the aqueous layer with a 3:1 mixture of chloroform and isopropyl alcohol which was evaporated. The residue was dissolved in a small volume of methanol and on addition of water a precipitate, 1.6 g, was obtained. After two recrystallizations from benzene the compound, 1.3 g, had m.p. 149.5°–150°. (Found: C 76.17; H 6.58; N 8.09. Calc. for $C_{11}H_{11}NO$: C 76.25; H 6.42; N 8.19).

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The C-Terminal Amino Acids of Three Variants of Horse Myoglobin

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In previous communications [1,2] the isolation and analysis of three different horse myoglobins have been reported. The almost identical analytical data for the three components, including the amino acid composition, indicated this to be a case of microheterogeneity, where the difference between the three components consists of an exchange of one amino acid for another or a different distribution of amino acids or charged groups within the molecule. As myoglobin has only one N-terminal amino-acid group [3,4], it is fairly certain to be built up of a single polypeptide chain and identification of the end groups in the three variants would in this case be of singular interest. The N-terminal amino acid has been determined in all three myoglobins and found to be the same [3], while the C-terminal amino acids have been determined only in unseparated horse myoglobin, where one of the components accounts for about 80 % of the material. By following the rate of liberation of amino acids from the apomyoglobin on hydrolysis with carboxypeptidase A the sequence of four C-terminal amino acids was determined [5]. This paper reports the determination of four C-terminal amino acids in the three different myoglobins, using the same method, but with a somewhat different result.

Experimental. The three myoglobin components, designated MbI, II_1 and II_2, were prepared as described previously.[2] Carboxy peptidase A * (CP A) was a commercial product and was treated with diisopropylfluorophosphate (DFP) before use. As the rate of action of CP A on the native as well as denatured myoglobins is extremely slow, the apoproteins were used for the degradation experiments. The latter were prepared by splitting the myoglobins with acid acetone in the cold as described earlier [1]. The native apoproteins, however, proved almost as resistant to attack by the enzyme as unsplit myoglobin. To render them accessible to carboxypeptidase action, the apoproteins were denatured by heating in saltfree solution at pH 8.5 and 90°C for 4 min. Under these conditions a uniform suspension of a finely divided precipitate was obtained, which did not aggregate visibly during the time of hydrolysis. The DFP-treated CP A was dissolved in a 10 % LiCl solution immediately before use.

The hydrolyses were carried out in an unbuffered medium at 37°C and pH 8.5 under continuous stirring. The weight ratio of enzyme to apoprotein was 1:20. The pH was kept constant by addition of 0.1 M NaOH and at suitable intervals 1 ml samples were withdrawn, transferred to centrifuge tubes containing 1 ml of trichloroacetic acid and immediately centrifuged. The supernatant was then analyzed for amino acids in an automatic amino acid analyzer according to Spackman *et al.* [6]. As asparagine and glutamine under these conditions appear exactly coincident with the serine peak on the chromatogram, while aspartic and glutamic acids are well resolved, it was necessary to analyze the supernatant before as well

* Carboxypeptidase, which is equivalent to carboxypeptidase A, was obtained from Worthington Biochemicals, Freehold, New Jersey, as a water suspension of three times recrystallized material.

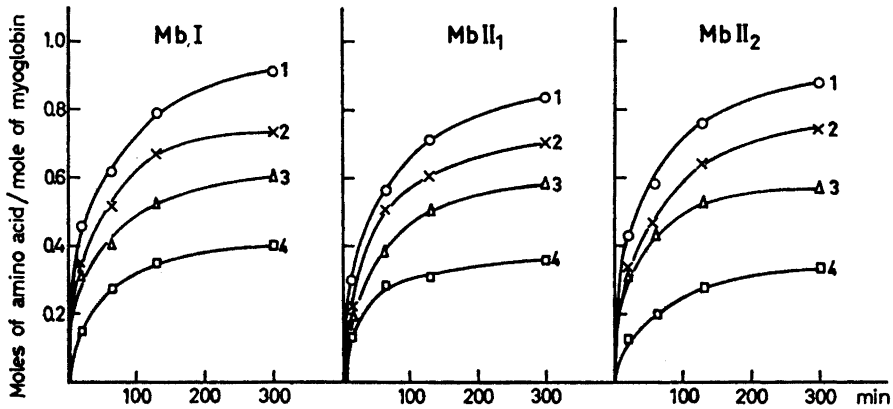

Fig. 1. Liberation of some amino acids from the apoproteins of MbI, $MbII_1$, and $MbII_2$ by carboxypeptidase A. 1 = gly, 2 = glu(NH_2), 3 = phe and 4 = leu.

as after acid hydrolysis. One aliquot was analyzed immediately while another aliquot was hydrolyzed for 3 h with 5 M HCl at 100°C in an evacuated and sealed tube. Most of the HCl was then removed by evaporation *in vacuo* and the hydrolyzed sample analyzed for amino acids as above. Samples of the three denatured apomyoglobins as well as corresponding amounts of CP A were incubated separately under the conditions used for the actual hydrolyses and analyzed for liberated amino acids the same way. In all cases only traces of a few amino acids were released even after prolonged incubation.

Results and discussions. The results of hydrolysis of the apoproteins of the three myoglobins by CP A are shown in Fig. 1.

In addition to the amino acids included in the figure, around 0.2 moles of alanine and tyrosine were released per mole of each apoprotein after 300 min. Their relative rates of release were the same. Smaller amounts of asparagine, isoleucine, threonine, valine and serine in decreasing order were liberated also from all three apomyoglobins. On prolonged hydrolysis for 36 h the molar ratios of amino acid to protein increased to approximately 1 for glycine and glutamine. The corresponding figure for phenylalanine was 0.7 and for leucine 0.6, the values being about the same for all three apoproteins. However, alanine later exceeded tyrosine, the values having increased to 0.4 and 0.3, respectively.

These results show clearly that the sequence of the six C-terminal amino acids is identical in the three myoglobin variants. The similar order of appearance in the different hydrolysates, albeit in rather small quantities, of the other amino acids found, suggests that the likeness stretches even further along the peptide chain. This identity in C-terminal amino acid sequence confirms our earlier conclusion [2] that the three myoglobin variants are distinct, preformed proteins and not artifacts produced during purification, *e.g.* by splitting off or modifying terminal amino acids.

The sequence of the four terminal amino acids can now be established with certainty and it is most probable that the next amino acid is alanine, followed by tyrosine. Investigations of the action by CP A on different peptides [7] have shown that amino acids with short aliphatic side chains are released at a much slower rate than those with an aromatic side chain. It thus seems improbable that the rates of liberation of alanine and tyrosine would be nearly the same if tyrosine was released first. The C-terminal sequence of the three myoglobins would thus be:
(Tyr-Ala-) Leu-Phe-Glu(NH_2)-Gly(COOH).
This sequence differs, however, in several respects from the one reported by Holleman and Biserte [6] for unseparated horse apomyoglobin. They found the sequence leu-asp(NH_2)-phe-gly(COOH) followed by glu-thr or thr-glu, and they evidently did not find tyrosine among the liberated amino acids, and alanine only in negligible quantities. As regards the two penultimate amino acids, the discrepancies between the

two sequences very likely arise from the fact that glutamine and not asparagine is released on the enzymic hydrolysis. Holleman and Biserte converted the liberated amino acids to their DNP-derivatives and analyzed them by paper chromatography. This method does not, however, distinguish between asparagine and glutamine, and even though they, evidently arbitrarily, chose asparagine to represent the amide in the sequence, they really left the question open. The fact that glutamine is the amide in question also explains why they found phenylalanine as the penultimate amino acid instead of glutamine. The present analyses indicated that a large part of the liberated glutamine was converted to pyrrolidone carboxylic acid during the digestion, and thus escaped detection, unless reconverted to glutamic acid by acid hydrolysis.

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