Protective antigen (PA) is a central virulence factor of *Bacillus anthracis* and a key component in anthrax vaccines. PA binds to target cell receptors, is cleaved by the furin protease, self-aggregates to heptamers, and finally internalizes with a complex of either lethal or edema factors. Under mild room temperature storage conditions, PA cytotoxicity decreased ($t_{1/2} = 7$ days) concomitant with the generation of new acidic isoforms, probably through deamidation of Asn residues. Ranking all 68 Asn residues in PA based on their predicted deamidation rates revealed five residues with half-lives of <60 days, and these residues were further analyzed: Asn$^{162}$ in the 20-kDa region, Asn$^{306}$ at $P_6$, vicinal to the furin cleavage site, Asn$^{506}$ in the pro-pore translocation loop, and both Asn$^{713}$ and Asn$^{719}$ in the receptor-binding domain. We found that PA underwent spontaneous deamidation at Asn$^{162}$ upon storage concomitant with decreased susceptibility to furin. A panel of model synthetic furin substrates was used to demonstrate that Asn$^{162}$ deamidation led to a 20-fold decrease in the bimolecular rate constant ($k_{ca}/K_m$) of proteolysis due to the new negatively charged residue at $P_6$ in the furin recognition sequence. Furthermore, reduced PA cytotoxicity correlated with a decrease in PA cell binding and also with deamidation of Asn$^{713}$ and Asn$^{719}$. On the other hand, neither deamidation of Asn$^{10}$ or Asn$^{306}$ nor impairment of heptamerization could be observed upon prolonged PA storage. We suggest that PA inactivation during storage is associated with susceptible deamidation sites, which are intimately involved in both mechanisms of PA cleavage by furin and PA-receptor binding.

The Gram-positive spore-forming bacterium *Bacillus anthracis*, the causative agent of anthrax, produces a bipartite A/B-type toxin. The B subunit is the 83-kDa protective antigen (PA)$^3$ receptor-binding moiety (named for its use as a vaccine), and the two catalytic A subunit moieties are edema factor (EF; 89 kDa) and lethal factor (LF; 90 kDa) (1). EF is a $Ca^{2+}$- and calmodulin-dependent adenylate cyclase (2). LF is a $Zn^{2+}$ protease that cleaves and inactivates mitogen-activated protein kinase-1 and -2 (3, 4). Following PA binding to cell receptors (5–8), it is converted to an $83 kDa$ (domain I, residues 1–258) prevents premature PA polymerization and harbors the furin cleavage site, which is located in an unstructured flexible loop; domain II (residues 259–487) is involved in heptamerization and is in the membrane insertion loop; domain III (residues 488–595) is also involved in heptamerization (18); and domain IV (residues 596–735) is the C-terminal receptor-binding domain, with a small and a large sub-domain loop. Mutagenesis studies implied the involvement of the small loop (residues 679–693), but not the large one (residues 704–722), in PA-receptor binding (21). Other studies have shown the involvement of the large loop in binding (20). Recent x-ray crystallography studies of the PA-receptor complex revealed two contact regions of PA with the receptor, residues 681–688 and 714–716, suggesting the contribution of both the small and large loops, respectively, in PA binding (22).

The currently approved human vaccines against anthrax are prepared from crude bacterial supernatant enriched with PA in addition to other *B. anthracis*-related proteins such as LF and EF (23, 24). Previous studies have shown that protective immunity to anthrax disease correlates with induction of neutralizing anti-PA antibodies (25–27). “Second generation” recombinant anthrax vaccines are based on the recombinant protein as the sole or major *B. anthracis* antigenic component (28, 29) or on a live attenuated bacterial strain expressing recombinant PA (30, 31).

PA has been reported previously to be a thermally unstable protein, losing its *in vitro* cytotoxic activity upon storage at 37 °C within 48 h (32) or within few minutes above 40 °C as a result of aggregation (33). Protein loss of function under mild storage conditions is generally attributed to a variety of nonenzymatic modifications such as deamidation, isomerization, oxidation, and alternative disulfide pairings (34, 35). Spontaneous deamidation, which occurs mainly at Asn side chains and at a much slower rate at Gln residues (36), is a major and well documented degradation pathway in proteins. Deamidation rates depend on pH, temperature, primary sequence (“nearest neighbor” effect), and protein conformation (37–40). Deamidation proceeds by nucleophilic attack on the side chain carboxyl carbon of Asn by the nitrogen of the adjacent peptide bond, resulting in the formation of an unstable five-member succinimide ring, which is hydrolyzed to produce mainly l-Asp and l-isooaspartic acid at a ratio of 1:2 (41). Thus, deamidation can induce structural and functional perturbations in proteins through the introduction of a new negatively charged amino acid (Asp) and the insertion of an additional methyl residue in the polypeptide backbone (isoaspartic acid). As a result, generation of new acidic isoforms is considered a hallmark of deamidation (42, 43). It has been hypothesized that *in vivo* deamidation functions as an internal clock, regulating the half-life of proteins (39, 44), and may have significant physiological consequences,
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resulting in autoimmune diseases (45), Alzheimer disease (46), lens cata-
ракs (47–49), and in other physiological systems (43, 44, 50). Furthermore,
the deamidation process is of major concern in the production of
pharmaceutical proteins mainly during their purification and under
long-term storage, as reported for recombinant human growth hor-
mone (51), growth hormone-releasing factor (52), recombinant plas-
minogen activator (53), and recombinant human interleukin-11 (54),
and even in monoclonal antibody preparations following in vivo admin-
istration (55). In addition, it was demonstrated recently that deamida-
tion of tetanus vaccine under long-term storage results in the impair-
ment of antigen processing and presentation (56).

In this study, we aimed to clarify the molecular mechanism(s) by
which PA undergoes loss of function. We present evidence supporting
the notion that inactivation of PA upon storage is caused by spontane-
ous deamidation of at least three vulnerable Asn sites, perturbing crucial
steps in its mechanism of action.

EXPERIMENTAL PROCEDURES

Materials—Fmoc-derivatives were purchased from Novabiochem. Fmoc
amide resin (Applied Biosystems) was used as a solid phase. Human
furan (EC 3.4.21.75) was purchased from Sigma, trypsin (por-
cine; EC 3.4.21.4) from Promega, and endoprotease Asp-N (EC
3.4.24.33) from Calbiochem. All other reagents were analytical grade.

Production and Purification of PA and LF—PA and LF were purified
from B. anthracis strain V770-NP1-R (ATCC 14185) cultivated as
described previously (31, 55, 57) and stored at −20 °C until used.

PA Cytotoxicity Assay—Purified stocks of PA and LF were kept at
−20 °C until used. Cytotoxicity was determined in J774A.1 cells (Ameri-
tan Type Culture Collection, Manassas, VA) as described previously
(19, 27). In brief, cells (6 × 105 cells) were plated in 96-well cell culture
plates. The cytotoxicity assay was initiated by the addition of PA (in
serial dilutions) and LF, followed by incubation for 5 h at 37 °C under 5% CO2. Cell viability was monitored by the 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide assay (58). Cytotoxicity was deter-
mined by linear regression of each tested sample from a PA standard
curve and is expressed as percent activity at time 0.

PA Storage Conditions and Peptide Deamidation—PA was stored at
25 °C in 50 mM NH4HCO3 buffer (pH 8.0) for up to 30 days in sealed
tubes in a humid and sterilized (0.2-
m filtration) environment. Storage
conditions were selected on the basis of the PA purification methodol-
ogy (31, 57) and heat stability studies (33). Peptides were incubated for
a few days in sealed tubes at 37 °C essentially as described above in 50 mM
NH4HCO3 (pH 8.0). pH and sample volumes were stable throughout
the incubation period.

Isoelectric Focusing and SDS-Polyacrylamide Gels—Isoelectric focus-
ing was carried out at a pH range of 3–9, and SDS-polyacrylamide gels
were analyzed using precast gels (PhastSystem, Amersham Biosciences)
or by gel filtration on a Superdex 200 10/30 fast protein liquid chromatography column (Amersham Biosciences).

PA Biotinylation and Binding Assay—The PA heptamerization process
was followed essentially as described previously (17, 61) with the following
modifications. PA samples (2 mg/ml) were cleaved by trypsin (0.25
μg/ml) for 30 min at 37 °C in 50 mM NH4HCO3 (pH 8.0). The reaction
was stopped by 1 mM phenylmethylsulfonyl fluoride (final concen-
tration). LF was added to the PA samples at various molar ratios (25 °C),
and the mixtures were immediately separated by native gel (4–15%)
electrophoresis (PhastSystem) or by gel filtration on a Superdex 200
10/30 fast protein liquid chromatography column (Amersham Biosciences).

Peptide Synthesis and Purification—Simultaneous Fmoc solid-phase
peptide synthesis was performed manually in “T-bags” (59). Peptides
were purified on a semipreparative reversed-phase HPLC column
(Supelcosil LC-18-DB 300A, 5-μm pore size, 250 × 10 mm) using a
Waters system equipped with 600E delivery pumps, a 996 photodiode
array detector, and a 717 autosampler and controlled by Millenium
software. About 2 mg of each peptide were purified (>95%), dried
(Savant SpeedVac), and stored at −20 °C until used.

MALDI-TOF/MS and Amino Acid Analysis—Reversed-phase
HPLC-purified peptide samples were applied to a MALDI-TOF/MS
target in 0.1% trifluoroacetic acid as described previously (60). Peptide
quantitation was by amino acid analysis using vapor acid hydrolysis
(110 °C, 16 h) and phenylthiocarbamyl pre-column derivatization (phe-
nylthiocarbamyl workstation, Waters Corp.).

In-gel Asp-N Digestion—Coomassie Blue-stained protein bands
were excised from the gel and in gel-digested with 2 μg/ml endopro-
teinase Asp-N in 50 mM NH4HCO3 and 30% CH3CN. Following 2 h
of incubation at 37 °C, peptides were extracted in 20 μl of 1% triflu-
oracetic acid for 20 min at room temperature and analyzed by
MALDI-TOF/MS.

Enzyme Assays and Michaelis-Menten Constant Determination—
Km(app) and Vmax(app) were determined using GraphPad Prism soft-
ware. The data obtained were fitted to the hyperbolic Michaelis-
Menten rate equation. Peptide cleavage was initiated by the addition
of 1 μl of furin (~4 ng) to 100 μl of peptide solution containing 150
mM Tris- HCl (pH 7.4) and 1 mM CaCl2 over a peptide concentration
range of 0.05–20 μM at 37 °C, well within the affinity range of furin
for the peptide substrates used in this work. At 1–2 min intervals
(for up to 10 min), reactions were stopped by the addition of 10 μl of
10% trifluoroacetic acid, followed by injection into a Supelcosil
LC-318 analytical column (250 × 4.6 mm). The intact peptide and its
N-terminal cleaved product were identified by MALDI-TOF/MS.
Cleavage rates were calculated as the ratios of product peak area
versus the sum of the substrate and product peaks at a given reaction
time. The theoretical isotopic distribution of identical amino acid
sequences for each peptide was determined as 0% deamidation
(using Micromass MassLinx software).

PA Peptidase Digestion—The PA heptamerization process was
followed essentially as described previously (17, 61) with the following
modifications. PA samples (2 mg/ml) were cleaved by trypsin (0.25
μg/ml) for 30 min at 37 °C in 50 mM NH4HCO3 (pH 8.0). The reaction
was stopped by 1 mM phenylmethylsulfonyl fluoride (final concentra-
tion). LF was added to the PA samples at various molar ratios (25 °C),
and the mixtures were immediately separated by native gel (4–15%)
electrophoresis (PhastSystem) or by gel filtration on a Superdex 200
10/30 fast protein liquid chromatography column (Amersham Biosciences).

PA Biotinylation and Binding Assay—PA was labeled with biotin
(Pierce kit 21430), at a 1:50 biotin/PA molar ratio according to the
manufacturer’s instructions. Biotin substitution was evaluated follow-
ing PA dialysis at 4 °C in 50 mM NH4HCO3 for 1 h by the 2-hydroxy-
azonbenzene-4'-carboxylic acid assay (Pierce kit 21430). PA binding
studies were performed at 4 °C using biotinylated PA (1.3 mol of biotin/
mol of PA). Chinese hamster ovary cells were grown in 24-well plates to
1.5 × 105 cells/well in α-minimal essential medium and 10% fetal calf
serum in an CO2 atmosphere (62). The PA binding assay was performed
at 4 °C. Biotinylated PA was added at various concentrations (0.25–10
μg/ml) in the absence or presence of a 100-fold molar excess of unla-
beled PA (for nonspecific binding determination). Two h later, cells
were washed with cold phosphate-buffered saline and lysed in 250 μl of
cold buffer solution containing 50 mM Tris-HCl (pH 7.4), 1% Nonidet
P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 2 mM
MgCl2, 10 units/ml DNase, and protease inhibitor mixture (Sigma).

Extracted PA was applied to streptavidin-precoupled enzyme-linked
immunosorbent assay plates (Pierce). Biotinylated bound PA was quan-
titated using guinea pig anti-PA polyclonal antibodies.

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RESULTS

Loss of PA Cytotoxic Activity Correlates with Generation of Acidic Isoforms

PA cytotoxicity in the presence of LF decreased gradually upon storage at 25 °C and pH 8.0 with an apparent half-life of ~7 days (Fig. 1A). Loss of cytotoxic activity was accompanied by gradual appearance of acidic isoforms exhibiting pI values lower than the calculated value of 5.8 (Fig. 1B, right panel) with no apparent molecular breakdown (left panel), suggesting the involvement of a deamidation process (42, 63). Note the presence of some acidic isoforms at time 0 (Fig. 1B, right panel), suggesting that a certain level of deamidation had occurred already during PA isolation and purification (see Fig. 5). To determine putative deamidated residues, we first ranked all of the 68 Asn residues of PA (Protein Data Bank code 1ACC) according to their theoretical deamidation half-lives calculated on the basis of the algorithm of Robinson and Robinson (available at www.deamidation.org) (39), which takes into account empirical studies as well as primary, secondary, and three-dimensional structures of the protein, under standard experimental conditions. This ranking procedure resulted in predicted Asn deamidation half-lives ranging from 24 days (Asn713) to >2.5 × 10^6 days (Asn602). Based on the PA storage time frame used in this study, a cutoff half-life of 60 days was selected. Only five Asn residues with calculated deamidation half-lives of <60 days fall in this category (Fig. 2B). These include Asn10, with no known role in the mechanism of PA cytotoxicity (1); Asn162 at P6 in the cleavage recognition sequence of furin (9, 64); Asn206 in the heptameric pre-pore loop (19); and finally, Asn713 and Asn719 in the receptor-binding large loop domain (20), in close proximity to the PA-receptor direct contact interface region (Gly714–Thr716) (8, 22). Their actual deamidation upon PA storage and the relationships between deamidation processes and PA inactivation were further evaluated.

Analysis of the Five Asn Residues with Predicted Susceptibility to Deamidation

The five Asn residues in PA with the shortest predicted deamidation half-lives (Fig. 2B) were subjected to analysis for possible deamidation by mass spectrometry. During deamidation, one expects a 1-Da mass increase (NH₃ versus OH, Δ = 1 Da) of Asn-containing peptide fragments. This mass shift is used to evaluate the deamidation process at the selected Asn residues (65) as detailed below.

Asn¹⁰ and Asn¹⁶²—In an attempt to mass analyze the peptide fragments encompassing Asn¹⁰ and Asn¹⁶² in domain I, PA was cleaved by trypsin, followed by SDS-PAGE separation of the resulting 20-kDa polypeptide (cf. Fig. 6B). (Note that prior to this treatment, all stored PA preparations contained essentially intact 83-kDa polypeptide.) The 20-kDa band was subsequently gel-digested with Asp-N. MALDI-TOF/MS analysis resulted in three major peptides (suitable for isotopic mass resolution) corresponding to the predicted Asp-N cleavage products: Glu1–Ser25 (2877.4 Da), Asp42–Ser74 (3741.9 Da), and Asp149–Arg167 (2269.3 Da) (Fig. 3B). The Asp-N digestion product Glu¹–Ser²⁵ with a mass of 2877.4 Da displayed a stable isotopic distribution following 0, 14, and 30 days of PA storage (Fig. 3D), implying that Asn¹⁰ (located in this peptide) does not undergo detectable deamidation. On the other hand, the Asp¹⁴⁹–Arg¹⁶⁷ peptide of 2269.3 Da (Fig. 3C) clearly demonstrated a gradual increase in mass of 1 Da, as evident from the decreased intensity of the monoisoionic mass of 2269.3 Da, concomitant with an increased intensity of the successive isotopes, indicating progress in deamidation. The quantitation results indicated 28 and 70% deamidation following 14 and 30 days of PA storage, respectively, compared with time 0. The D¹⁴⁹NLQLPELKQSS¹⁶²SRKKR¹⁶⁷ peptide of 2077.2 Da (Fig. 3C) showed a mass increase of 2 Da, suggesting that at least a second Asn residue in the vicinity of this peptide undergoes deamidation. These results confirm the predicted occurrence of Asn¹⁰ deamidation, consistent with the previously established role of this residue as a deamidation site (66).

Asn⁷¹³ and Asn⁷¹⁹—To determine whether Asn⁷¹³ and Asn⁷¹⁹ underwent deamidation, we selected a 22-kDa Glu₁–Arg⁷⁴ polypeptide fragment of 2057.8 Da, which contains Asp₁⁴⁹–Arg₁⁶⁷, and its Asn⁷¹³ and Asn⁷¹⁹ counterparts, Glu₁–Asp⁴⁲ (2245.9 Da) and Glu₁–Asp²⁰⁶ (2470.9 Da), respectively (Fig. 3B). MALDI-TOF/MS analysis showed that Glu₁–Asp⁴⁲ and Glu₁–Asp²⁰⁶ displayed stable isotopic distributions upon storage, indicating that these peptides do not undergo deamidation. In contrast, Glu₁–Arg⁷⁴ displayed an increase in mass of 1 Da upon storage, suggesting that Asn⁷¹³ and Asn⁷¹⁹ undergo deamidation, consistent with the predicted occurrence of Asn⁷¹³ deamidation (65).

Asn²⁰⁶—To confirm the occurrence of Asn²⁰⁶ deamidation, we selected a 22-kDa Glu₁–Arg⁷⁴ polypeptide fragment of 2057.8 Da, which contains Asp₁⁴⁹–Arg₁⁶⁷, and its Asn²⁰⁶ counterpart, Glu₁–Asp²⁰⁶ (2470.9 Da) (Fig. 3B). MALDI-TOF/MS analysis showed that Glu₁–Asp²⁰⁶ displayed a stable isotopic distribution upon storage, indicating that this peptide does not undergo deamidation. In contrast, Glu₁–Arg⁷⁴ displayed an increase in mass of 1 Da upon storage, suggesting that Asn²⁰⁶ undergoes deamidation, consistent with the predicted occurrence of Asn²⁰⁶ deamidation (65).

In conclusion, we have demonstrated the occurrence of Asn¹⁰ deamidation, consistent with the previously established role of this residue as a deamidation site (66). Moreover, we have provided evidence for the occurrence of Asn⁷¹³ and Asn⁷¹⁹ deamidation, consistent with the predicted occurrence of these residues as deamidation sites (65). These findings are significant because they suggest that deamidation of PA may play a role in the inactivation of PA, potentially by altering its ability to interact with its target cells.
peptide has in fact two Asn residues at positions 150 and 162, which may represent two potential deamidation sites. Based on the algorithm of Robinson and Robinson (39), however, the predicted half-lives of Asn150 and Asn162 are 21 and 38 days, respectively. It is therefore likely that Asn 162 is fully responsible for the observed deamidation. This assumption was further confirmed experimentally using a model peptide of identical sequence, which was deamidated, cleaved by trypsin, and analyzed by mass spectrometry (data not shown).

Asn306—To test the possible deamidation of Asn306 (predicted half-life of 53 days) (Fig. 2), located in heptamerization domain II (residues 259–487) (19), PA samples were digested with endoproteinase Asp-N. Fingerprint MALDI-TOF/MS analysis was used to identify the Asp-N peptide product Asp283–Phe314 at time 0 and following 14 days of PA storage (Fig. 4). The similarity between the spectra obtained and the expected isotopic model distribution of the peptides presented in B with masses of 2269.3 and 2877.4 Da (arrows), corresponding to the fragments carrying Asn162 and Asn10, respectively. Percent deamidation was calculated as the ratio of the monoisotopic intensity value versus the sum of all isofrom intensities of the same peptide.

Within the exposed "large loop" of 20 amino acids (positions 703–722), which was suggested to be involved in PA-receptor interaction (8, 20, 22), and in close proximity to one of the PA-receptor contact interfaces (amino acids 714–716) (22). Tryptic mass fingerprints of native PA at time 0 and following 7 and 14 days of storage were analyzed and compared with the model isotope distribution of an identical peptide sequence. As shown in Fig. 5A, the theoretical monoisotopic ratio of the expected tryptic amino acid peptide sequence, 0.32 (Fig. 5A and legend to Fig. 3C), was used as a reference point for 0% deamidation. Unexpectedly, the monoisotopic ratio of the tryptic fragment from the PA sample at time 0 was 0.14, indicating >50% deamidation. This suggests that a deamidation process had already occurred in this region during PA isolation and purification (see acidic isoform distribution in Fig. 1B at time 0). It should be noted that this analyzed tryptic peptide, E704NTIINPENGDTSTNGIKK723, has in fact four potential deamidation sites at Asn705, Asn709, Asn713, and Asn719, with predicted short deamidation half-lives of 24 and 49 days (Fig. 2), respectively, are located...
ollowing incubation for 10 days at 37 °C resulted in a 2-Da gain in mass (data not shown), indicating complete deamidation of two Asn residues. This deamidated peptide was further analyzed by Edman amino acid sequence analysis. As shown in Fig. 5B, the phenylthiohydantoin (PTH)-Asn signals at P2 and P6 (Asn705 and Asn709) were not affected upon deamidation compared with the control untreated peptide, whereas the PTH-Asn residues at cycles 10 and 16 (corresponding to Asn713 and Asn719) were completely replaced with PTH-Asp residues, clearly indicating a deamidation process at these specific Asn sites (Fig. 5B).

**Possible Relationship between Deamidation and PA Functional Impairment**

The Furin Cleavage Step—Cleavage of PA by furin at RKKR167↓ is a crucial step in the PA mechanism of action, allowing its heptamerization and co-internalization with either LF or EF into target cells (9, 64). To examine whether PA inactivation during storage correlates with deamidation of Asn162 in the P6 recognition sequence (Fig. 6A), we first examined the susceptibility of PA to furin cleavage at different storage times. As shown in Fig. 6 (B and C, left panels), the native PA fraction could be readily cleaved by furin (time 0). However, stored fractions became progressively insensitive to cleavage. To ensure that reduced PA sensitivity to furin upon storage is not caused by hindering its furin cleavage site due to nonspecific protein denaturation and aggregation (33), we subjected the same stored samples to tryptic digestion. Trypsin cleavage efficiency was not significantly affected by PA storage (Fig. 6, B and C, right panels). The limited cleavage of PA by trypsin mainly at Arg167 is also consistent with the conclusion that the overall conformation of PA is preserved.

**FIGURE 4.** Monitoring Asp306 deamidation by mass analysis of the Asp-N peptide fragment Asp283–Phe314. PA samples (2 mg/ml) at time 0 and after 14 days of storage were digested with 2 μg/ml Asp-N for 3 h at 37 °C and analyzed by MALDI-TOF/MS. The peptide of interest with a calculated mass of 3532.7 Da was identified in both samples and compared with the isotopic model (Micromass MassLinx software). Isotopic distribution of the model overlaps the experimental distribution of the peptides at time 0 and following 14 days of PA storage, suggesting that PA does not undergo deamidation at Asn306 during storage. Monoisotopic, monoisotopic.

**FIGURE 5.** Monitoring the peptide fragment Glu704–Lys723 following tryptic digestion of stored PA. A, PA samples (2 mg/ml) at time 0 and following 7 and 14 days of storage were denatured at 95 °C for 2 min in 0.2% SDS and digested with trypsin (25 ng/ml) for 2 h at 37 °C. Digests were analyzed by MALDI-TOF/MS at 1:10 and 1:100 dilutions in 0.1% trifluoroacetic acid. The calculated monoisotopic ratios and percent deamidation are listed. The unexpected low monoisotopic ratio at time 0 compared with the isotopic model indicates deamidation of this peptide in the native PA preparation. B, Edman N-terminal sequencing (Applied Biosystems Model 492 sequencing system) of the native and deamidated model synthetic peptides. The arrows indicate sequence direction. X indicates unidentified PTH-derivatives. PTH-Asp and PTH-isosuccinic acid co-eluted under the separation conditions of on-line HPLC. The deamidated peptide showed no traces of Asn residues in cycles 14 and 19.
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Asn\textsuperscript{162} Deamidation Reduces Furin Cleavage Efficiency—To examine whether the reduced sensitivity of stored PA fractions to furin, from 95% to only 50% cleavage upon 30 days of PA storage (Fig. 6C), is indeed due to the observed deamidation at P\textsubscript{6} in the furin recognition sequence, a model peptide (L\textsubscript{156}KQKSSRKKKR\textsubscript{170}) encompassing the furin cleavage site in PA was synthesized as a substrate for furin. This peptide was further subjected to deamidation \textit{in vitro} (see “Experimental Procedures”). Deamidation of this peptide is clearly manifested by a typical decrease in the percent PA cleavage by furin or trypsin as determined by densitometric quantitation of PA and PA-derived protein bands in \(B\).

Heptamerization of PA Is Not Affected by Prolonged Storage—Heptamerization, involving domains II and III of the PA molecule (16, 18, 19), has a central role in PA cytotoxicity. Although no evidence for Asn\textsuperscript{306} deamidation could be detected (Fig. 4), it may still be possible that other deamidation processes could be indirectly involved in PA heptamerization impairment, which may in turn contribute to the

![FIGURE 6. Effect of PA storage on its sensitivity to cleavage by furin and trypsin. A, shown is the amino acid sequence surrounding the furin/trypsin cleavage site (arrowheads). Ser\textsuperscript{160} and Ser\textsuperscript{170} are indicated for orientation. B, PA samples were cleaved by either furin (left panel) or trypsin (right panel) following PA storage for the indicated time periods and analyzed by SDS-PAGE (Coomassie Blue stain). Intact PA (83 kDa) and its cleaved products (63 and 20 kDa) are indicated. C, shown is the percent PA cleavage by furin or trypsin as determined by densitometric quantitation of PA and PA-derived protein bands in \(B\).](image-url)

\(V\)
FIGURE 7. Testing the native and deamidated peptides as potential substrates for furin. A, the model peptide used as the synthetic furin substrate (TABLE ONE, Peptide 1) was stored for the indicated time periods and analyzed by MALDI-TOF/MS, resulting in a deamidated peptide (Deam. Pep.) substrate (Peptide 2). A deamidation $t_{1/2}$ of 2.6 days was calculated based on first-order kinetics. B, the native and deamidated peptides (10 μM each) were incubated in the presence of furin (40 ng/ml) for 60 min, and the reaction mixtures were analyzed by MALDI-TOF/MS. The arrows indicate the peptide products. C, the furin cleavage rates of the native and deamidated peptides was monitored by reversed-phase HPLC. The native and deamidated peptides (10 μM each) were incubated in the presence of 40 ng/ml furin for 10 min. The arrowheads indicate the N-terminal fragment product Ac-LKQSSN5SRKKR peak (identified following collection and analysis by MALDI-TOF/MS; not shown). The indicated percent cleavage was calculated as the substrate peak area versus the sum of peak areas of the product and the remaining substrate peaks. The lower curve represents the initial furin (40 ng/ml) cleavage rates of the native and deamidated peptides versus peptide substrate concentrations.
observed inactivation of PA. The effects of storage on the PA heptamer-
cleaved PA samples upon the addition of LF toxin at increasing
LF/PA molar ratios (0–1.5 mol/mol) (see “Experimental Procedures”).
No apparent differences could be observed between the oligomerization
patterns of the native versus 85% inactivated (14 days) stored samples as
monitored by native gel analysis (18) and gel filtration experiments (data
not shown). We thus conclude that the heptamerization process does
not contribute to PA inactivation upon storage.

**PA Binding Is Impaired during Storage**—As shown above, the two
susceptible sites (Asn173 and Asn179) were already partially deamidated
at time 0 and underwent further deamidation upon PA storage. These
residues are located in close proximity to one of the receptor interface
segments (Gly714–Thr716) (22). This proximity might affect the effi-
ciency of binding of stored PA to cell receptors, impairing its cytotoxic
activity. For binding assays, PA was first labeled with biotin at a bio-
tin/PA molar ratio of 2, resulting in the substitution of 1.3 mol of biotin/
mol of PA, which had no significant effect on its cytotoxic activity (data
not shown). Binding experiments using biotinylated PA samples after 0,
7, and 14 days of storage were then performed. As shown in Fig. 8, the
maximum binding site value for native PA (stored at −20 °C) was found
to be 13,000 PA molecules/Chinese hamster ovary cell, in agreement
with a previous study (62). A sharp decrease in PA binding of 47 and 85%
was observed following 7 and 14 days of storage, respectively, concom-
itant with a similar loss of cytotoxic activity (Fig. 1).

**DISCUSSION**

PA is of great pharmacological importance as a vaccine against
anthrax. In addition, PA is also an attractive model system for studying
the impact of deamidation on protein-protein interaction and the
mechanism of action. PA has a relatively high abundance of Asn resi-
dues (Asn/total amino acid ratio of 0.09 versus a normal statistical dis-
tribution of 0.05) (21, 67). Furthermore, PA has a complex mechanism
of action, involving multiple forms of protein-protein interactions: a
substrate for furin, as a ligand to cell receptors, as a self-forming homo-
oligomer (heptamer), and as a complex with LF and EF (6, 16, 68). We,
as well as others (33, 69), have observed that, under various storage
conditions, PA loses its cytotoxic activity in macrophage cell line assays.
We have demonstrated here that, under mild conditions (25 °C, pH 8.0),
loss of activity is not due to proteolytic degradation or denaturation
because the inactivated PA preparations preserve (a) their molecular
integrity (Fig. 1B, left panel) and (b) their global conformation, as indi-
cated by their limited trypsin cleavage pattern (Fig. 6, B and C, right
panels), confined mainly to the Arg67–Ser168 furin cleavage bond. On
the other hand, loss of biological activity upon storage appears concom-
itant with the formation of new acidic isoforms (Fig. 1B). This phenom-
emon of multiple acidic isoform formation has been demonstrated pre-
viously to be the hallmark of deamidation (40, 42, 43, 54, 63). When all
PA Asn residues were ranked on the basis of their predicted deamida-
tion upon storage. The five Asn residues (Fig. 2) are scattered in protein
domains, which may be critical for PA biological function: two residues
are located in domain I (Asn10, in a region with no known function, and
Asn162 at the proximity of the furin cleavage site); one residue (Asn306)
is located in domain II within the heptamerization insertion loop; and
two residues (Asn713 and Asn719) are located in domain IV in the PA
receptor-binding region. Heptamerization, cell receptor binding effi-
ciency, and cleavage susceptibility to furin were therefore monitored in
an attempt to correlate functional impairment with Asn deamidation.

**Heptamerization Process**—According to the current model (70),
furin cleavage enables the polymerization of PA as heptamers on the cell
surface, which then interact with either LF or EF. Because this hepta-
merization involves the interaction of domains II and III (16, 18), it
was of interest to evaluate whether this complex process is impaired
upon 14 days of PA storage concomitant with the deamidation of Asn306
and cytotoxic inactivation. As shown in Fig. 4, Asn306 did not appear to
undergo deamidation. In addition, heptamerization proceeded similarly
in the fully activated PA as well as inactivated stored PA preparations as
judged by native gel analysis and size exclusion chromatography exper-
iments (data not shown). We conclude that heptamerization is probably
not related to the observed PA inactivation upon storage.

**PA Binding to Cell Receptors**—PA binding to cell receptors involves
its C-terminal domain IV (8, 16, 20, 22). Studies of the PA-receptor
complex implied direct interaction of PA with the receptor at the con-
tact region between positions 714 and 716 (8, 22). We observed that
both Asn713 and Asn719, with predicted short half-lives of 24 and 49
days, respectively, underwent deamidation (Fig. 5) with concomitant
impairment of PA binding (Fig. 8) and reduced cytotoxicity (Fig. 1). It is
thus tempting to speculate that the close vicinity of the two susceptible
Asn residues to a PA-receptor contact region may impair PA binding
upon deamidation, resulting in a decrease in LF-mediated PA cytotoxi-
city. Yet, in view of site-directed mutagenesis studies (21) in which
Asn713 and Asn719 replacement with Ala had no substantial effect on PA
toxicity, it is difficult to determine the contribution of deamidation at
these positions to the overall decrease in PA cytotoxicity.
PA Proteolytic Cleavage by Furin—Asn\textsuperscript{162}, a predicted candidate residue for deamidation (Fig. 2), is located six amino acids (P\textsubscript{6}) upstream of the site of serine cleavage by furin. Furin typically cleaves protein substrates at the consensus sequence Arg-Xaa-(Lys/Arg)-Arg (9, 71). Although the requirements for Arg at P\textsubscript{4} and P\textsubscript{6} have been documented extensively in the majority of furin-processed proproteins, those at P\textsubscript{4} are less characterized. Yet, in furin natural protein substrates, cleavage requirements are restricted merely to alkaline residues at either P\textsubscript{4} or P\textsubscript{6} (72–74). Thus, the importance of the amino acid at P\textsubscript{6} is expressed mainly when a basic residue at P\textsubscript{4} is missing. We have demonstrated here that, upon storage, PA sensitivity to furin decreases concomitant with substantial deamidation of Asn\textsubscript{162}. To test the hypothesis of the causal relationship between deamidation at P\textsubscript{6} and PA inactivation, we compared the efficiency of furin cleavage of a synthetic peptide substrate in its native state and following deamidation. In addition, we generated a series of peptide substrate analogs to directly investigate the major determinants affecting furin cleavage efficiency upon deamidation. In quantitative kinetic studies, we have demonstrated that deamidation impairs furin cleavage efficiency due to the insertion of a negatively charged residue at P\textsubscript{6} in the furin recognition sequence (TABLE ONE). These results are consistent with the notion that PA inactivation upon storage is at least partially due to PA deamidation at Asn\textsubscript{162}, resulting in its reduced sensitivity to furin (Figs. 6 and 7). Indeed, the important role of P\textsubscript{6} in the RKRK recognition sequence (in which P\textsubscript{4} is positively charged) has been addressed previously (75). In addition, a recent three-dimensional x-ray model of furin demonstrates a canyon-like crevice of the active site with a negative surface potential (Glu\textsuperscript{230} and Asp\textsuperscript{233}) adjacent to P\textsubscript{6} of the substrate (16). Although deamidation of Asn\textsubscript{162} may reduce the efficiency of PA cleavage by furin and contributes to the overall reduced PA cytotoxicity upon storage, it cannot explain the extensive reduced PA cytotoxicity (75%) (Fig. 1) following 14 days of storage because, at this time, Asn\textsubscript{162} underwent only 28% deamidation (Fig. 3B), with an ∼30% reduced cleavage by furin (Fig. 6C). It is thus conceivable that additional factors, such as binding impairment, are involved in the PA inactivation mechanism (76).

Physical Factors Affecting PA Deamidation Rates—In good agreement with the predicted rules (39), we have found that the three Asn residues with the predicted shortest half-lives indeed undergo deamidation. It should be noted that the "standard storage conditions" applied for theoretical predictions were 37 °C at pH 7.4 (39), whereas we applied 25 °C at pH 8.0. In addition to the nearest neighbor effect of the intrinsic protein and its three-dimensional structure, deamidation susceptibility is highly dependent on various incubation conditions, such as temperature and pH, which were shown to have the most profound effect on deamidation. For example, it has been shown recently that deamidation of glutamate dehydrogenase produced in Escherichia coli can be substantially reduced by temperature decreases during growth and during protein isolation (63). Additional studies have indicated the pH effect on the deamidation rate (77). Indeed, preliminary studies in our laboratory clearly indicate a decrease in the deamidation rate upon PA storage (as deduced from the reduction in the appearance of acidic isoforms) at pH 7.4 and at lower temperature coconcomitant with an improved biological stability of the protein.

In summary, five of the 68 Asn residues in B. anthracis PA were selected and analyzed based on their predicted deamidation half-lives for their possible involvement in protein inactivation upon storage. Indeed, three of these residues have been demonstrated here to undergo spontaneous deamidation. These deamidation events could be correlated with a decrease in PA cytotoxicity, caused by impairment of two distinct steps in PA cytotoxicity: cell receptor binding and furin cleavage efficiency. The possible effect (if any) of PA deamidation on the induction of protective immunity by PA-based vaccines remains to be determined.

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