IDENTIFICATION OF THE N-TERMINAL BLOCKING GROUPS OF TROUT HEMOGLOBINS BY MASS SPECTROMETRY

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1. Introduction

Low resolution mass spectrometry is now established as a technique for peptide sequence analysis [1]. In addition, the method is uniquely useful for sequence analysis of peptides with blocked N-terminal residues [2–4]. We describe here the sequence analysis of the pentapeptides from the N-termini of the α-chains of Hb trout I and Hb trout IV.

In view of the well established role of the amino-termini of the α-chains in the Bohr effect and in CO_2 transport in human HbA [5], this structural information on the Hb components from trout represents a step foreward in understanding their structure–function relationship [6].

2. Materials and methods

Peptides originating from the N-termini of the α-chains were isolated from tryptic digests of Hb trout I (carboxymethylated globin) and Hb trout IV (carboxymethylated α-chain) prepared according to conventional procedures [7]. Peptide purification by gel filtration, ion-exchange chromatography and paper techniques, peptide characterization by thin-layer analytical chromatography, amino acid analysis and carboxypeptidase digestion were performed according to procedures described [8].

Peptides (0.7–1.0 μmol) were acetylated [9] and permethylated for mass spectrometry [1]. Acetylation was carried out using [$^2H_6$]acetic anhydride (99 atom%, BOC Ltd, London SW19 3UF,). Permethylation with methyl sulphinyl carbanion [10] and methyl iodide was allowed to proceed for only 2 min as in the rapid procedure [11].

Mass spectra were recorded at 70 eV using an AEI MS12 mass spectrometer over a range of source temperatures from 190–240°C.

3. Results

3.1. *N-terminus of the α-chain from Hb trout IV*

From a tryptic digest of this chain a peptide with the following composition: Lys (0.8), Ser (1.7), Ala (1.0) and Leu (0.9) was isolated in 46% yield after gel filtration and ion-exchange chromatography. The N-terminal residue was blocked, while results of digestion with carboxypeptidase C and consideration of the specificity of trypsin suggested lysine as being the C-terminal residue of the peptide and alanine the penultimate residue. Furthermore, from a chymotryptic digest of the α-chain, a peptide with the sequence Ser–Ala–Lys–Asp–Lys–Ala–Asn–Val–Lys–Ala–Ile–Trp, corresponding to the residues from 3–14, was isolated and from a thermolytic redigestion of the N-terminal CNBr-fragment a peptide

corresponding to residues from 2–9 having leucine as the N-terminal residue was also isolated. The sum of these results gave indirect evidence for the following sequence for the N-terminal tryptic peptide: *X*-Ser-Leu-Ser-Ala-Lys, where *X* represents a blocking group.

In order to confirm this sequence and determine the nature of the blocking group a sample of the chemically modified peptide was analysed by mass spectrometry. The presence of the C-terminal lysine residue made it necessary to acetylate the peptide before permethylation. The mass spectrum of the peptide observed at source temperatures between 200°C and 240°C is shown in fig.1. The point of immediate interest is the occurrence of an abundant ion at *m/e* 158. This corresponds to the derivative of serine in which the amino group carries an isotopically normal acetyl substituent. Since acetylation was carried out with [$^2H_6$]acetic anhydride, the acetyl group on serine must have been present in the native peptide. This establishes the nature of the natural blocking group on the α-chain of Hb trout IV.

The complete amino acid sequence of the peptide could be deduced from the results in fig.1 using the data on ion masses and fragmentation patterns given [1]. The parent ion was at *m/e* 717; the mass of the C-terminal residue (232) corresponded to that of the [$^2H_3$]acetyl derivative of lysine as expected. All sequence ions had associated ions (*m/e* values of 126, 253, 368, 453) due to loss of CH_3OH from serine. A complex pattern of signals was observed at the high mass end of the spectrum due to loss of CH_3OH, $-OCH_3$ and CD_2CO in various combinations from the parent ion.

3.2. *N-terminus of the α-chain from Hb trout I*

From a tryptic digest of carboxymethylated globin prepared from Hb trout I a blocked N-terminal peptide with the following amino acid composition: Lys (0.9), Thr (1.1), Ser (1.0). Ala (1.0) and Leu (0.9) was isolated in 30% yield after gel filtration, ion-exchange chromatography and paper chromatography. An aliquot of this peptide was digested with chymotrypsin and after high-voltage paper electrophoresis at pH 6.5 two fragments were isolated; one, with the N-terminal residue blocked, was composed of serine and leucine, the other had the sequence Thr–Ala–Lys.

From considerations of homology with α-chain sequences of Hb components of other fish (including Hb trout IV) and also because from the same tryptic digest a peptide with the sequence Val–Glu–Trp–Thr–Asp–Ala–Glu–Lys, clearly originating from the N-terminus of the β-chain was isolated in high yield, the blocked tryptic peptide was assigned to the N-terminal region of the α-chain from Hb trout I and the following structure was proposed: *X*–Ser–Leu–Thr–Ala–Lys. A sample of the chemically modified peptide was analysed by mass spectrometry.

The mass spectrum observed at source temperatures between 200°C and 230°C is shown in fig.2. The pro-

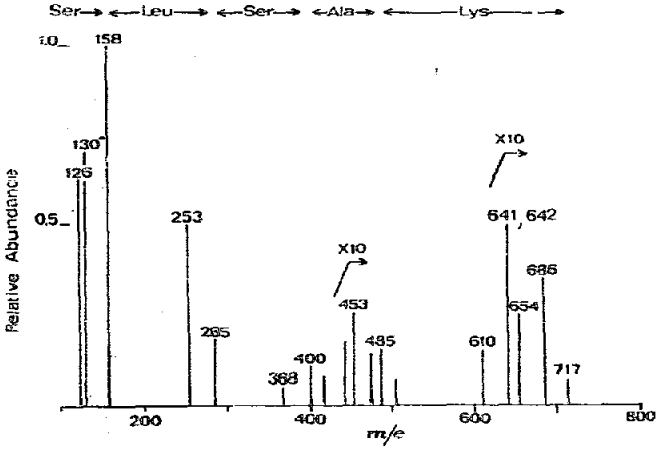

Fig.1. Mass spectrum of the tryptic N-terminal peptide from the α-chain of Hb trout IV.

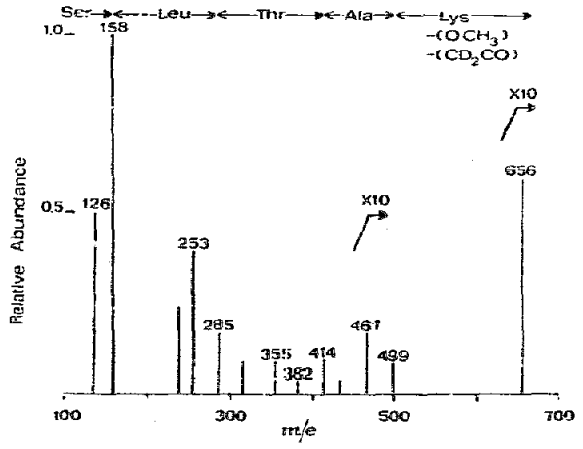

Fig.2. Mass spectrum of the blocked tryptic peptide from Hb trout I.

	HbA	Val–Leu–Ser–Pro–Ala
	Carp	Ac-Ser–Leu–Ser–Asp–Lys
α-chain	*Catostomus*	Ac-Ser–Leu–Ser–Asp–Lys
	Trout I	Ac-Ser–Leu–Thr–Ala–Lys
	Trout IV	Ac-Ser–Leu–Ser–Ala–Lys
	HbA	Val–His–Leu–Thr–Pro–Glu–Glu–Lys
β-chain	Trout I	Val–Glu–Trp–Thr–Asp–Ala–Glu–Lys
	Trout IV	Val–Asp–Trp–Thr–Asp–Ala–Glu–Arg

Fig.3. Comparison of the N-terminal amino acid sequences of HbA (human) [12] and fish hemoglobins [13,14]. The N-terminus of the carp α-chains is acetylated (Ac-) and the *Catostomus* protein is also blocked. Since acetic acid was released from the latter protein upon hydrolysis, Powers and Edmundson assumed that the protein is blocked with an acetyl group [14].

minent ion at *m/e* 158, corresponding to the serine derivative with an isotopically normal acetyl group, establishes that in this case also the natural blocking group is acetyl. Sequence ions at *m/e* 285, 4[illegible] and 499, together with the ions corresponding to the loss of 32 mass units (CH_3OH) in each case, confirmed the amino acid sequence to the penultimate residue. It is of interest that an ion was observed at *m/e* 355 corresponding to the loss of the entire side chain (59 mass units) of the threonine residue; loss of side chain minus H (58 mass units) is usually to be expected [1] The spectrum was not clear at the high mass end; ions were observed in the region expected for the parent ion (*m/e* 731), but could not assigned with certainty. A clear signal was observed at *m/e* 656 which probably arose from loss of $-OCH_3$ and CD_2CO from the parent ion; a similar ion was observed in the spectrum of the tryptic peptide from the Hb trout IV α-chain.

4. Discussion

Considerations of the functional significance of blockage of the α-amino groups of the α-chains can be based on the observation that in all fish hemoglobins analyzed to date the N-terminus of these chains is Ac-serine (fig.3). These include, besides the two components of trout blood, the following: Carp Hb [13], *Catostomus* Hb [14] (both components), *Cichlasoma cyanoguttatum* Hb [15] (in this case only evidence for a blocked N-terminal residue is available).

It is known that in mammalian hemoglobins the N-terminal amino group of the two α-chains is involved directly in the heterotropic regulation of O_2 transport [5]. Thus it has been shown that this group is:

(i) One of the residues which contribute the alkaline O_2 Bohr effect.

(ii) One of the residues which binds and transports CO_2 in the form of carbamate [16,17].

From the 2.5 Å electron density map of human deoxy Hb (Arnone et al. (1975) quoted [5]) there is evidence for the participation of the α-amino groups of the α-chains in a cluster of charges contributed by residues of the same or the other α-chain, i.e., Val $1\alpha_1$ interacts with: Arg $141\alpha_2$, Lys $127\alpha_1$ and Asp $6\alpha_1$.

In addition, recent crystallographic data on human Hb (Arnone, A., personal communication) have shown that the N-terminal amino group is also part of the small anion binding sites on the α-chains. In particular the new data have indicated that CO_2 may be bridging between the Val $1\alpha_1$ amino group and the guanidinium group of Arg $141\alpha_2$.

Although we have shown that Arg $141\alpha_2$, Lys $127\alpha_1$ and Asp $6\alpha_1$ are maintained in both components of trout Hb ([7,18] unpublished data) acetylation of the α-amino group will abolish these interactions, as well any direct contribution to the linkage between protons and CO_2 on one side and oxygen on the other.

In Hb trout I this chemical modification, together with the substitution of His 146 (β) for Phe [18], accounts almost completely for the absence of a Bohr

effect [19]. In Hb trout IV acetylation of the N-terminal serine of the α-chains is not incompatible with the very extreme pH effects observed since evidence already available has strongly suggested that the molecular basis of the Root effect may lie on the nature of the subunit interactions at the interfaces (Brunori et al., in preparation).

On the basis of this new structural information, it may be of great interest to explore more quantitatively than previously attempted the effect of CO_2 on O_2 transport by fish Hbs, to elucidate more fully the possible significance of the presence in fish of acetyl-serine as the terminus of the α-chains.

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