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Research Article

Chemical Pathways of Peptide Degradation. I. Deamidation of Adrenocorticotropic Hormone

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Deamidation of Asn residues is one of the major chemical pathways of degradation of proteins and peptides. Adrenocorticotropic hormone (ACTH), a 39-amino acid polypeptide with a single Asn residue, was shown in this study to be a useful model polypeptide for the study of the effects of pH and buffer concentration on the rate and pathway of deamidation. The disappearance of ACTH and appearance of deamidated ACTH were monitored by isoelectric focusing (IEF), and ammonia production was monitored spectrophotometrically using a coupled enzymatic assay. Using these analytical methods, the deamidation of ACTH was shown to follow pseudo-first-order kinetics and was dependent on pH and buffer concentrations. The separation of the deamidated ACTHs (Asp-ACTH and isoAsp-ACTH) from ACTH was successful, but attempts to separate Asp-ACTH from isoAsp-ACTH using cation-exchange HPLC and IEF were unsuccessful. Using bovine protein carboxymethyltransferase (PCM), which selectively methylates the carboxyl group of isoAsp residue, the isoAsp-ACTH could be detected at pH 7.0 and 9.6 but not at pH 1.9. These data support the hypothesis that under neutral and alkaline conditions, deamidation of ACTH proceeds through the formation of a cyclic imide intermediate (slow step), followed by its hydrolysis to the Asp-ACTH and isoAsp-ACTH (fast step). Under acidic conditions, the reaction appears to proceed via direct hydrolysis of the Asn residue to form Asp-ACTH without the formation of a cyclic imide intermediate.

KEY WORDS: deamidation; aspartyl, asparaginyl; isoaspartyl, ACTH; protein carboxymethyltransferase; cyclic imide.

INTRODUCTION

With the advent of biotechnology, the large-scale preparation of proteins for pharmaceutical purposes is now feasible. Development of rational strategies for stabilization of these biotechnology products will require an understanding of the many physical and chemical pathways by which proteins degrade.

Deamidation of Asn[3] residues is one of the major chemical pathways of degradation of proteins and peptides (1–4). Deamidation of Asn residues in proteins and peptides results in formation of a normal α-linked Asp residue and/or a β-linked Asp residue (isoAsp) (5). Asp residues resulting from deamidation of Asn residues were detected in the early sequence studies of bovine ribonuclease (6) and pseudomonas cytochrome c-551 (7). Deamidated forms of insulin (8) and bovine seminal ribonuclease (9) have also been reported.

Deamidation of specific Asn residues has been linked to changes in the biological activity of proteins (2–4,10–14). For example, deamidation of Asn residues was found to be a major pathway for degradation of yeast triosephosphate isomerase (4), hen egg-white lysozyme (2), ribonuclease (3), epidermal growth factor (13), and calmodulin (14). A unique stabilization strategy for yeast triosephosphate isomerase involving the replacement of Asn^{14} and Asn^{78} by Thr^{14} and Ile^{78}, respectively, was recently reported (4). This genetically engineered protein was much more stable to heat inactivation than the native form of the enzyme. Deamidation of Asn residues not only occurs *in vitro*, but the biological significance of this reaction *in vivo* is currently under intense investigation (15).

At alkaline and neutral pHs, the deamidation of proteins and peptides containing Asn residues is believed to proceed through a cyclic imide intermediate formed by the intramolecular attack of the peptide bond nitrogen of the succeeding peptide group on the side-chain carbonyl carbon of Asn residue with the liberation of ammonia (5,16). The cyclic imide is spontaneously hydrolyzed to form a mixture of Asp and isoAsp peptides in which the peptide backbone is attached to the Asp residue via an α-carboxyl linkage and a β-carboxyl linkage, respectively. Limited information is available concerning the mechanism of hydrolysis of Asn residues under acidic conditions. Meinwald *et al.* (17) have reported that the dipeptides, Asn-Gly and isoAsn-Gly, yield only Asp-Gly and isoAsp-Gly, respectively, when incubated in 1 *N* HCl at 37°C. These data suggest a direct hydrolytic mechanism rather than formation of a cyclic imide intermediate.

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[3] Unless otherwise noted, all amino acids listed are L-enantiomers of the 20 common amino acids and are referred to by their three-letter abbreviations.

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Most of the detailed deamidation studies have used small, synthetic peptides (1,5,18,19). By selecting small, synthetic peptides, it has been possible to separate the individual deamidated products (Asp and isoAsp peptides) by HPLC (5,19). NMR has also been employed to study the deamidation of small, synthetic peptides (18). With larger peptides and proteins, most investigators have employed protein carboxymethyltransferase (PCM) for specific methylation, thus identifying the isoAsp-containing peptide (15,20–24).

From the studies reported to date, various factors influence the rates of deamidation of Asn residues in proteins and peptides. These include (a) exogenous factors such as pH, temperature, and buffer concentrations (1–5,10–14); (b) the conformation of the protein (18,25–27); and (c) the nature of the amino acid residue on the C-terminal side of the Asn residues (1,5,17). Very little information is available concerning the influence of exogenous factors, conformation, and adjacent amino acids on the distribution of the deamidated products (e.g., ratio of isoAsp- to Asp-containing peptides) (5,19). In this study we have selected adrenocorticotropic hormone (ACTH), a polypeptide with 39 amino acid residues including a single Asn residue, as a model peptide to study the deamidation reaction. Attempts were made to develop methodology for the separation of native ACTH from the deamidated ACTHs, as well as for the separation of Asp-ACTH from isoAsp-ACTH using cation-exchange HPLC and isoelectric focusing (IEF). PCM was also employed to detect the presence of the isoAsp-containing peptide. Using these analytical procedures we have studied the influence of pH and buffer concentration on the rate of deamidation of ACTH and the distribution of the deamidated products.

MATERIALS AND METHODS

Materials

Reagents and chemicals were purchased from the following commercial sources: *S*-adenosyl-L-[*methyl*-^{3}H]-methionine ([^{3}H]S-AdoMet), 15 Ci/mmol (ICN Pharmaceuticals, Inc., Irvine, CA); porcine ACTH (70–90 IU per mg), urea (98+%), Coomassie Brilliant Blue G, trichloroacetic acid (anhydrous, crystalline), DL-aspartic acid, γ-globulin, and AdoMet (Sigma Chemical Company, St. Louis, MO); carboxymethylcellulose (CMC) (preswollen, microgranular, cation exchanger CM52) Whatman Ltd., Maidstone, Kent, England); ammonium acetate (HPLC grade) and ACS-grade buffer components (Fisher Scientific, St. Louis, MO); acrylamide, *N,N'*-methylenebisacrylamide, ammonium persulfate (all ultrapure), Ripel Silane, GelBond PAG films (124 × 258 mm), Mylar sheets, and electrode strips (LKB, Bromma, Sweden); Pharmalyte (pH 3–10) and analytical IEF plastic sample applicators (Pharmacia, Uppsala, Sweden); Amberlite (MB-6) (Serva Feinbiochemica, Heidelberg/New York); and ammonia assay kit (Sigma Diagnostics, St. Louis, MO). Deionized, distilled water was used in all the experiments.

Purification of ACTH, AdoMet, and Protein Carboxymethyltransferase (PCM)

ACTH was purified to homogeneity by chromatography on CMC according to the method of Aswad (20). AdoMet was purified to homogeneity by chromatography on CMC according to the method of Chirpich (28). The type I isozyme of PCM was purified from bovine brain as described by Aswad and Deight (29). The purified enzyme had a specific activity of 5000–8000 pmol/min/mg at 30°C, with γ-globulin as the methyl-accepting substrate, and contained no detectable endogenous methyl-accepting activity.

Kinetic Experiments

All incubations for kinetic studies were carried out in Pierce Reacti-Vials. The kinetic studies were done in duplicate using 10 m*M* HCl (pH 1.9), 100 m*M* sodium acetate (pH 5.0) 5 m*M* sodium phosphate (pH 7.0), and 10, 50, and 100 m*M* glycine (pH 9.6) buffers. The ionic strength was maintained at 0.1 *M* using NaCl. The pH of all buffers at 37°C was determined using a digital pH/mV meter (Model 701A) from Orion Research, Inc. The glassware used to make the solutions, the pipetting syringes, the Reacti-Vials, and the vial seals were autoclaved, and the buffers and water were filtered through 0.45-μm filter paper for all the kinetic experiments. The solutions were made and pipetted into the vials under aseptic conditions. The concentration of ACTH was maintained at about 3 mg/ml in all experiments. From a solution of pure ACTH in sterile water, samples were pipetted into the vials for IEF analysis (50 μl), PCM assay (50 μl), and ammonia assay (250 μl). The samples were immediately frozen in dry ice–acetone mixture and lyophilized. The deamidation reaction was started by adding an appropriate amount of buffer, and the tightly sealed vials were incubated in a 37°C water bath. The reaction was terminated by removing duplicate vials from the water bath at appropriate time intervals, freezing in dry ice–acetone mixture, and storing at −70°C until analysis. After the incubations were completed, the collected samples were analyzed by IEF to monitor the disappearance of ACTH and the appearance of deamidated ACTH. The appearance of ammonia was monitored spectrophotometrically. The appearance of the isoAsp peptide was monitored by subjecting the samples to the PCM assay procedure described below.

Analytical Procedures

Isoelectric Focusing. For a 0.4-mm-thick gel, 5 ml of stock solution (29.1% acrylamide + 0.9% *N,N'*-methylenebisacrylamide), 1.26 ml of Pharmalyte (pH 3–10), water (1.06 ml), and an aqueous solution (11.99 ml) of urea (6 *M*, pretreated with Amberlite) were mixed and degassed for 15 min. Just before casting, an aqueous solution (112 μl) of ammonium persulfate (0.025 g in 250 μl) was mixed and the gel was cast on GelBond PAG film, using an Ultramound Gel Casting Unit, and run on an electrophoresis unit (2117 Multiphor 11), all supplied by LKB (Bromma, Sweden). The power supply unit (Model 494) was from ISCO Inc., Lincoln, Nebraska. The gels were maintained at 7.8°C using a refrigerated constant-temperature bath and circulator (Model 9000) from Fisher Scientific (St. Louis, MO). DL-Aspartic acid (0.04 *M*) was the electrolyte at the anode and NaOH (0.5 *M*) was the electrolyte at the cathode. The gel was prefocused for 45 min at 1000 V, 20 mA, and 6 W. Then the power supply was turned off and 7 μl of samples (21 μg of peptide)

was applied on the gel surface using Hamilton micropipette syringes at predetermined positions for each buffer. The focusing was carried out for 45 min at 2000 V and 13 W. The gels were run at constant wattage. On completion of focusing, the gels were stained with Coomassie Brilliant Blue G by the procedure of Righetti and Chillemi (30) and quantitated using a system consisting of a dual-wavelength gel densitometer (CS-930) and a data recorder (DR-2), supplied by Shimadzu Corporation, Kyoto, Japan).

Ammonia Assay. The coupled enzymatic ammonia assay was performed spectrophotometrically at 340 nm by following the exact manual procedure (No. 170-UV) provided by Sigma Diagnostics (St. Louis, MO), using a UV-VIS recording spectrophotometer (UV-260) from Shimadzu Corporation (Kyoto, Japan). The reductive amination of 2-oxoglutarate to form glutamate, using glutamate dehydrogenase (GLDH) and reduced nicotinamide adenine dinucleotide (NADH), is coupled with the oxidation of NADH to NAD on a molar equivalent basis as shown in the following equation.

$$\text{2-oxoglutarate} + NH_3 + \text{NADH} \xrightarrow{\text{GLDH}} \text{glutamate} + \text{NAD}$$

PCM Assay. Methylation reactions were carried out at 30°C for 45 min in 1.5-ml microfuge tubes as described by Aswad and Deight (29). In a final volume of 50 μl, the mixture contained citrate/phosphate/EDTA buffer (pH 6.0), 4 μmol of purified PCM, 200 μmol of [^{3}H]S-AdoMet (200–250 dpm/pmol), and appropriate amounts of peptide (10 μl of 1:10 diluted ACTH solution for pH 9.6 and 5 μl of ACTH solution for pH 1.0 and 7.0). The reaction was initiated by adding the [^{3}H]S-AdoMet. Blanks lacked the peptide substrate. All reactions were stopped by the addition of 1 ml of 10% (w/v) TCA. γ-Globulin [20 μl (25 mg/ml)] was added as a carrier to assure effective precipitation of ACTH. After repeated centrifugation and resuspension, the final TCA pellet was dissolved in 100 μl of 0.5 *M* NaOH:0.05% (w/v) Triton X-100:1% (w/v) methanol. One milliliter of scintillation fluid was added directly to the tube for counting. All assays were performed in duplicate.

Kinetic Calculations

As the reaction kinetics were pseudo-first order, the rate of degradation of ACTH was obtained by the semilogarithmic plots of percentage of ACTH remaining versus time, taking the quantitative reading for the ACTH band at zero time as 100%. The value of the slope of these plots is the rate constant. The rates of appearance of deamidated ACTH and ammonia were obtained by using the following equation:

$$\ln [(A_t - A_f)/(A_i - A_f)] = -kt$$

where A_f = the total theoretical amount of the product, assuming a total conversion of ACTH into deamidated ACTH and, in the case of ammonia, the total theoretical amount of ammonia expected from ACTH; A_t = the amount of the product at any time t; A_i = the initial amount of the product at $t = 0$; t = time; and k = the rate constant.

RESULTS AND DISCUSSION

Purification of ACTH

ACTH was purified by chromatography on CMC using the procedure described by Aswad (20). To check the purity of ACTH, IEF experiments were conducted. As shown in Fig. 1, the ACTH which had been purified by chromatography on CMC exhibited a single band with a pI = 7.8. The pI value obtained in our laboratory for ACTH is consistent with a value of 7.9 reported in the literature (31). The purity of ACTH was further checked by chromatographing a sample of purified ACTH onto a CMC column (data not shown). Elution of a single peak having the same retention volume as

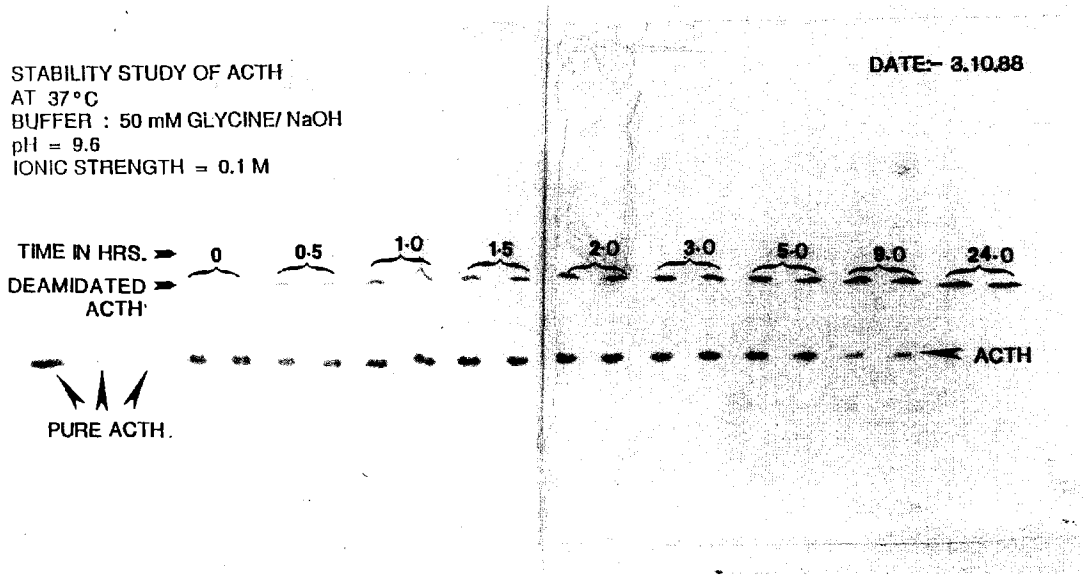

Fig. 1. Typical isoelectric focusing gel, showing separation of ACTH from deamidated ACTHs (isoAsp-ACTH + Asp-ACTH) and the time course of deamidation of ACTH at pH 9.6, 50 m*M* glycine buffer (I = 0.1 *M* with NaCl) at 37°C.

previously observed further verified the purity of the ACTH and demonstrated that the ACTH was not undergoing deamidation during chromatographic purification.

Separation of Native Peptide from Deamidated Peptides

Separation of native ACTH from the deamidated ACTHs (mixture of isoAsp and Asp peptides) was achieved using IEF (Figs. 1 and 2) and cation-exchange HPLC (data not shown). The optimum IEF conditions for achieving sharp, clean separation of the native ACTH from the deamidated ACTHs were found to be 1000 V, 6 W, and 20 mA during prefocusing and 2000 V and 13 W during focusing. Successful separation of the native ACTH from the deamidated ACTHs resulted because of the additional negative charge associated with the Asp (or isoAsp) residue in the deamidated peptides compared to the native ACTH. Unfortunately, we were unsuccessful in separating the two deamidated ACTHs (e.g., Asp peptide from isoAsp peptide) by either IEF (Figs. 1 and 2) or cation-exchange HPLC (data not shown).

A hexapeptide (Val-Tyr-Pro-Asn-Gly-Ala) derived from residues 22–27 of ACTH and its two deamidated peptides (Asp- and isoAsp-containing) have been separated by HPLC (5,19). In these smaller peptides, the differences in the physical characteristics (e.g., pK_as of Asp and isoAsp residues, solution conformations, hydrophobicity, dipole moment, etc.) must be sufficient to allow for separation of these peptides. These differences in the physical characteristics of the intact ACTH must be masked by other physicochemical characteristics of the peptides which prevent their separation by IEF and HPLC.

Detection of isoAsp Containing ACTH Using PCM

PCM is known to methylate selectively the carboxyl group of an isoAsp residue, whereas it does not methylate the carboxyl group of an Asp residue (9,15,20,21). When pure ACTH was deamidated at pH 7.0 (5 m*M* phosphate) and at pH 9.6 (50 m*M* glycine), the time-dependent formation of the isoAsp peptide could be detected using the PCM assay (Fig. 3). However, the quantitation of isoAsp and Asp peptides was unsuccessful due to the high standard deviation observed in the PCM assay.

The detection of β-isomerized deamidation products of many proteins and peptides has implied cyclic imide formation as an intermediate in the deamidation reaction at alkaline and neutral pHs (5,9,17,23). Thus the appearance of the isoAsp form of deamidated ACTH at pH 7.0 (Fig. 3A) and pH 9.6 (Fig. 3B) suggests that the deamidation of ACTH at alkaline and neutral pHs proceeds via formation of a cyclic imide, which hydrolyzes to give the Asp and isoAsp peptides (Fig. 4).

Cyclic imides could also be formed directly from the Asp and isoAsp peptides as shown in Fig. 4; however, this interconversion is much slower than the deamidation of the Asn peptides at the pHs chosen for these deamidation studies (5). The possibility of initial formation of an isoimide (Fig. 4) cannot be ruled out in our study, because it is known that rearrangement reactions may convert isoimide products to cyclic imides (32,33). The isoimide could be formed as a result of the nucleophilic attack of the peptide bond carbonyl oxygen on the carbonyl carbon of the side-chain amide linkage of Asn peptide (34).

The isoAsp form of deamidated ACTH was not detected when deamidation of ACTH was carried out in pH 1.9 (10

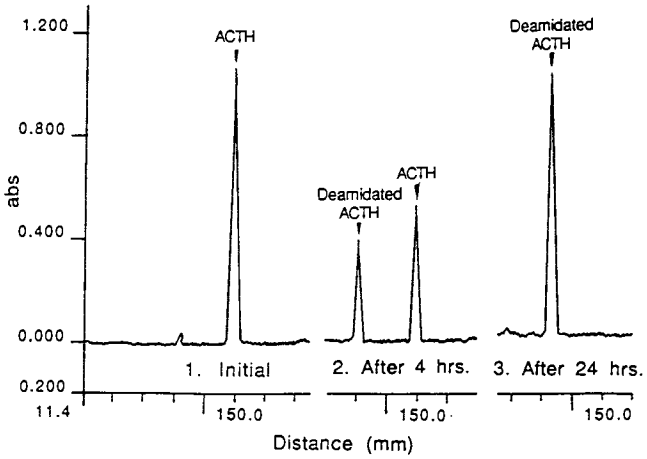

Fig. 2. Typical chromatogram obtained from scanning the IEF gel shown in Fig. 1 using a gel densitometer. Separation of ACTH (disappearing peak) from deamidated ACTHs (appearing as isoasp-ACTH + Asp-ACTH) is clearly illustrated. Reaction conditions are those described in Fig. 1.

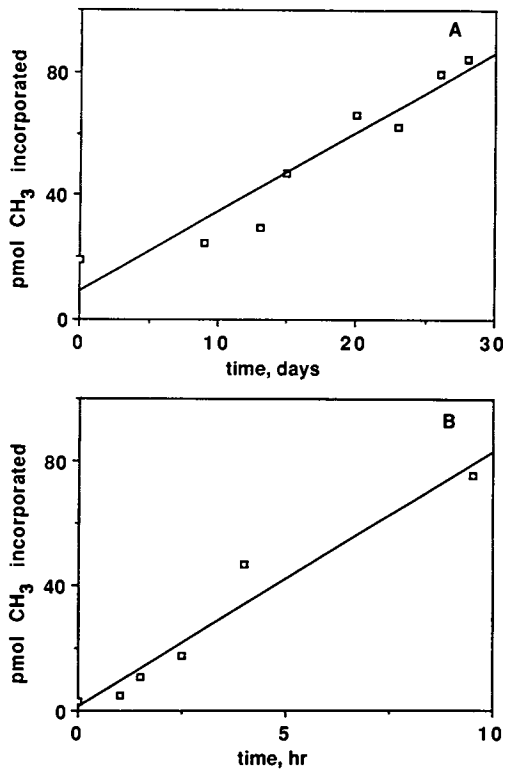

Fig. 3. Time course for appearance of isoAsp residue in deamidated ACTH as determined by PCM-catalyzed methylation reaction. (A) Deamidation of ACTH (3284 pmol of ACTH/5 μl of stock solution) at pH 7.0, 5 m*M* phosphate buffer (I = 0.1 *M*) at 37°C. (B) Deamidation of ACTH (657 pmol of ACTH/10 μl of stock solution) at pH 9.6, 50 m*M* glycine buffer (I = 0.1 *M*) at 37°C. PCM analysis was conducted as described under Materials and Methods.

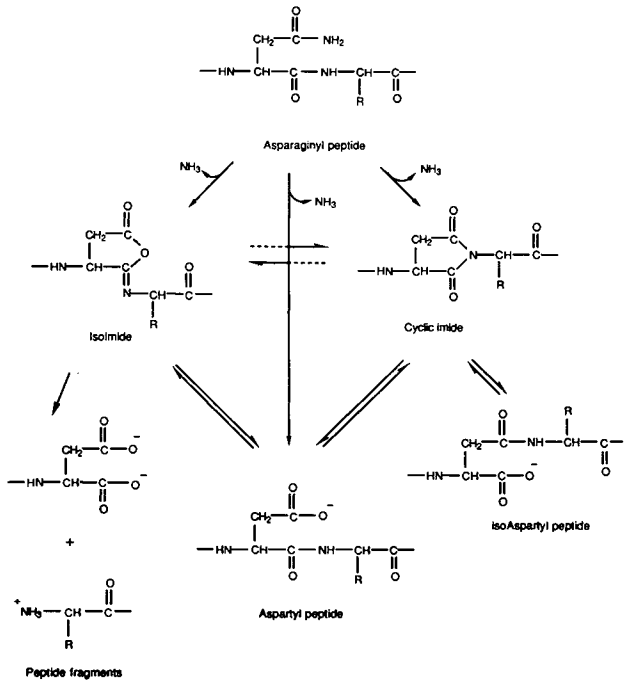

Fig. 4. Proposed pathway for degradation of Asn peptide. The cyclic imide intermediate has been shown by Geiger and Clarke (5) to undergo racemization forming a mixture of the D- and L-cyclic imide peptides. This phenomenon has not been illustrated in this figure.

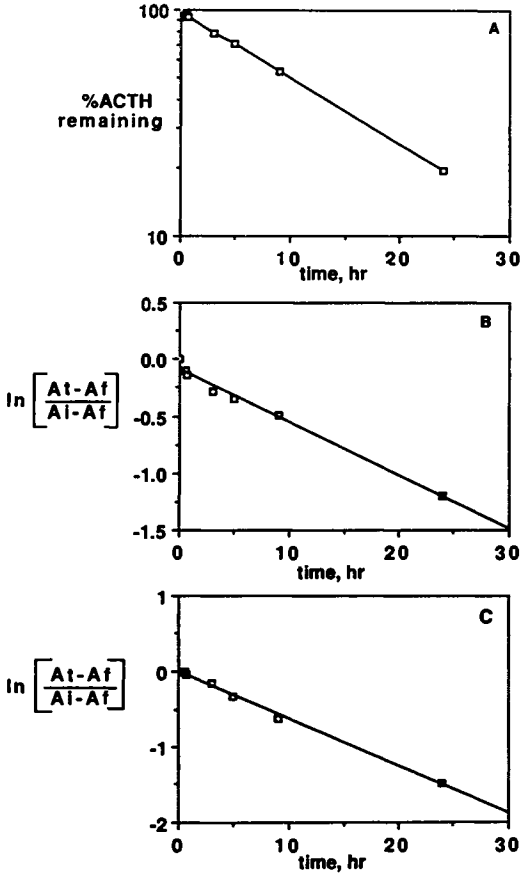

Fig. 5. Pseudo-first-order kinetics of deamidation of ACTH at pH 9.6 (50 m*M* glycine, I = 0.1 *M*) at 37°C. (A) Disappearance of ACTH; (B) appearance of deamidated ACTH; (C) appearance of ammonia. See Materials and Methods for experimental details.

m*M* HCl) at 37°C (data not shown). This result suggests that at acidic pH ACTH is undergoing deamidation via a pathway which is different from that at alkaline or neutral pH. This observation is consistent with the observation by Meinwald *et al.* (17) that the dipeptides, Asn-Gly and isoAsn-Gly, yield only Asp-Gly and isoAsp-Gly, respectively, when incubated in 1 *N* HCl at 37°C. Using a model Asn-hexapeptide, we have recently shown that only the Asp-hexapeptide is produced upon deamidation at acidic pHs. The formation of only the Asp-hexapeptide appears to result from direct hydrolysis of the side-chain amide linkage of the Asn residue (19).

Effects of pH and Buffer Concentrations on the Rate of Deamidation of ACTH

At all pHs and buffer concentrations studied, the disappearance of ACTH follows pseudo-first order kinetics (Fig. 5A), which is consistent with the reports in the literature that nonenzymatic deamidation of Asn (or Gln residues) in proteins and peptides follows first-order kinetics (5,35). The degradation of ACTH as monitored by the appearance of deamidated ACTH (Fig. 5B) or ammonia (Fig. 5C) also followed first-order kinetics.

Table I lists values of the observed rate constants and half-lives for the disappearance of ACTH at various pHs and buffer concentrations studied at 37°C. The rate of disappearance of ACTH was observed to increase with increasing pH, which again is consistent with the findings that the rate of deamidation of Asn residues is pH dependent (1). At pH 1.9 (10 m*M* HCl), the deamidation of ACTH was extremely slow but detectable.

The deamidation of ACTH was also found to be dependent upon buffer concentrations as shown in Table II. The effects of buffer concentrations on the rates of disappearance of ACTH and appearance of deamidated ACTH and ammonia at pH 9.6, 37°C (I = 0.1 *M*) are shown in Table II. In addition, it should be noted that at all three buffer concentrations the rates measured for disappearance of ACTH, appearance of deamidated ACTH, or appearance of ammo-

Table I. Observed Rate Constants for the Disappearance of ACTH at 37°C and Various pHs and Buffer Concentrations (I = 0.1 *M*)

pH	Buffer	Buffer concentration (m*M*)	k_{obs} (hr^{-1})[a]	$t_{1/2}$ (hr)
1.9	HCl	10	3.8×10^{-4}[b]	1824[b]
5.0	Acetate	100	7.5×10^{-4}	924
7.0	Phosphate	5	1.9×10^{-3}	365
9.6	Glycine	10	6.6×10^{-2}	11
9.6	Glycine	50	1.4×10^{-1}	5
9.6	Glycine	100	2.3×10^{-1}	3

[a] Each value is the mean of two experimental values, the values being within 5% of each other.

[b] About 41% degradation of ACTH was completed at the time of analysis, thus the k_{obs} and $t_{1/2}$ reported are apparent values.

Table II. Effect of Buffer Concentrations on the Rate Constants for Disappearance of ACTH and Appearance of Deamidated ACTH and Ammonia at pH 9.6 (10, 50, and 100 m*M* Glycine; I = 0.1 *M*) at 37°C

	k_{obs} (hr^{-1})[a]		
	10 m*M*	50 m*M*	100 m*M*
Disappearance of ACTH	6.6×10^{-2}	1.4×10^{-1}	2.3×10^{-1}
Appearance of deamidated ACTH	4.7×10^{-2}	1.4×10^{-1}	2.6×10^{-1}
Appearance of ammonia	6.5×10^{-2}	1.6×10^{-1}	2.9×10^{-1}

[a] Each value is the mean of two experimental values, the values being within 5% of each other.

nia are similar. The similarity in the rates of disappearance of ACTH and appearance of the deamidated ACTH and ammonia suggests that the rate-limiting step in the deamidation of ACTH is the formation and not the breakdown of cyclic imide intermediate. Meinwald *et al.* (17) have reported similar results using peptides containing Asn-Gly and isoAsn-Gly. However, with peptides containing Asp esters, Meinwald *et al.* (17) have reported that the rate-limiting step is the hydrolysis of cyclic imide.

In a separate study of deamidation, we have used a hexapeptide (Val-Tyr-Pro-Asn-Gly-Ala) which is a segment of the ACTH molecule from residues 22–27 (19). The deamidation of this model hexapeptide showed a marked dependence on pH, temperature, and buffer composition (19). At neutral to alkaline pH the hexapeptide deamidated exclusively via a cyclic imide intermediate with the formation of both the Asp- and the isoAsp-hexapeptides. Buffer catalysis was also observed in the pH range of 7 to 11. However, at acidic pH the deamidation was very slow and produced only the Asp-hexapeptide upon deamidation. A direct comparison of the deamidation kinetic parameters for the hexapeptide and ACTH at pHs 1.9 (10 m*M* HCl), 7.0 (5 m*M* phosphate), and 9.6 (50 m*M* glycine) at 37°C (I = 0.1 *M*) is shown in Table III. It is interesting to note that the rates for deamidation of these two peptides at a specific pH and buffer concentration are quite similar (Table III). Since Asp and isoAsp forms of the deamidated hexapeptide have been detected at neutral and alkaline conditions (19), and the observed rate constants for disappearance of both ACTH and hexapeptide are within the same order of magnitude (the difference is only two- to threefold), it can be concluded that the deamidation of ACTH at alkaline and neutral pHs involves a cyclic imide intermediate (Fig. 4). Similar to the results observed for ACTH under acidic conditions of deamidation, the Asn hexapeptide yields only the Asp peptide (19). These data support a deamidation pathway for ACTH and hexapeptide at acidic conditions that involves either the direct hydrolysis of the amide side chain or the intermediate formation of an isoimide (Fig. 4). In conclusion, we have shown in these studies that, depending upon the pH, different deamidation products are generated from ACTH. Under basic conditions one gets a mixture of Asp- and isoAsp-containing peptides, whereas under acidic conditions one observes only the Asp-containing peptide. Maximum stability of ACTH to deamidation can be achieved by avoiding strongly basic conditions and high buffer concentrations.

Table III. Observed Rate Constants for the Disappearance of Asn-Hexapeptide and ACTH at 37°C and Various pHs and Buffer Concentrations (I = 0.1 *M*)

pH	Buffer	Buffer concentration (m*M*)	k_{obs} (hr^{-1})[a]	
			Hexapeptide[b]	ACTH
1.9	HCl	10	1.6×10^{-4}[c]	3.8×10^{-4}[d]
7.0	Phosphate	5	6.8×10^{-3}	1.9×10^{-3}
9.6	Glycine	50	3.2×10^{-1}	1.4×10^{-1}

[a] Each value is the mean of two experimental values, the values being within 5% of each other.
[b] Data taken from Ref. 19. Hexapeptide sequence was Val-Tyr-Pro-Asn-Gly-Ala.
[c] About 25% degradation of hexapeptide was completed at the time of analysis, thus the k_{obs} is apparent.
[d] About 41% degradation of ACTH was completed at the time of analysis, thus the k_{obs} is apparent.

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