. (1956). *Analyt. Chem.* **28**, 836.
Pfleiderer, G. & Celliers, P. G. (1963). *Biochem. Z.* **339**, 186.