Studies on the N-Terminal Sequence of Eel Hemoglobin—I. Valyl Polypeptide Chain*

Hideomi AMANO, Kanehisa HASHIMOTO, and Fumio MATSUURA**

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N-Terminal amino acid sequences of the valyl polypeptide chains of the four hemoglobin components (F₁, F₂, S₁, and S₂) of eel, *Anguilla japonica*, were analyzed by the PTC-DNP method and the leucine aminopeptidase method. Results obtained are summarized as follows: (1) The N-terminal sequence of the valyl polypeptide chain was Val-Glu-Thr-Asn... for components F₁ and F₂, and Val-Gln-Ser-Ala... for components S₁ and S₂. As far as the present results are concerned, therefore, there are no differences between components F₁ and F₂ nor between S₁ and S₂. In the four components, Val was the only common residue. (2) It seemed impossible to judge, based on the present results, which chain of the α and β chains of higher vertebrate Hb is comparable to the valyl chain of each Hb component of eel.

In the previous paper,¹⁾ the authors reported that the four hemoglobin (Hb) components of eel all have two valyl and two acetylated amino acid residues at N-termini, and indicated that fish Hb is rather specific in this respect because the higher vertebrate Hb usually does not have any masked N-terminus.

On the other hand, the presence of such four N-termini in the fish Hb suggests that the fish Hb is composed of one pair each of two types of polypeptide chains like the higher vertebrate Hb. In this connection, it is recognized with the latter Hb that in the N-terminal sequence one of both types of chains is fairly resembling to the α chain of human Hb and the other to the β chain, irrespective of animal species.²⁾ As for the fish Hb, HILSE *et al.*³⁾ recently have elucidated the sequence of one of the two types of polypeptide chains of carp Hb, and proposed that the said chain could be comparable to the α chain of higher vertebrate Hb. No other papers of the same kind have been published so far. Hence it seemed interesting from the comparative biochemistry of Hb to extend the N-terminal sequence analysis, and the present series of investigation was undertaken. This paper communicates the sequences of valyl polypeptide chains of the four Hb components of eel.

Experimental

Materials: The blood of eel, *Anguilla japonica*, was collected from many live in-

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** Lab. of Marine Biochem., Fac. of Agr., The Univ. of Tokyo, Bunkyo-ku, Tokyo (天野秀臣・橋本周久・松浦文雄: 東京大学農学部)

dividuals and combined. Erythrocytes were separated by centrifugation and the clear hemolyzate was prepared therefrom as described previously.¹⁾ From the hemolyzate thus obtained, each of the four Hb components (F₁, F₂, S₁, and S₂) was isolated in cyanmet form by the method reported previously.¹⁾ After checking their homogeneity by starch gel electrophoresis,⁴⁾ those components were dialyzed until salt-free and treated with a HCl-acetone mixture to eliminate the heme moiety. The globin precipitates formed were collected by centrifugation, dried *in vacuo*, and directly used for N-terminal sequence analysis of the valyl polypeptide chain. Besides, where occasion permitted, the four Hb components were employed without elimination of the heme. Hb group F consisting of components F₁ and F₂, and Hb group S consisting of S₁ and S₂ were used in some experiments.

In addition, crystalline myoglobins (Mb's) prepared by the method reported before⁵⁾ from the heart muscle of skipjack, *Katsuwonus pelamis*, and from the skeletal muscle of sperm whale, *Physeter catodon*, were used to examine the reliability of the technique of N-terminal sequence analysis employed here.

Methods of analysis:

Determination of N-terminal sequence by PTC-DNP method. The N-terminal amino acid sequence was analyzed by THOMPSON'S PTC-DNP method with some modifications as outlined below: Globin of each Hb component was dissolved in a pyridine-water mixture (1:1, v/v). The globin solution was adjusted to pH 8.6, and to it was added the phenylisothiocyanate (PTC) reagent. The reaction mixture was vigorously stirred at room temperature for 2 hr and then extracted with benzene to remove the excessive PTC and pyridine.

To the aqueous layer was added large amounts of acetone. The greyish PTC-globin formed was collected by centrifugation and suspended in a concentrated formic acid at 25°C for 4 hr. The formic acid was removed by evaporation *in vacuo*, and the dry residue obtained was extracted with ethyl acetate to remove the phenylthiohydantoin (PTH) derivative of N-terminal amino acid. Then the residue from each component was divided into two portions. One portion was used for the analysis by the DNP method for the newly exposed N-terminal residue, in other words, for the second residue of the original globin. Another portion was used for the analysis of the third residue by the same procedure and so on.

Identification of Glx and Asx. For final identification of Glx and Asx residues found in the N-terminal sequences, the leucine aminopeptidase method was employed after TAKAHASHI⁶⁾ with some modifications: The solution of each Hb component which was previously dialyzed against a 0.005 M MgCl₂-0.005 M tris buffer, pH 8.5, was heated at 90°C for 30 sec. To the denatured Hb suspension, leucine aminopeptidase (Boehringer

Mannheim) was added at an enzyme to substrate ratio of 1:100, and incubated at 37°C for 23 hr. During and after the digestion, aliquots were taken at due intervals and analyzed by the DNP method for amino acid liberated.

Results and Discussion

As reported previously,¹⁾ each Hb component of eel is composed of two valyl and two acetylated polypeptide chains, and hence, it was expected that when the PTC-DNP method is applied to each component, only the valyl chains could be involved in the reaction, the acetylated chains being not. As control experiments in this respect, the PTC-DNP method was applied in the present manner to the following two pigments: One was the skipjack Mb the N-terminus of which is masked with acetyl group⁶⁾ unlike the N-terminus of mammalian Mb, and the other was the Hb component F₂ of eel which was previously dinitrophenylated to mask the N-termini of valyl chains. According to the results obtained, both pigments did not react with the PTC reagent at all, supporting the above expectation.

In the next place, the sperm whale Mb was analyzed for N-terminal sequence in order to check reliability of the experimental technique in the present PTC-DNP method. Results showed that the N-terminal sequence of Val-Leu-Ser-Glx-Gly-... which well agreed with that reported by EDMUNDSON.⁷⁾ In passing, it was noticed that yields of those residues were generally low, ranging from 0.10–0.77 mole/mole of Mb. Similarly poor yields of respective residues have been encountered in the cases of other proteins as well.^{8,9)} It was also noteworthy that in the determination of the fifth residue, Gly (yield, 0.14 mole/mole of Mb), unlike in those of the other residues, Glx at 0.04 mole/mole was simultaneously detected. The Glx residue detected here could be interpreted to be from the unreacted portion of the fourth residue, Glx, since several amino acids including Glx are much more difficultly cyclized into phenylthiohydantoin derivative than are the other amino acids.¹⁰⁾

N-Terminal sequences of valyl chains of eel Hb components as determined by PTC-DNP method: Then, the four Hb components of eel were analyzed by the PTC-DNP method for N-terminal amino acid sequences of their valyl polypeptide chains. Tentative results obtained are given in Table 1. As seen in the table, yields of respective residues were generally poor, compared to the theoretical value for each residue, 2 moles/mole of Hb. Particularly poor were those of the third residues, Glx, ranging from only 0.13 to 0.32 mole/mole of Hb. In this connection, trace amounts of Thr in the cases of components F₁ and F₂ and trace amounts of Ser in the cases of S₁ and S₂ were detected in addition to Glx in the determination of the third residue. As far as those tentative sequences are concerned, Thr is the fourth residue of component F₁ or F₂, and Ser, that of S₁ or S₂, as Table 1 shows. Thus, considering also the similar experience with sperm whale Mb

Table 1. Tentative N-terminal sequences of valyl polypeptide chains of the four Hb components of eel, as analyzed by the PTC-DNP method. Under respective amino acid residues are given the determined values of residues in moles/mole of Hb.

Hb component	Tentative sequence				
F ₁	Val —Glx —Glx —Thr —Asx —...	1.73	0.37	0.13	0.55 0.49
F ₂	Val —Glx —Glx —Thr —Asx —...	1.74	1.42	0.32	0.74 0.78
S ₁	Val —Glx —Glx —Ser —Ala —...	1.92	1.13	0.15	0.28 0.35
S ₂	Val —Glx —Glx —Ser —Ala —...	1.91	1.20	0.24	0.19 0.29

as described before, further examination seemed to be required as to whether Glx is present as the third residue through all the components, and it was performed by use of the N→O acyl migration¹¹⁾ as follows.

To begin with, each of the Hb groups, F and S, was dinitrophenylated, and the DNP-Hb produced was suspended in 100-fold volumes of the constant-boiling HCl. The DNP-Hb was then partially hydrolyzed by heating for 15 min under reflux. After completion, the hydrolyzate was extracted with ether to separate the DNP-peptides fraction. The fraction from either Hb group presented a major and a minor yellow spot in LEVY's two-dimensional paper chromatography. Both spots were extracted from the chromatogram, completely hydrolyzed with a constant-boiling HCl, and analyzed for amino acid composition. Results showed that the major spot was the DNP derivative of a peptide composed of Val and Glx. The molar ratio of Val to Glx in the peptide was 1:0.84 and 1:0.77 for the Hb groups, F and S, respectively. Therefore it was supposed that both amino acids are present at an equimolar ratio in the peptide. On the other hand, the minor spot was demonstrated to be DNP-Val. Since the peptide linkage involving the α -amino group of Thr or Ser is selectively split by the N→O acyl migration when hydrolyzed for a short time in a concentrated acid, the above results strongly suggest that there is only one Glx residue between the N-terminal Val and the Thr or Ser residue in each Hb component. Thus, the N-terminal sequences of the valyl chains must be Val-Glx-Thr-Asx... for components F₁ and F₂, and Val-Glx-Ser-Ala... for S₁ and S₂.

Identification of Glx and Asx in the sequence: Final identification of Glx and Asx detected in those N-terminal sequences was then attempted by the leucine aminopeptidase method. In Fig. 1 are given the amounts of respective amino acids liberated as a function of incubation time. In the cases of components F₁ and F₂, four spots as identified to be Val, Glu, Thr, and Asn were detected under the conditions applied. Irrespective of incubation time, the amount of Val almost always was highest, followed by Glu, Thr, and Asn. This order of the four amino acids coincides with the one expected from the N-terminal

sequence of valyl chain of component F_1 or F_2 . Because of the results, the second (Glx) and the fourth residue (Asx) in either component were finally identified to be Glu and Asn, respectively.

Fig. 1. Liberation of amino acids when the four Hb components were incubated with leucine aminopeptidase at 37°C. The amino acids liberated were identified and determined by the DNP method. See the text for further details.

On the other hand, four kinds of free amino acids, Val, Gln, Ser, and Ala, were found in the aminopeptidase hydrolyzate of each of components S_1 and S_2 . In the case of component S_2 , Val was dominant, followed by Gln, Ser, and Ala at most incubation times, as expected from the N-terminal sequence of its valyl chain. Hence the second residue (Glx) in the sequence was identified as Gln. In the case of component S_1 , the same four kinds of amino acids were detected again. However, the amounts of Ser liberated gradually exceeded Gln and even Val with the lapse of incubation time. Though the reason remains unclear, it seemed reasonable to identify the Glx in question as Gln.

Thus, the N-terminal sequences of the valyl chains of the four Hb components could be concluded as given in Table 2. The terminal sequences inward to the fourth residue are quite same between components F_1 and F_2 , and also between S_1 and S_2 . N-Terminal Val is the only common residue throughout the four Hb components, as far as the present results are concerned. It is pointed out that the α polypeptide chains of higher vertebrate Hb's rather resemble one another in amino acid sequence, as do their β polypeptide chains.²⁾

However, it seems impossible to judge from the limited results obtained here which of both chains of higher vertebrate Hb is comparable to the valyl chain of each Hb component of eel.

Table 2. N-Terminal amino acid sequences of valyl polypeptide chains of the four Hb components.

Hb component	N-Terminal sequence
F ₁	Val —Glu —Thr —Asn —...
F ₂	" " " "
S ₁	Val —Gln —Ser —Ala —...
S ₂	" " " "

In this connection, HILSE *et al.*³⁾ recently have elucidated the whole amino acid sequence of the acetylated polypeptide chain of carp Hb, indicating that the chain is comparable to the α chain of higher vertebrate Hb. As for another polypeptide chain the N-terminus of which has been demonstrated to be Val,¹²⁾ they have proposed it to compare to the β chain, though no sequence analysis of that chain has been performed as yet. Therefore, if one follows HILSE *et al.*, the valyl chains of eel Hb components might be regarded as the β chain. In this respect, further discussion will be presented in the succeeding paper which will deal with sequence analyses on the acetylated chains of eel Hb components.

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