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Amino-terminal processing of proteins: Hemoglobin South Florida, a variant with retention of initiator methionine and N^{α}-acetylation

(initiation/cotranslational modification/aminopeptidase/mutant hemoglobin)

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ABSTRACT **The hemoglobin variant South Florida has been shown by protein sequencing and fast-atom-bombardment mass spectroscopy to have a substitution of methionine for the NH_2-terminal valine of the β-globin chain. In addition, there was complete retention of the initiator methionine on the mutant polypeptide. Approximately 20% of the protein was acetylated at the NH_2 terminus of the β chain. A search of a comprehensive data bank of protein and gene sequences revealed 84 unrelated vertebrate proteins that have not undergone cleavage of leader sequences. A highly nonrandom distribution of residues at the NH_2 termini of these proteins predicts removal of the initiator methionine as well as NH_2-terminal acetylation. Proteins that undergo removal commonly have serine, alanine, glycine, or valine, as the NH_2-terminal residues. The first three residues favor N^{α}-acetylation. Proteins that retain the initiator methionine commonly have a charged residue or methionine at the second position. Information on Hb South Florida and other hemoglobins coupled with this survey of primary sequence provides insights into the NH_2-terminal processing of proteins.**

The biological properties of a large number of proteins are significantly altered by cotranslational and posttranslational modifications. In eukaryotes, the initiation of translation takes place at an AUG codon on mRNA (1). The NH_2-terminal methionine residue is generally cleaved from the nascent polypeptide by an aminopeptidase (2). Soon thereafter the new NH_2-terminal residue is often acetylated at the α-amino group (3, 4). Only fragmentary information on NH_2-terminal processing is available and, therefore, the molecular mechanism is still poorly understood. It has been suggested that cleavage of the initiator methionine is prevented when the adjacent residue is a charged amino acid (5). The collective body of information on hemoglobins has provided a wealth of insight into a variety of genetic and cellular events. In this report, we describe a human hemoglobin variant, Hb South Florida, in which an amino acid replacement of what is normally the NH_2-terminal valine of the β chain causes retention of the initiator methionine. Moreover, the mutant subunit is partially acetylated at the NH_2 terminus. Thus, a single amino acid difference can determine whether cleavage of methionine and/or acetylation occur. To gain a better understanding of the rules that govern cleavage of the initiator methionine and subsequent N^{α}-acetylation, we have taken advantage of the large bank of data collected to date on protein and DNA sequences. This survey, in conjunction with inferences drawn from Hb South Florida, permits the identification of residues that determine NH_2-terminal processing.

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MATERIALS AND METHODS

Phosphate-free hemolysates (6) were prepared from heparinized blood samples. Hemolysate (1–2 g of hemoglobin) was analyzed on a column (5 × 22 cm) of Bio-Rex 70 (Bio-Rad) cation-exchange resin as described (7). The "Hb A_{Ic}" fraction was dialyzed vs. 0.25 M ammonium acetate, pH 8.5, and rechromatographed on a column (2 × 6 cm) of Glycogel B boronate affinity resin (Pierce) (8). The non-retained Hb fraction from the affinity column and Hb A_0 fraction from Bio-Rex chromatography were converted to globin by acid/acetone precipitation (9) and separated into α and β subunits by carboxymethyl-cellulose chromatography in 8 M urea. After aminoethylation (10), the isolated chains were digested with trypsin (11).

The tryptic peptides were analyzed by reverse-phase HPLC (RP-HPLC) on an ultrasphere ODS Altex column (2 mm × 25 cm) protected by a C_{18} guard column. A Gilson (Villiers-le-Bel, France) model 320 apparatus was used. The peptides were eluted by developing a nonlinear gradient of acetonitrile (Pierce, HPLC grade), 0–50% in 49 mM KH_2PO_4, 5.4 mM H_3PO_4, pH 2.95. Peptides were purified on an analytical Aquapore RP 300 column (Brownlee Laboratories, Santa Clara, CA) using a 0.1 M ammonium acetate (pH 5.7)/acetonitrile system with a flow rate of 1.5 ml/min.

After hydrolysis in 6 M HCl and 0.05% 2-mercaptoethanol, the amino acid composition of the peptides was determined on an HPLC Micro System IV (American Research, Beltsville, MD). Alternatively, peptides isolated by HPLC were digested with leucine aminopeptidase (Sigma) for 24 hr at 37°C. The progress of the digestion was monitored by RP-HPLC. The amino acid sequences of the tryptic peptides were analyzed by using manual solid phase degradation. DITC-glass (*p*-phenylene diisothiocyanate-glass, 30 mg, Pierce) was treated for 12 hr with 20 nmol of peptide. Coupled peptides were then subjected to the multiple steps of the 4-*N*,*N*-dimethylaminoazobenzene-4′-isothiocyanate/phenylisothiocyanate (DABITC-PITC) method described by Chang (12). 4-*N*,*N*-dimethylaminoazobenzene-4′-thiohydantoin derivatives (DABTH) of the amino acids were identified by thin layer chromatography on polyamine sheets (2.5 × 2.5 cm) with a solvent system consisting of acetic acid/water (1:3, vol/vol) for the first dimension and toluene/*n*-hexane/acetic acid (2:1:1, vol/vol) for the second.

Fast-atom-bombardment mass spectrometric (FAB-MS) analyses were carried out using a Varian MAT 731 high-resolution mass spectrometer equipped with both Ion Tech EI/FI/FD ion source and fast-atom-bombardment gun. Tryptic peptides were dissolved in 0.5% acetic acid/glycerol, 1:1 (vol/vol) solution. About 2 μg of the sample was applied per analysis.

Abbreviations: FAB-MS, fast-atom-bombardment mass spectroscopy; RP-HPLC, reverse-phase HPLC.

RESULTS

Case Report. A 9-year-old Caucasian male was found to have a high level of Hb A_{Ic} (14.8%; normal, 4.4 ± 0.6%) as determined by rapid cation exchange chromatography. Further investigations, conducted at the University of South Florida Diabetes Center, revealed a discrepancy between the Hb A_{Ic} values obtained after Bio-Rex 70 chromatography and analyses by a colorimetric method using thiobarbituric acid or by affinity chromatography on a Glyc-Affin column (Isolab, Akron, OH) (Hb A_{Ic} = 6.9%). The glucose tolerance test was normal. Routine electrophoreses at acidic or alkaline pH were normal. The isoelectric focusing pattern showed only increased Hb A_{Ic}. A family survey revealed similar elevations in chromatographic Hb A_{Ic} in 11 other individuals. These observations suggested the presence of an abnormal nonglycated hemoglobin fraction that coelutes with Hb A_{Ic} on cation exchange chromatography. More details concerning the index patient and the family history will be presented elsewhere (S.C.S., J.I.M., J.-P.B., and T.K., unpublished data).

We have completed structural analysis of hemoglobin from the grandfather of the propositus, who carries the same abnormality.

Structural Analyses. Approximately 12% of the total hemoglobin applied to a preparative Bio-Rex 70 column eluted in the Hb A_{Ic} peak. Only 17.5% of this hemoglobin bound to the boronic acid affinity resin, so that glycated Hb A_{Ic} is about 2% of the total hemoglobin. Globin from the fraction not bound to the affinity resin and globin from the Hb A_o purified on Bio-Rex were separated into α and β chains by chromatography on CM-cellulose in 8 M urea. An identical chromatographic behavior was observed for both α chains, but the β chain from the nonglycated Hb A_{Ic}-like fraction eluted from the column slightly before the Hb A_o β chain, indicating an increased negative charge.

Both of the β-chain fractions were aminoethylated prior to trypsin digestion. Fig. 1 shows the elution profiles of the various tryptic digests when analyzed by RP-HPLC. When compared to a normal β-A chain hydrolysate, the pattern obtained with the Hb A_{Ic}-like fraction revealed the absence of normal βT-1 and a new peptide (βT-1X') that eluted just before the βT-12a peptide. The separation of the tryptic peptides from β-A_o showed that all peptides could be found at their normal position, including βT-1, but an additional peak (βT-1X) eluted before the βT-3 peptide A.

Peptide purifications were conducted by RP-HPLC in volatile buffers. When compared with the normal βT-1, the amino acid composition of the two abnormal peptides (T-1X and T-1X') gave similar results: the disappearance of a valine residue and the presence of two new methionines (Table 1).

Thus, the structure of the two abnormal βT-X and -X' peptides corresponds to the substitution β1 (NA1) Val → Met. [β1 (NA1) Val is the NH_2-terminal residue of the normal β chain.] This mutation prevents cleavage of the initiator methionine, leading to an elongated β chain with two NH_2-terminal methionine residues. This structure was confirmed by manual sequencing of βT-1X. The sequence for βT-1X was Met-Met-His-Leu-(Thr-Pro-Glu-Glu-Lys). This variant has not been described and was named Hb South Florida (S.C.S., J.I.M., J.-P.B., and T.K., unpublished data). The sequence for βT-1A was Val-His-Leu-(Thr-Pro-Glu-Glu-Lys).

The unsuccessful attempts at manual sequencing of the T-1X' peptide suggested the existence of an NH_2-terminal blocking group. Comparative studies of leucine aminopeptidase digestion on both βT-1X and -X' variant peptides supported the presence of such a blocking group at the NH_2-terminal side of βT-1X'. Indeed, although βT-1X was rapidly and completely digested, βT-1X' remained intact even after 24 hr as demonstrated by RP-HPLC analyses.

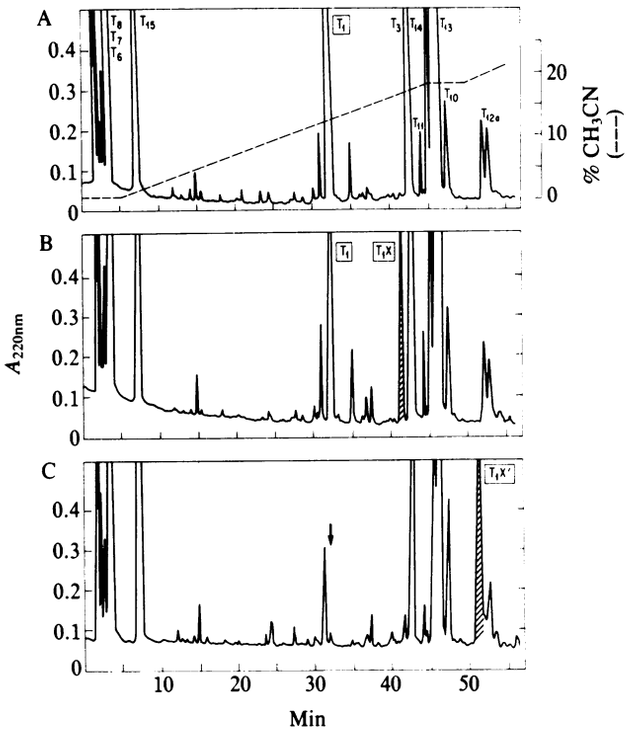

FIG. 1. Elution patterns of hydrophilic β chain tryptic peptides. Separation was achieved by RP-HPLC on an ultrasphere ODS Altex column using a nonlinear gradient of acetonitrile in 49 mM KH_2PO_4, pH 2.95, with a 1.5 ml/min flow rate. (*A*) Normal β-A chain, (*B*) β chains from the Hb A_o fraction, and (*C*) β-chain from the nonglycated Hb A_{Ic}-like fraction. The arrow indicates the elution area of the normal T-1 peptide.

Identification of the NH_2-Terminal Blocking Group. N^{α}-acetylation is by far the most common modification of NH_2-terminal methionine residues in eukaryotes. Indirect evidence of the presence of such an acetyl group in the modified βT-1X' peptide was provided by the RP-HPLC behavior of this peptide, when compared with the elution of the nonblocked βT-1X peptide (14). FAB-MS were obtained for βT-1X and its blocked analog βT-1X' (Fig. 2). The calculated molecular weight of the unblocked peptide was 1114. Molecular protonated ion were observed at m/z 1115 for the native NH_2-terminal peptide and m/z 1157 for the modified. These results indicate a shift of 42 units in the first residue of the modified peptide and, therefore, are consistent with the presence of a covalently attached *N*-acetyl group on

Table 1. Amino acid composition of the βT-1 peptides

Residue	Molar ratio: Observed βT-1X	βT-1X'	βT-1A	Theoretical for βT-1A
Thr	1.0	1.0	0.9	1
Glu	2.0	2.1	2.0	2
Pro	0.8	0.9	0.9	1
Val	—	—	**1.1**	1
Met	**1.7**	**1.6**	—	0
Leu	1.0	1.1	0.9	1
His	0.8	0.9	0.8	1
Lys	0.9	1.0	1.0	1

Boldface numbers identify residues that are substituted in variant Hbs.

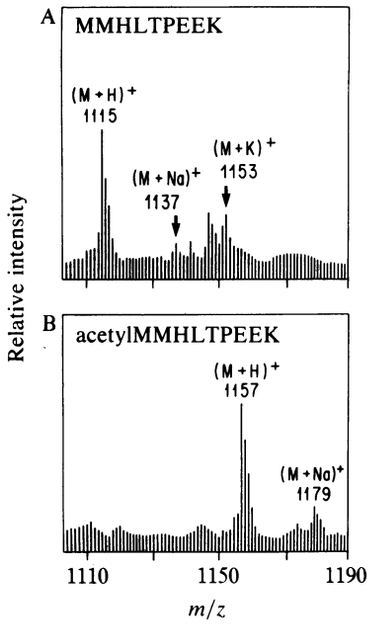

FIG. 2. FAB-MS of βT-1X (*A*) and βT-1X modified (T-1X′) (*B*) showing the region corresponding to their molecular ion clusters. Molecular weight 1114 corresponds to βT-1X and molecular weight 1156 corresponds to βT-1X′.

the NH_2-terminal methionine. FAB-MS also confirmed the sequence determined by Edman degradation.

It is interesting to note that this variant, Hb South Florida was first detected only by its acetyl derivative, a slightly anionic species which migrates like A_{Ic}. The valine to methionine substitution and the nonrelease of the initiator methionine do not change the hemoglobin charge. Therefore, the unblocked variant coelutes with Hb A on cation-exchange resins.

As calculated from the RP-HPLC profiles, Hb South Florida comprised about 40% of the total hemoglobin. Only 20% of the variant undergoes NH_2-terminal acetylation.

DISCUSSION

In prokaryotes, protein synthesis begins with a formylated amino acid, *N*-formylmethionine. This residue is removed through the successive action of two enzymes, a deformylase and then a peptidase (15). In eukaryotic cells, the initiator methionine, though not formylated, also undergoes cleavage by an aminopeptidase (2). Studies of hemoglobin synthesis in rabbit reticulocyte and yeast cell-free systems have shown that the methionine is normally removed when the nascent chain contains about 30 amino acids (16, 17), leaving the penultimate valine as the final NH_2-terminal residue. The existence of a specific enzyme that hydrolyzes the NH_2-terminal methionine has been demonstrated in rabbit reticulocytes (2). Blocking of the methionine amino group with a formyl group inhibits the action of the peptidase (18).

The single amino acid substitution, β1 (NA1) Val → Met, in Hb South Florida prevents cleavage of the initiator methionine. Preservation of the initiator methionine also occurs in several other hemoglobins: α chains from newt, platypus, chicken (α-1), and duck (α-2); fetal and adult β chains from bovine, sheep, and goat. Published gene sequences of chicken and ruminant proteins confirm the retention of the initiator methionine in the mature protein. Comparison of these sequences suggests that the NH_2-terminal methionine peptidase exhibits marked specificity and that its action depends on the nature of the amino acid(s) adjacent to this residue.

Table 2. Occurrence of NH_2-terminal residues in 84 proteins

NH_2-terminal amino acid	Proteins, no.	NH_2-terminal frequency,* %	Total frequency,† %	Acetylated residues, no.
Met	25	29.8	2.6 ± 1.5	13
Ser	25	29.8	6.5 ± 2.5	16
Ala	21	25.0	7.4 ± 3.1	15
Gly	9	10.7	7.4 ± 2.4	4
Val	6	7.1	6.8 ± 2.2	0
Others	2	2.4		1

A total of 1764 protein sequences were obtained from a data bank supplied by the National Biomedical Research Foundation (see ‡ text footnote). The above tabulation was established for 84 vertebrate protein sequences selected from the data bank. In 15 proteins both amino acid and nucleotide sequences have been published. All the proteins belonging to super families known to be further processed have been discarded—e.g., ester, glycosyl, and peptide hydrolases; protease inhibitors; growth factors and hormones; and toxins and immunoglobulin related proteins. In addition, all mitochondrial proteins have been discarded. When several sequences were available for the same protein, only the most frequent NH_2-terminal amino acid was entered. In four cases, two NH_2-terminal residues were observed with equal frequency; therefore, this table contains 88 entries. To avoid bias, proteins derived from the duplication of an ancestral gene were allocated to only one entry. A complete list of the proteins, including NH_2-terminal sequence and amino acid composition, is available on request.

*The frequency with which each residue occurs at the NH_2-termini among the 84 proteins selected.

†The frequency with which each residue, in the composite of the 84 proteins selected appears in the total amino acid compositions of these proteins.

To elicit rules governing cleavage of the initiator methionine, we analyzed 1764 known NH_2-terminal sequences of eukaryotic proteins. This computer-based survey was obtained from the National Biomedical Research Foundation, Washington, DC (May 1985).‡ The fact that all of the proteins synthesized in mitochondria and chloroplasts have an NH_2-terminal methionine residue suggests that the specific aminopeptidase may not be located in these cellular components. Alternatively, since *N*-formylmethionyl-tRNA is present in these organelles (19), the peptidase may be inhibited by *N*-formylation of the initiator methionine.

The nonrandomness of NH_2-terminal residues can be deduced from sequence comparisons. For this purpose we have identified 84 vertebrate proteins, which have arisen from distinct gene families and which have not undergone propeptide cleavage (Table 2). The distribution of the various NH_2-terminal residues of these proteins indicates that removal of the initiator methionine occurs preferentially when it directly precedes a serine, alanine, glycine, or valine. In Hb Raleigh, the β1 valine is replaced by alanine with complete removal of the initiator methionine (20).

In contrast, NH_2-terminal methionine is present in 25 of the 84 proteins and, presumably, represents the noncleaved initiator residue, as clearly established for 5 of these proteins in which both amino acid and nucleotide sequences have been published. Table 3 shows that the cleavage appears to be inhibited if a charged residue (aspartic acid, glutamic acid, arginine, or lysine) directly follows the initiator methionine. Recent information on viral proteins is in complete accord with this specificity of the NH_2-terminal methionine peptidase. Kamps *et al.* (21) have prepared two mutants of the transforming protein $p60^{src}$, having substitutions of alanine and glutamic acid for the normal NH_2-terminal glycine

‡National Institutes of Health (1983) *Genetic Sequence Databank* (Research Systems Div., Bolt, Baranek, and Newman, Boston), Tape Release 15.

Table 3. Amino acids adjacent to NH_2-terminal methionine residue

Residue	Proteins, no.	Positional frequency,* %	Total frequency,† %
Asp-2	8	32	5.6 ± 2.3
Glu-2	4	16	8.2 ± 3.9
Arg-2	3	12	4.8 ± 2.3
Lys-2	1	4	
Leu-2	3	12	8.9 ± 3.1
Asn-2	2	8	
Pro-2	2	8	
Met-2	2	8	
Cys-2	1	4	
Gln-2	1	4	
Pro-3	4	16	4.6 ± 2.8

The 25 proteins with NH_2-terminal methionine indicated in Table 3 were compared according to the residue(s) adjacent to the NH_2 terminus. Since two of the methionine-retaining proteins displayed two possible second residues, a total of 27 adjacent amino acids is indicated.

*The frequency of the second (or the third) amino acid among the 25 proteins is tabulated.

†The frequency with which each residue appears in the combined amino acid composition of the 25 NH_2-terminal methionine proteins.

residue. The former undergoes full cleavage of the initiator methionine whereas the latter does not. In addition, the NH_2-terminal sequence of pr 76^{gag}, a structural protein of avian retroviruses, is Met-Glu- (22). Again, the negatively charged glutamic acid residue prevents cleavage of the initiator methionine.

Cleavage is also prevented when methionine follows the initiator methionine. In addition to Hb South Florida two other NH_2-terminal Met-Met sequences have been found.

The third residue following the initiator methionine also appears to be an important determinant of cleavage. Recently, two laboratories have independently discovered another variant, Hb Long Island/Marseille (β2 His → Pro) (23, 24) that also results in an NH_2-terminal elongated β chain owing to the preservation of the initiator methionine. In contrast, the other known variants at the β2 site, Hb Deer Lodge (His → Arg) (25) and Hb Okayama (His → Gln) (26) undergo complete cleavage of the initiator methionine.

Besides specific primary sequences at the cleavage site, other structural constraints on the peptide chains may be required to ensure the subsequent removal of the initiator residue. Accordingly, predictive calculations based on the Chou and Fasman rules (27) were also done for the proteins in which both protein and nucleotide sequences were known. No specific secondary structure appears to be required for NH_2-terminal methionine peptidase recognition. In proteins which have undergone methionine cleavage, 48% β-sheet structures and 18% α-helix structures can be predicted from the NH_2-terminal amino acid sequences; equal frequencies of α and β structures (37%) are expected when the methionine is retained in the mature protein sequence.

About 20% of the β chains of Hb South Florida are N^{α}-acetylated. NH_2-terminal acetylation is an enzyme-catalyzed event in which the acetyl group is derived from acetyl CoA (28). N^{α}-acetyltransferases have been discovered in a variety of tissues (28–32) and do not display either cellular or phylogenetic specificity. N^{α}-acetylation is commonly encountered in intracellular proteins of eukaryotes but also in certain secreted proteins such as ovalbumin (33). In all of these proteins, the addition of the acetyl group to the NH_2-terminal residue occurs cotranslationally when the polypeptide chain reaches a length of approximately 50 residues (34–36). However, in other membrane associated proteins or secreted proteins such as corticotropin (ACTH) (32) N^{α}-acetylation is a posttranslational event.

Only two other human hemoglobins are known to be acetylated. The NH_2-terminal glycine of the γ chain of Hb F is partially (15%) acetylated (37) and the substituted NH_2-terminal alanine of the β chain of Hb Raleigh (20) is fully (90%) acetylated. Among animal hemoglobins, *N*-acetylserine is present in α chains of fish and β chains of tadpole and cat Hb B. The NH_2-terminal residues of these hemoglobins are quite representative of favored sites for acetylation. Glycine, alanine, serine, and methionine account for the great majority (80%) of the NH_2-terminal residues of acetylated proteins (3). However, not all proteins having these NH_2-terminal amino acids are so modified. In eukaryotes, the two cotranslational amino-processing events, cleavage of the initiator methionine and N^{α}-acetylation, seem to be closely related. If the initiator methionine is preserved, this residue is often acetylated. It is of interest that the most frequently acetylated residues are those that allow methionine cleavage (e.g., serine, alanine, glycine). Valine seems to be an exception, but one N^{α}-acetylvaline has been observed (38). N^{α}-acetylaspartic acid is found in a few proteins and appears to involve an alternate NH_2-terminal processing pathway (39, 40).

A unique initiator methionine tRNA is required for the recognition of the specific site on mRNA at which translation begins. Enzymatic removal of the initiator methionine allows the protein considerably more diversity of structure and presumably of function. This cleavage as well as subsequent N^{α}-acetylation is determined primarily by the NH_2-terminal sequence of the protein. A free NH_2-terminal amino group on a polypeptide may render the protein susceptible to proteolytic attack. Moreover, its low pK_a makes the amino group vulnerable to nonenzymatic modification by reactive metabolites such as glucose (41) and cyanate (13). N^{α}-acetylation may confer protection against these unwanted events.

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