A METHOD FOR PURIFYING METHIONINE-CONTAINING PEPTIDES BY RADIOACTIVE LABELLING

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1. Introduction

In the determination of the primary structure of proteins considerable use is made of the method of selective chemical cleavage of polypeptide chains at methionine residues [1]. This has the advantage in most cases of giving a small number of large fragments which may be purified and are amenable to sequence analysis by normal methods. The problem of arranging the fragments in their original order remains, however, and methods of specifically purifying the overlapping peptides containing methionine would be of considerable value. Such a method has recently been described by Tang and Hartley [2] who made use of a diagonal method based on the specific alkylation of methionyl residues with iodoacetamide at low pH [3] to give a positively charged sulphonium salt and hence a net change in the charge of the peptide. After purification the peptides containing CM-methionine* residues were cleaved into two fragments by conversion of the CM-methionine into homoserine [2]. This method requires the enzymic digestion to precede the alkylation reaction and would seem to limit its usefulness to tryptic digests since peptide bonds C-terminal to methionine residues are susceptible to chymotrypsin [4]. It seemed, therefore, that it might be an advantage to alkylate the methionine residues in the intact protein as the positively charged CM-methionine might be expected to be resistant to chymotryptic cleavage. Furthermore the use of radioactively labelled iodoacetamide would allow the methionine-containing peptides to be identified more easily.

In order to test the feasibility of this method the sequences around the two methionine residues in sperm whale myoglobin [5] were investigated.

2. Materials and methods

Apomyoglobin was prepared from chromatographically purified sperm whale metmyoglobin as described previously [6]. Iodo (1-^{14}C) acetamide (sp. act. 17.2 mC/mM) was obtained from the Radiochemical Centre (Amersham, Bucks). Amino acid analyses were performed with a Locarte amino acid analyser using a 25 cm column eluted stepwise with sodium citrate buffers of pH 3.25, 4.25 and 6.65 from which the basic as well as acidic and neutral amino acids are eluted. Autoradiography of the CM-methionine-containing peptides after separation by high voltage electrophoresis was carried out overnight using Kodak "Blue Brand" medical X-ray film. Other methods used were as described previously [7].

3. Results and discussion

Apomyoglobin was dissolved in 6 M urea 0.2 M sodium formate buffer, pH 3.5, at a concentration of 20 mg/ml. An aqueous solution of iodo (1-^{14}C) acetamide was added to give a concentration of 0.5 mole/mole of methionine, and the mixture was incubated at 37° for 48 hr. After this period an aqueous solution of cold iodoacetamide was added to give a 10-fold

* CM-methionine = S-carboxamidomethylmethionine.

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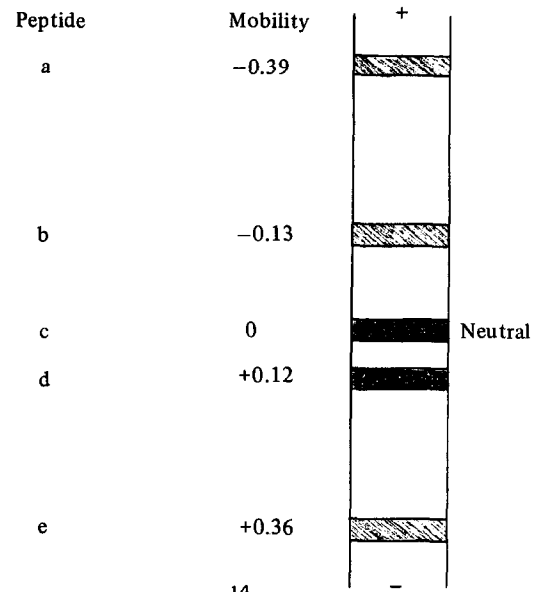

Fig. 1. Autoradiograph of ^{14}C-CM-methionine peptides from a tryptic/chymotryptic digest of myoglobin after electrophoresis at pH 6.5.

molar excess over methionine and incubated for a further 24 hr at 37°. Excess reagent was removed by dialysis against water and the protein was finally dialysed against 1% ammonium bicarbonate, at which point it was insoluble.

It is possible, although unlikely, that lysine and histidine residues might also be alkylated during the reaction. In order to check this point samples of myoglobin were analysed for the basic amino acids before and after alkylation; there was no difference, however, in the ratio of lysine and histidine to arginine.

The alkylated myoglobin was digested for 5 hr at 37° with a mixture of 2% (by weight) of trypsin and 2% (by weight) of chymotrypsin. After a short while the solution became clear, and on completion of the digestion it was applied to a column (1.7 × 145 cm) on Sephadex G50 and eluted with 0.05 M ammonia. One major radioactive peak was present in the chromatogram and this was pooled, freeze-dried and subjected to high voltage electrophoresis at pH 6.5.

Autoradiography showed a pattern of five radioactive bands as shown in fig. 1, two of the bands, *c* and *d*, were very much more intense than the other three. The bands were cut out, sewn to fresh sheets of paper, and purified by electrophoresis at pH 3.5. the purity being ascertained by staining guide strips with ninhydrin. The purified peptides were eluted, analysed and their N-terminal amino acids determined by the "dansyl" method. The results, together with their mobility at pH 6.5 are shown in table 1. No attempt was made to convert the CM-methionine to homoserine [2] before analysis and consequently peaks were observed in the positions of homoserine and methionine with a third being eluted between proline and glycine, which is probably S-carboxymethylhomocysteine [8].

The sequences of the peptides were ascertained by comparing the data presented in table 1 with the published sequence of myoglobin [5], and these sequences are shown in table 2. All five are sequences around one or other of the methionine residues and it is clear that there has been no chymotryptic cleavage C-terminal to the CM-methionine residues. Peptides *a* and *c* contain the first methionine residue while peptides *b*, *d* and *e* contain the second methionine residue; peptides *b* and *d* are idential in composition and their difference in mobility may be due to deamidation of glutamine and asparagine residues, whether this took place during the modification reaction or is due to heterogeneity in the myoglobin [9] is uncertain.

The specific activities of the purified peptides were in good agreement with each other and it is clear that the modification reaction, under the conditions used, is specific for methionine residues and gives an equal degree of labelling on each residue. Iodoacetamide was used for alkylation as it seemed advantageous for subsequent purification steps to introduce an extra positive charge, but there is no reason why iodoacetic acid should not be used if a neutral substituent is desired. As no other reactive group is present during the reaction the radioactive label is incorporated exclusively into the protein and thus preparations with a high specific activity may be obtained, enabling the methionine-containing peptides to be easily identified and isolated. This method should provide a useful alternative to that of Tang and Hartley [2].

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Table 1
Amino acid compositions of the radioactive peptides isolated from a tryptic/chymotryptic digest of myoglobin, alkylated at the methionine residues with iodo 1-^{14}C acetamide.

	Composition (moles of amino acid/mole peptide)				
Amino acid	*a*	*b*	*c*	*d*	*e*
His	–	0.9	–	0.9	–
Lys	2.2	0.7	1.2	0.9	1.1
Asp	1.2	3.0	–	3.1	2.1
Thr	1.0	–	0.8	–	–
Ser	0.8	–	–	–	–
Glu	3.6	1.1	2.0	1.1	1.1
Pro	–	0.9	–	1.0	–
Gly	–	3.0	–	2.9	1.6
Ala	2.0	2.9	1.0	2.9	2.8
Leu	0.9	–	–	–	–
Phe	–	1.0	–	0.9	–
N-terminal amino acid	Thr	His	Thr	His	Gly
Mobility at pH 6.5	−0.39	−0.13	0	+0.12	+0.36
Yield	2.7%	1.8%	19%	16%	1.6%

Mobility was measured relative to aspartic acid = −1. Yields were calculated from the recoveries of the peptides after final purification.

Table 2
Amino acid sequences of the radioactive methionine peptides of table 1.

Peptide	
a	Thr-Glu-Ala-Glu-CMMet-Lys-Ala-Ser-Glu-Asp-Leu-Lys
c	Thr-Glu-Ala-Glu-CMMet-Lys
b and *d*	His-Pro-Gly-Asn-Phe-Gly-Ala-Asp-Ala-Gln-Gly-Ala-CMMet-Asn-Lys
e	Gly-Ala-Asp-Ala-Gln-Gly-Ala-CMMet-Asn-Lys

The sequences are taken from ref. [5].

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