**Deamidation of Human Erythrocyte Protein 4.1: Possible Role in Aging**

By Mutsumi Inaba, Kailash C. Gupta, Mikinori Kuwabara, Tsuneo Takahashi, Edward J. Benz Jr, and Yoshimitsu Maede

The human erythrocyte membrane protein 4.1 exists in two major electrophoretic forms: 4.1a (80 Kd) and 4.1b (78 Kd). Mass spectrometry and amino acid analysis of the proteolytic peptides derived from carboxyl-terminal regions of these proteins indicate that they differ by deamidation of two asparagine residues at positions 478 and 502. Electrophoretic analysis of carboxyl-terminal peptides has shown that the mobility difference between the two polypeptides is due to the deamidation of Asn502 and not that of Asn478.

**THE PROTEIN 4.1** is a predominant component of the cytoskeletal network of the erythrocyte. Protein 4.1 in human erythrocyte membranes consists of two polypeptides designated 4.1a and 4.1b. These two polypeptides are closely related in their primary structure and function, but differ in apparent molecular masses (Mr) by 2 Kd on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Laemmli buffer system. Several lines of evidence suggest that a posttranslational event(s) causes the conversion of 4.1b to 4.1a. This is primarily based on the observation that the ratio between protein 4.1a to 4.1b increases as red blood cells age. As in human erythrocytes, protein 4.1 in most mammalian erythrocytes consists of a doublet of polypeptides, suggesting that the posttranslational modification of the protein 4.1 and its various isoforms is a common theme in mammalian cells during aging. However, the nature of posttranslational modification(s) remains unclear. Some possible posttranslational events that could cause alterations in Mr of proteins on SDS-PAGE, like phosphorylation, methylation, glycation, and ubiquitination, have been proposed for the protein 4.1 modification. An understanding of the nature of the posttranslational modification of the protein 4.1 may provide information about the molecular events leading to erythrocyte aging.

Leto and Marchesi have shown that the difference in 4.1a and 4.1b can be attributed to the 22- and 24-Kd carboxyl-terminal fragments produced by restricted digestion of these proteins with chymotrypsin. Previously, we demonstrated that glycosylation of Ser or Thr with N-acetyl-D-glucosamine (O-GlcNAc) moiety attached to the 22-/24-Kd carboxyl-terminal regions had no relation to the difference in the Mr of 4.1a and 4.1b. However, our results suggested that the putative posttranslational event(s) probably occurs on the peptide portion proximal to either the amino- or carboxyl-terminus of 22-/24-Kd fragments. The present study describes the molecular basis of difference between protein 4.1a and 4.1b. Here, we demonstrate that deamidation occurs at two Asn residues (at positions 478 and 502) in the carboxyl-terminal region of protein 4.1 with different rates, and that deamidation at the 502nd Asn occurs rather slowly and is the sole cause of conversion of 4.1b to 4.1a. Our results also suggest that deamidation at this position creates a conformational change of protein 4.1 molecule.

This observation was confirmed by converting a congener of the protein 4.1b to 4.1a by site-directed mutagenesis of Asn502 to Asp. These results unambiguously demonstrate that deamidation of Asn502 is responsible for conversion of protein 4.1b to 4.1a. Since the conversion of protein 4.1b to 4.1a, under physiological conditions, occurs in a time-dependent manner, our study clearly shows that deamidation is an excellent marker for red blood cell aging.

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**MATERIALS AND METHODS**

In the present study, amino acid residues were numbered according to the predicted amino acid sequence of the erythroid protein 4.1, which includes sequence motifs I, II, and III (see Fig 5).

**Purification of the carboxyl-terminal fragments of protein 4.1.** Human erythrocyte protein 4.1 (mixture of 4.1a and 4.1b) was highly purified as reported previously. Protein concentration was determined by the method of Bradford with a Bio-Rad (Richmond, CA) protein assay kit using bovine serum albumin as the standard. Protein 4.1 (35 nmol) was lyophilized and dissolved in 1.0 mL of 70% formic acid. To this was added 40 mg of solid cyano gent bromide (CNBr). The CNBr digestion was performed in the dark at 25°C for 48 hours followed by evaporation under a stream of nitrogen gas. Cleavage products were dissolved in a small volume of 8 mol/L urea and 100 mmol/L Tris-C1 (pH 7.0), and separated on a gel permeation chromatography column of YMC-Pack Diol-200 (8 × 500 mm; YMC, Kyoto, Japan), which had been equilibrated with the same buffer. The 25- to 30-Kd carboxyl-terminal fragments, as deduced by amino acid sequencing (described below), were further purified on a reversed-phase high-performance liquid chromatography (HPLC) column of Chemosorb 300-7C4 (4 × 250 mm; Chemco, Osaka, Japan). The column was equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with a...
linear gradient of 0.1% TFA to 0.08% TFA in 80% CH$_3$CN at a flow rate of 1 mL/min. Eluates were monitored at 215 nm and 280 nm, and peaks were collected manually.

**Proteolytic digestions of the carboxyl-terminal fragments (CB25-30).** The lyophilized carboxyl-terminal peptides (CB25-30) were dissolved in a small volume of 8 mol/L urea and were digested with lysylendopeptidase (Wako Pure Chemical Industries, Osaka, Japan) in the presence of 2 mol/L urea and 100 mmol/L Tris-Cl, pH 8.8, with a molar ratio of enzyme to substrate of 1:200. Digestion was continued for 6 hours at 30°C and quenched by acidifying the reaction with TFA. Resultant peptides were separated on a reversed-phase HPLC column of Chemcosorb 300-7C8 (4 x 250 mm) with a linear gradient of 0.1% TFA to 0.09% TFA in 40% CH$_3$CN.

Digestion of CB25-30 with arginylendopeptidase (Takara, Kyoto, Japan) was performed at 37°C for 6 hours in the presence of 1 mol/L urea in 100 mmol/L Tris-Cl (pH 8.0), with a molar ratio of enzyme to substrate of 1:100. Proteolysis was stopped by the addition of phenylmethylsulfonyl fluoride to yield a final concentration of 8 mmol/L. Digestion products were separated on an HPLC column of Chemcosorb 300-7C4.

$[^3]$Hgalactose labeling of peptides. O-N-acetyl-D-glucosamine residue on protein 4.1 and its proteolytic digests were labeled enzymatically with UDP-[^3]$H$galactose (20 Ci/mmol = 740 MBq/mmole; Amersham International, Amersham, UK) in the presence of bovine milk galactosyltransferase (Sigma, St Louis, MO) as described previously.$^9$ Enzymatic glycosylation exists within Thr$_{544}$-Ala-GlcNAc region.$^9$ The 25- to 30-Kd CNBr fragments (CB25-30) and a major fragment produced by their digestion with arginylendopeptidase (CB25-30/R) contained the O-GlcNAc region and exhibited a positive reaction to XAR-5 film without drying, illustrating the structural fragments determined by reversed-phase HPLC. These results suggested the possibility of posttranslational modification(s) in the remaining small portion (amino- and/or carboxyl-proximal regions) of the 22- to 24-Kd fragments.$^{10}$

**RESULTS**

**Localization and characterization of posttranslational modification site(s) in protein 4.1a and 4.1b.** A previous study using chymotryptic digestion of protein 4.1a and 4.1b showed that the difference in protein 4.1a and 4.1b can be attributed to their 22- and 24-Kd carboxyl-terminal fragments.$^9$ We further demonstrated that the glycosylated 20-Kd chymotryptic peptides of these proteins, although identical in their electrophoretic mobility, nested in the 22- and 24-Kd fragments.$^9$ This observation suggested the possibility of a posttranslational modification(s) in the remaining small portion (amino- and/or carboxyl-proximal regions) of the 22- to 24-Kd fragments (Fig 1).

To precisely determine the modification site(s), we used methanol/10% acetic acid, brief staining with Coomassie blue, and destaining with 30% methanol/10% acetic acid. The gels were exposed to XAR-5 film without drying, using intensifying screens, at 4°C.

**Plasmids and site-directed mutagenesis.** Recombinant DNA manipulations were performed as described by Sambrook et al.$^9$ The EcoRI-XbaI fragment of pLym encoding full-length protein 4.1$^{1.10}$ was ligated to the vector plasmid pGfP4.$^9$ Site-directed mutants pGfP4.D502, pGfP4.D478, and pGfP4.D502/478 were created as described previously$^9$ using the 19-mer mutagenic oligonucleotide primers, 5'-GATAATGCCGATGGTGA-3' and 5'-CTTACACATCGATGGGAA-3' to change Asn502 and Asn478 to Asp, respectively. Mutations were confirmed by sequencing the vectors using a 19-mer (5'-GATGATGTTCTGGTCTAG-3') complementary to the sequence approximately 50-nucleotide upstream from Asn502 using Sequenase Version 2 kit (US Biochemical, Cleveland, OH).

In vitro transcription and translation. Capped sense transcripts were synthesized using T7 promoter within the vector by the procedure described by Sambrook et al.$^9$ The transcripts were translated in a rabbit reticulocyte lysate system (Bethesda Research Laboratories, Bethesda, MD) in the presence of $[^3]$Hmethylamine (Tran$^{35}$S-label, ICN, Irvine, CA) as detailed elsewhere.$^{17,18}$ Translation products were analyzed by SDS-PAGE followed by exposure to Kodak XAR-5 films.

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![Fig 1. Maps and partial sequences of peptides derived from the carboxyl-terminal region of human erythrocyte protein 4.1. Top alignment illustrates the structural fragments determined by restricted proteolysis by chymotrypsin. Predicted amino acid sequence of the carboxyl-terminal region are numbered corresponding to the full-length human erythroid protein 4.1, which includes three discrete peptides joined by RNA splicing. The O-GlcNAc moiety accessible to enzymatic glycosylation exists within Thr$_{544}$-Ala-...-Lys$_{563}$ (O-GlcNAc region). The 25- to 30-Kd CNBr fragments (CB25-30) and a major fragment produced by their digestion with arginylendopeptidase (CB25-30/R) contained the O-GlcNAc region and exhibited heterogeneity in the Mr, reflecting the difference of Mr between 4.1a and 4.1b.](image-url)
Fig 2. Purification of the 25- to 30-Kd carboxyl-terminal peptide from human erythrocyte protein 4.1. (A) The fragments produced by CNBr cleavage were separated on a YMC-Pack Diol-200, monitored at 280 nm. Eluates were analyzed by SDS-PAGE using 13.5% gels (insert). Lane M includes standard proteins; from top to bottom, Mr = 92,500, 66,200, 45,000, 21,500, and 14,400, respectively. Lane I, intact human erythrocyte protein 4.1; lanes 42, 44, . . ., 50, proteins eluted at 42 to 43, 44 to 45, . . ., 50 to 51 minutes, respectively. Eluates containing the 25- to 30-Kd fragments (shaded area) were pooled. (B) The 25- to 30-Kd fragments were further purified on a reversed-phase HPLC column of Chemcosorb 300-7C4. The elution gradient was linearly increased from A to 20% B in 10 minutes and then from 20% B to 70% B in 50 minutes at a flow rate of 1 ml/min (solution A was 0.1% TFA and solution B was 0.08% TFA in 80% CH3CN). (C) Major peak (shaded area in B) was collected and analyzed by SDS-PAGE (CBZS-30/CBB) using Laemmlli’s system. The 25- to 30-Kd polypeptides (CB25-30) were labeled at high specific activity with [3H]galactose (CB25-30/[3H-Gal]).

Fig 3. Separation of lysylendopeptidase digest of CB25-30 by reversed-phase HPLC. Proteolytic peptides of CB25-30 generated by lysylendopeptidase were separated on a Chemcosorb 300-7C8 column with a linear gradient of 0.1% TFA to 0.09% TFA in 40% CH3CN in 100 minutes at a flow rate of 1 ml/min. Full scale corresponds to 1.0 absorbance unit at 215 nm. The peaks were numbered LE1 through LE33 and used for chemical analyses. LE7 and LE9 contained pure peptide corresponding to Thr492- . . . -Lys505 (Table 1). LE30 contained the peptide Arg465- . . . -Lys491. Both LE31 and LE32 consisted of two peptides: Arg465- . . . -Lys491, and Thr522- . . . -Lys663 (or Lys566). LE30, LE31, and LE32 were further treated with arginylendopeptidase to obtain smaller peptides with the same sequence Thr474- . . . -Lys491 (LE30/R2, LE31b/R2, and LE32/R2; Table 1).
arginylendopeptidase. The major peak of the digests on a reversed-phase HPLC (data not shown) mainly contained two polypeptides with Mr of 22,000 and 18,500, which were amenable to labeling with [3H]galactose (Fig 2C). Again, despite the heterogeneity of polypeptides observed on the gel, the sequence of these polypeptides was determined to be Thr474-Leu-...Arg573 (or Arg577, Fig 1) based on the amino acid composition and amino-terminal sequence. The difference between Mr in these two polypeptides (~3,500) roughly corresponded with the difference of Mr between protein 4.1a and 4.1b. Based on these results and the previous finding that the 20-Kd glycosylated fragments from the amino-terminal region of the 22-/24-Kd fragments (Fig 1) with the amino acid sequence of Thr474-Leu-...Leu-Leu543.

To test our deduction and to determine the basis of this difference, we obtained proteolytic fragments from the amino-terminal region of CB25-30. Approximately 27 nmol of CB25-30 was proteolyzed with lysylendopeptidase and the digests were separated by reversed-phase HPLC (Fig 3). The peptides detected by absorption at 215 nm and 280 nm were collected and analyzed for their amino acid compositions, amino acid sequences, and accurate masses. Interestingly, LE7 and LE9, both having the identical amino acid composition and corresponding to Thr492-Gln-...Lys505, showed slight but consistent differences in their mass numbers. As shown in Table 1, amino acid sequence analysis of LE7 and LE9 showed that the eleventh amino acid residue, which corresponds to the 502nd position in the full-length erythroid protein 4.1, is different. The eleventh residue was Asn in LE7. This is in agreement with the cDNA-deduced sequence. However, LE9 had Asp at the corresponding site (Table 1). Therefore, the difference of mass number between LE7 (1,461) and LE9 (1,463) was most likely due to Asn (132.1) in LE7 and Asp (133.1) in LE9.

We then analyzed the electrophoretic profiles of these peptides after labeling with 125I to determine if the alteration at the 502nd amino acid residue indeed generated the difference of Mr by 2,000 as observed in the parent proteins 4.1a and 4.1b. Surprisingly, the LE9 (the 502nd residue = Asp) migrated significantly slower than LE7 (the 502nd residue = Asn) on a conventional SDS gel with a Laemmli buffer system (Fig 4). The Mr of LE9 was calculated to be approximately 6,500, whereas LE7 showed a Mr value of 1,800. This difference of electrophoretic mobilities in LE7 and LE9 was due to the intrinsic difference in these peptides and not because of any effect of the labeling agent ([125I]-labeled Bolton-Hunter reagent), since both peptides were labeled at the identical two amino acid residues (amino-terminal Thr, and carboxyl-terminal Lys). These results clearly indicated that a substitution of Asp for Asn502 remarkably affected the electrophoretic mobility of peptides and possibly that of the polypeptide (protein 4.1) involving this site.

### Table 1. Amino Acid Sequences and Accurate Molecular Masses of LE7, LE9, LE30/R2, LE31b/R2, and LE32/R2

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Peptide yield* (nmol)

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<th>Cycle No.</th>
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<th>LE30/R2</th>
<th>LE31b/R2</th>
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The phenylthiohydantoin-derivatives in each cycle of the Edman degradation and their yields in picomoles are presented. The yields and accurate masses of each peptide are also shown. Deamidated residues are underlined.

**Abbreviations:** AA, amino acid; ND, not done.

*The yields of the peptides were determined based on amino acid compositions of the collected fraction when 27 nmol of CB25-30 was digested and separated by reversed-phase HPLC (Fig 3). Therefore, the yields of LE30/R2, LE31b/R2, and LE32/R2 are shown as those of their precursor peptides corresponding to Arg465-...-Lys491 (see legend to Fig 3).
Our analysis also found a second site of alteration at 478 residue. Mass numbers of peptides carrying the 478 residue (LE30/R2, LE31b/R2, and LE32/R2) were slightly higher than those ascertained from the primary structure, representing the substitution of Asp for Asn478 (Table 1). Amino acid sequencing analysis of these peptides confirmed this substitution as shown in Table 1. However, the replacement of Asn478 by Asp had no significant effect on the electrophoretic mobility of these peptides (Fig 4).

Taken together, we conclude that a posttranslational deamidation of Asn to Asp occurs at two specific positions (Asn478 and Asn502) at the carboxyl-terminus of human erythrocyte protein 4.1 (Fig 5A) and deduced that deamidation of Asn502 affects the electrophoretic mobility of protein 4.1. Further, based on the mobilities of LE7 and LE9, deamidated protein was assigned to be protein 4.1a. These observations suggest that deamidation of Asn502 in protein 4.1b results in protein 4.1a during red blood cell aging. Since deamidation of Asn478 was detected in all peptides carrying this residue, we believe that deamidation of Asn478 occurs immediately after the synthesis of protein 4.1.

The electrophoretic mobilities of site-directed mutants of a congener of protein 4.1. To confirm our deductions and to test that deamidation of residue 502 is, indeed, a cause of the difference in protein 4.1a and 4.1b, we created mutants of a congener of protein 4.1 in which either one or both of Asn502 and Asn478 were changed to Asp by oligonucleotide-directed mutagenesis. For this purpose, we used cDNA clone, pGfP4.1, of the protein 4.1. Although this clone lacks the sequence motifs I, III, and V, it includes most of the sequence of protein 4.1, particularly the putative region of deamidation (Fig 5B).

SDS-PAGE of translation products of the wild-type (WT) and mutant (D502) transcripts showed that the product of D502 (76 Kd) was larger than that of the WT (74 Kd) by approximately 2,000 (Fig 6). This difference is in good agreement with the mobility difference between 4.1a and 4.1b. No further change in the mobility of the protein occurred when both Asn502 and Asn478 were changed to Asp (D502/D478). In contrast, when Asn478 alone was changed to Asp (mutant D478), no mobility change was observed in translation product of the mutant as compared with the WT. In addition to the expected translation product, a 135-Kd isoform, which starts at the ATG codon within motif IV (Fig 5B), was also detected. Another translation product, a 60-Kd polypeptide, is most likely one of the truncated isoforms of protein 4.1 generated by alternate splicing. Interestingly, 135-Kd and 60-Kd polypeptides behaved similarly to the above translation products in their migration in SDS-PAGE. That is, mutants D502 and D502/D478 showed proteins which migrated slower than WT and mutant D478 proteins. That all of these polypeptides were indeed translation products of the protein 4.1 cDNA was confirmed by immunoprecipitation.
shown) using anti-canine protein 4.1 IgGs. These results unambiguously demonstrate that the slower migration of the protein 4.1a is due to alteration of Asn502 into Asp.

DISCUSSION

Systematic chemical and mutational analyses showed that carboxyl-termini of protein 4.1a and 4.1b differed by two residues at the positions of 478 and 502. The possibility that these proteins originate from two closely related genes is ruled out by the finding that 4.1 is a single-copy gene in the human. In addition, the predicted primary structure of the human protein 4.1 reported by two independent groups left no doubt that amino acid residues at 478th and 502nd positions are Asn. These findings lead to the conclusion that substitution of Asp for Asn502 alone causes the increase in Mr of protein 4.1b by 2,000 in SDS-PAGE (Fig 6). Our study also demonstrated that the different isoforms of protein 4.1, including the 135-Kd and 60-Kd polypeptides, form similar doublets of proteins on deamidation. This provided evidence that they contain the sequence motif Pro487-...-Asn502-...-Gln529 encoded by one of the multiple exons of protein 4.1 gene. Hence, we suggest that the relative amounts of polypeptides in a doublet of the protein 4.1 isoform can also be used as a measure of cellular aging, especially in long-lived cells such as lens cells.

It is now believed that protein deamidation at Asn residue under physiological conditions proceeds through a five-membered succinimide ring intermediate, which automatically hydrolyses to give rise to a mixture of L- and D-normal aspartyl and L- and D-isoaspartyl peptide linkages. Since deamidation requires succinimide formation, its rate is influenced by amino acid residue on its carboxyl side. Hence deamidation is preferred at Asn-Gly sequence, which most likely occurs at the flexible region of a molecule. However, deamidation at Asn-Ala sequence is expected to be rather slow. Our results showed that deamidation of the protein 4.1 occurred at sequence Asn478-Gly479 and Asn502-Ala503. The Asn478 was converted to Asp immediately after protein synthesis, whereas deamidation of Asn502 progressed slowly as red blood cells aged during their 120-day life span. The in vivo half-life of Asn502 appears to be approximately 41 days based on our previous calculation. Interestingly, in contrast to the fact that a significant degree of deamidation occurred at Asn502-Ala503, there was no detectable deamidation at the neighboring same sequence of Asn500-Ala501 (Table 1). This suggested that the sequence Asn502-Ala503 and Asn478-Gly479, but not the adjacent Asn500-Ala501, occurs in a flexible region of erythrocyte protein 4.1. It is likely that deamidation at these sites caused the conformational change of protein 4.1. Conformational change of protein 4.1 by deamidation may alter characteristics and functions of protein 4.1 during red blood cell aging. A major function of the erythrocyte protein 4.1 is to maintain cellular integrity and elasticity by forming a stable complex with spectrin-actin complexes and by anchoring these skeletal proteins to the membrane. Goodman et al have reported that 4.1a and 4.1b bind spectrin2 and glycophorin3 with equal efficiency. However, since protein 4.1 functions through binding with many cellular proteins such as spectrin-actin,25,26 glycophorin,27 band 3,25,26 calmodulin,29 and myosin,31 determination of the role of deamidation of protein 4.1 on these interactions could uncover the biological significance of this reaction. Moreover, since deamidation is a common reaction that proceeds in a time-dependent manner at physiological temperature and pH and is likely to modify many proteins,22,32 its role in other cellular activities cannot be ignored.

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