Deamidation of Human Erythrocyte Protein 4.1: Possible Role in Aging

By Mutsumi Inaba, Kailash C. Gupta, Mokinori 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 (M_r) 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 human erythrocytes, protein 4.1 in the 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 M_r 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.

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. This was added 40 mg of solid cyanogen 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-Cl (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 Chromasorb 300-7C4 (4 × 250 mm; Chemco, Osaka, Japan). The column was equilibrated with 0.1% trifluoroacetic acid (TFA) and eluted with a gradient of 0.1% TFA to 50% acetonitrile.

For personal use only.
linear gradient of 0.1% TFA to 0.08% TFA in 40% CH₂CN 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 300-7C4 (4 × 250 mm) with a linear gradient of 0.1% TFA to 0.09% TFA in 40% CH₂CN.

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 mol/L. Digestion products were separated on an HPLC column of Chemcosorb 300-7C4.

**[3H]Galactose labeling of polypeptides.** O-N-acetyl-D-glucosamine residue on protein 4.1 and its proteolytic digests were labeled enzymatically with UDP-[3H]galactose (20 Ci/mmol = 740 MBq/mmol; Amersham International, Amersham, UK) in the presence of bovine milk galactosyltransferase (Sigma, St Louis, MO) as described previously. 15

**[3H]Labeling of peptides.** Some of the peptides generated from CB25-30 by lysylendopeptidase digestion were labeled with [125I] using [125I]-labeled Bolton-Hunter reagent (2,200 Ci/mmol = 81.4 TBq/mmol; DuPont-New England Nuclear, Boston, MA). One hundred picomoles of each peptide was reacted with 50 μCi of [125I]-labeled Bolton-Hunter reagent in 10 μL of 40 mmol/L sodium phosphate (pH 7.7) for 1 hour at 0°C. After that, 10 μL of 1 mol/L Tris-Cl (pH 7.5) was added and allowed to stand for 30 minutes at 0°C. [125I]-labeled peptides were dried, dissolved in 8 mol/L urea, and subjected to SDS-PAGE.

**Chemical analyses.** Amino acid composition of the proteolytic digests was determined after gas-phase hydrolysis in the presence of 5.7 mol/L HCl at 110°C for 24 hours. Amino acids were labeled with phenylisothiocyanate and analyzed using a 130A phenylthiocarbamoyl (PTC)-derivative analyzer (Applied Biosystems, Foster City, CA).

The amino acid sequences of the peptides were determined using a model 447A pulse liquid-phase protein sequenator equipped with a model 120A phenylthiobydantoin (PTH)-derivative analyzer (Applied Biosystems).

Positive fast-atom bombardment-mass spectrometric analysis of the peptides was performed using a JEOL JMS-HX 110 mass spectrometer (Japan Electron Optics Laboratories, Tokyo, Japan). The peptides were dried onto the bottom surface of Eppendorf tubes, kept at −20°C, and dissolved in 2% TFA before analysis. Glycerol was used as the matrix.

**SDS-PAGE, fluorography, and autoradiography.** SDS-PAGE was performed using Laemmli’s discontinuous buffer system. 4 The Mr values of polypeptides and peptides were determined using marker proteins from Bio-Rad (Mr = 14,400 to 93,000) and Pharmacia-LKB (Uppsala, Sweden) (Mr = 1,700 to 17,000) as the standards.

The gels separating the polypeptides labeled with [3H]galactose were processed for fluorography with ENHANCE (DuPont-New England Nuclear), dried, and exposed to Kodak XAR-5 film (Rochester, NY) with intensifying screens (DuPont) at −80°C. The peptides labeled with [125I] were visualized by autoradiography.

After electrophoresis, the gels were fixed with 5% glutaraldehyde for 15 minutes, followed by repeated washing with water and 30% 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. 15 The EcoRI-XbaI fragment of pLYm encoding full-length protein 4.1 15 was ligated to the vector plasmid pG111 17 using EcoRI and XbaI sites to construct a wild-type plasmid pGFP4.1. Site-directed mutants pGFP4.1D502, pGFP4.1D478, and pGFP4.1D502/478 were created as described previously 17 using the 19-mer mutagenic oligonucleotide primers 5'-GATAATGCCGATGCTGTGA-3' and 5'-CTTACAATCGATGGGAAAA-3' to change Asn502 and Asn478 to Asp, respectively. Mutations were confirmed by sequencing the vectors using a 19-mer (5'-GTGATATGGTCTTTGCTTAC-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. 15 The transcripts were translated in a rabbit reticulocyte lysate system (Bethesda Research Laboratories, Bethesda, MD) in the presence of [35S]methionine (TranScan-label, ICN, Irvine, CA) as detailed elsewhere. 15,18 Translation products were analyzed by SDS-PAGE followed by exposure to Kodak XAR-5 films.

**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.3 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. 10 This observation suggested the possibility of a posttranslational modification(s) in the remaining small portion (amino- and/or carboxyl-proximal regions) of the 22-/24-Kd fragments (Fig 1).

To precisely determine the modification site(s), we used
DEAMIDATION OF PROTEIN 4.1

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 1, 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 300-7 C8. 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. All of these polypeptides were accessible to enzymatic galactosylation as shown in Fig 2C, indicating that they contained the O-GlcNAc–attached region as described previously. (C) Major peak (shaded area in B) was collected and analyzed by SDS-PAGE (CB25-30/CBB) using Laemmli’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 300-7 C8 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. (A) 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- . . .-Lys563 (or Lys566). LE30, LE31, and LE32 were further treated with arginylen-dopeptidase to obtain smaller peptides with the same sequence Thr474- . . .-Lys491 (LE30/R2, LE31b/R2, and LE32/R2; Table 1).

Chemical cleavage of the carboxyl-terminal region of protein 4.1 with CNBr. Based on the primary structure of the protein 4.1 deduced from its cDNA sequence, we expected to obtain two fragments (Glu452-. . .-Met606, and Ser607-. . .-Glu622) from its carboxyl-terminal region (Fig 1). The CNBr cleavage products were separated on a gel permeation chromatography column followed by reversed-phase HPLC separation (Fig 2A and B). The eluates in the major peaks on the C4 column contained several polypeptides (CB25-30) with Mr of 25,000, 27,000, 28,000, and 30,000 on SDS-PAGE (Fig 2C). All of these polypeptides were accessible to enzymatic galactosylation as shown in Fig 2C, indicating that they contained the O-GlcNAc–attached region as described previously. (Fig 1). Despite the heterogeneity in their Mr, these polypeptides showed identical amino-terminal sequence of Glu-Ser-Val-Pro-Glu-Pro-Arg-Pro-Ser-Glu- . . . corresponding to the sequence which starts at Glu452 of erythroid protein 4.1 as deduced from its cDNA sequence (Fig 1). In addition, analysis of the hydrolyzate of CB25-30 showed that the amino acid composition of CB25-30 was nearly identical to that of the polypeptide Glu452-. . .-Met (homoserine or homoserine lactone)606. Apparently, 25- and 27-Kd polypeptides appeared to be a set of doublet polypeptides derived from Glu452- . . .-Met606 of protein 4.1a and 4.1b as shown in Fig 1. Likewise, a larger pair of 28- and 30-Kd fragments could be attributed to polypeptide chains (Glu452- . . .-Glu622) of 4.1a and 4.1b produced by incomplete CNBr cleavage.

The CB25-30 was further analyzed by digestion with
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 4.la and 4.lb. Based on these results and the previous finding that the 20-Kd glycosylated fragments from the protein 4.la and 4.lb were identical,10 the posttranslational modification was deduced to be located within 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.11,19 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 Laemmi 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

<table>
<thead>
<tr>
<th>Cycle No.</th>
<th>LE7</th>
<th>LE9</th>
<th>LE30/R2</th>
<th>LE31b/R2</th>
<th>LE32/R2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AA</td>
<td>Yield</td>
<td>AA</td>
<td>Yield</td>
<td>AA</td>
</tr>
<tr>
<td>1</td>
<td>Thr</td>
<td>66.2</td>
<td>Thr</td>
<td>73.6</td>
<td>Thr</td>
</tr>
<tr>
<td>2</td>
<td>Gin</td>
<td>97.4</td>
<td>Gin</td>
<td>74.0</td>
<td>Leu</td>
</tr>
<tr>
<td>3</td>
<td>Thr</td>
<td>147.3</td>
<td>Thr</td>
<td>116.7</td>
<td>Asn</td>
</tr>
<tr>
<td>4</td>
<td>Val</td>
<td>171.1</td>
<td>Val</td>
<td>131.1</td>
<td>Ile</td>
</tr>
<tr>
<td>5</td>
<td>Thr</td>
<td>74.7</td>
<td>Thr</td>
<td>93.7</td>
<td>Asp</td>
</tr>
<tr>
<td>6</td>
<td>Ile</td>
<td>80.3</td>
<td>Ile</td>
<td>93.7</td>
<td>Gly</td>
</tr>
<tr>
<td>7</td>
<td>Ser</td>
<td>24.5</td>
<td>Ser</td>
<td>31.4</td>
<td>Gin</td>
</tr>
<tr>
<td>8</td>
<td>Asp</td>
<td>30.6</td>
<td>Asp</td>
<td>53.5</td>
<td>Ile</td>
</tr>
<tr>
<td>9</td>
<td>Asn</td>
<td>26.0</td>
<td>Asn</td>
<td>32.5</td>
<td>Pro</td>
</tr>
<tr>
<td>10</td>
<td>Ala</td>
<td>21.0</td>
<td>Ala</td>
<td>41.0</td>
<td>Thr</td>
</tr>
<tr>
<td>11</td>
<td>Asn</td>
<td>19.0</td>
<td>Asp</td>
<td>19.4</td>
<td>ND</td>
</tr>
<tr>
<td>12</td>
<td>Ala</td>
<td>14.8</td>
<td>Ala</td>
<td>16.8</td>
<td>ND</td>
</tr>
<tr>
<td>13</td>
<td>Val</td>
<td>12.2</td>
<td>Val</td>
<td>9.1</td>
<td>ND</td>
</tr>
<tr>
<td>14</td>
<td>Lys</td>
<td>1.5</td>
<td>Lys</td>
<td>0.6</td>
<td>ND</td>
</tr>
<tr>
<td>15</td>
<td>ND</td>
<td>ND</td>
<td>Pro</td>
<td>9.5</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>ND</td>
<td>ND</td>
<td>Leu</td>
<td>13.9</td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>ND</td>
<td>ND</td>
<td>Val</td>
<td>3.2</td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>ND</td>
<td>ND</td>
<td>Lys</td>
<td>0.7</td>
<td></td>
</tr>
</tbody>
</table>

Peptide yield* (nmol) 8.1 5.9 3.0 7.3 8.8
Mass
Observerved
Theoretical 1,461 1,463 1,849 1,850 1,849

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).
DEAMIDATION OF PROTEIN 4.1

Our analysis also found a second site of alteration at 478 residue. Mass numbers of peptides containing 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 (data not shown).

**Fig 4.** SDS-PAGE analysis of 14N-labeled peptides. 14N-Labeled peptides (10 to 20 pmol) were dried and dissolved in 2 to 4 µL of 8 mol/L urea. To this was added an equal volume of 2x Laemmli's sample buffer. SDS-PAGE was performed using Laemmli's buffer system. The gels used consisted of 20% acrylamide. Lanes 30, 7, 9, and 32 contained 14N-labeled LE30, LE7, LE9, and LE32, respectively. Molecular weights of the standards (k) are 17,201, 14,632, 8,235, 6,556, and 1,695 from top to bottom.

**Fig 5.** Strategy of mutational analysis. (A) Deamidation of Asn (N) into Asp (D) at two different positions. Results of the chemical analysis are summarized. Human erythrocyte protein 4.1 (78- and 80-Kd) includes all three sequence motifs I, II, and III, corresponding to 21, 34, and 35 amino acid residues. (B) Design of site-directed protein 4.1 mutants and structure of the products. Two AAT codons for Asn478 and Asn502 in the plasmid pGFP4.1 (WT) were changed to GAT independently (D502 and D478) and then two mutations were combined (D502/478). The protein 4.1 gene in the construct contained sequence motifs II and IV (closed blocks), but lacks motifs I, III, and V (open blocks), creating an open-reading frame that starts at an AUG codon within the motif IV and encodes a 135-Kd polypeptide. However, initiation at the downstream AUG codon was still obtained in reticulocyte lysate system as shown in Fig 6 and as observed by Tang et al. This resulted in the expression of a 74-Kd polypeptide because of the absence of motifs I and III.

From www.bloodjournal.org by guest on October 23, 2017. For personal use only.
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-Gln479 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 spectrin and glycophorin with equal efficiency. However, since protein 4.1 functions through binding with many cellular proteins such as spectrin-actin, glycophorin, band 3, calmodulin, and myosin, 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, its role in other cellular activities cannot be ignored.

ACKNOWLEDGMENT

We gratefully thank Dr T. Kumazaki (Faculty of Pharmaceutical Science, Hokkaido University) for his valuable discussion on this study and enthusiastic support in amino acid analysis, and Dr A. Kimura (Faculty of Agriculture, Hokkaido University) for his helpful comments and assistance in amino acid sequencing. We also thank Y. Tanakadate, K. Baba, and D. Menchaca for their secretarial assistance.
REFERENCES


From www.bloodjournal.org by guest on October 23, 2017. For personal use only.
Deamidation of human erythrocyte protein 4.1: possible role in aging

M Inaba, KC Gupta, M Kuwabara, T Takahashi, EJ Jr Benz and Y Maede

Updated information and services can be found at:
http://www.bloodjournal.org/content/79/12/3355.full.html
Articles on similar topics can be found in the following Blood collections

Information about reproducing this article in parts or in its entirety may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#repub_requests

Information about ordering reprints may be found online at:
http://www.bloodjournal.org/site/misc/rights.xhtml#reprints

Information about subscriptions and ASH membership may be found online at:
http://www.bloodjournal.org/site/subscriptions/index.xhtml