Rapid removal of acetimidoyl groups from proteins and peptides

Applications to primary structure determination

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Methylamine buffers can be used for the rapid quantitative removal of acetimidoyl groups from proteins and peptides modified by treatment with ethyl or methyl acetimidate. The half-life for displacement of acetimidoyl groups from fully amidinated proteins incubated in 3.44 M-methylamine/HCl buffer at pH 11.5 and 25°C was approx. 26 min; this half life is 29 times less than that observed in ammonia/HCl buffer under the same conditions of pH and amine concentration. Incubation of acetimidated proteins with methylamine for 4 h resulted in greater than 95% removal of acetimidoyl groups. No deleterious effects on primary structure were detected by amino acid analysis or by automated Edman degradation. Reversible amidination of lysine residues, in conjunction with tryptic digestion, has been successfully applied to the determination of the amino acid sequence of an acetimidated mouse immunoglobulin heavy chain peptide. The regeneration of amino groups in amidinated proteins and peptides by methylaminolysis makes amidination a valuable alternative to citraconoylation and maleoylation in structural studies.

Imidoesters, reagents that specifically derivatize the amino groups of proteins and peptides (Hunter & Ludwig, 1962, 1972), have recently found increased use in many areas of biochemistry. The derivatization reaction (amidination) has been used for extensive radiolabelling (Bates *et al.*, 1975a; Wood *et al.*, 1975), amino group protection (Demarchi *et al.*, 1978), introduction of special functional groups (Perham & Thomas, 1971; Riley & Perham, 1973; King *et al.*, 1978), studies of membrane architecture (Whiteley & Berg, 1974; Nemes *et al.*, 1980), and structure–function studies (Bates *et al.*, 1975b; Hucho *et al.*, 1975; Perham, 1977). The amidino group is normally introduced by such reagents as methyl or ethyl acetimidate in alkaline solution. Amidination offers a number of advantages over other approaches to amino group derivatization: (a) the reagent is freely water soluble, (b) the reaction is highly specific for amino groups, (c) derivatized peptides or proteins retain the positive charges of the amino groups, which maintains solubility in water and minimizes changes in physical and biological properties related to net charge, (d)

the amidine linkage is stable over a wide range of experimental conditions, and (e) reaction conditions can be found for selective modification of α -amino over ϵ -amino groups (Ludwig & Hunter, 1967; Hunter & Ludwig, 1972).

The removal of amidino groups has been traditionally carried out in slightly buffered (pH 11.5), concentrated NH_4OH solution (Hunter & Ludwig, 1972; Demarchi *et al.*, 1978). These harsh conditions needed for deblocking the protein or peptide are a drawback in the use of modifications by amidination because some side reactions have been observed during the reversal step (Ludwig & Hunter, 1967; Reynolds, 1968). Typically, such treatment requires periods of 18–24 h. Since we have been interested in the use of amidinated proteins for sequence analyses, it occurred to us that cleavage could be facilitated by the use of a reagent more strongly nucleophilic than ammonia, yet one which can also be removed readily by a volatilization process. In this report, we show that methylamine can be substituted for ammonia in the removal of acetimidoyl groups from proteins or peptides in

aqueous media, and that this reagent can displace amidine linkages at lower concentrations, at conveniently greater reaction rates, and without deleterious side effects.

Experimental

Proteins

Oxidized ribonuclease from bovine pancreas, equine heart myoglobin, and soya bean trypsin inhibitor were purchased from Sigma. Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase was obtained from Boehringer-Mannheim.

Chemicals

Methylamine, supplied as a 40% (w/v) aqueous solution, and NH_4HCO_3 were purchased from Fisher Scientific Co. Ethyl acetimidate hydrochloride and iodoacetamide were products of Aldrich. Iodoacetamide was recrystallized from toluene/ethanol (9:1, v/v) before use. Tris, dithiothreitol, and guanidine hydrochloride were Ultrapure reagents from Bethesda Research Labs. Triethylamine, Sequanal grade, was purchased from Pierce Chemical Co. Hydrochloric, acetic and formic acids, as well as NH_4OH , were Baker Analyzed Reagents. Ethyl[1- ^{14}C]acetimidate hydrochloride, 10 mCi/mmol, was obtained from The Radiochemical Centre, Amersham. Ultrogel AcA 34 gel filtration medium was supplied by LKB Instruments, Inc. Aquasol liquid scintillation cocktail was obtained from New England Nuclear.

Reduction and alkylation of proteins

Complete reduction and alkylation of proteins was performed as previously described (Robinson & Appella, 1979). The alkylation mixture was dialysed against 0.05 M- NH_4HCO_3 with 2.5 M-guanidine hydrochloride (two changes). After dialysis the sample was applied to an Ultrogel AcA 34 column (0.9 cm $\times$ 50 cm), equilibrated and eluted with 0.05 M- NH_4HCO_3 containing 2.5 M-guanidine hydrochloride. Fractions containing the bulk of protein were pooled, dialysed against 0.05 M- NH_4HCO_3 (three changes), and lyophilized.

Preparation of acetimidated proteins

The protein (100–700 nmol) was dissolved in 0.2 M-triethylamine/HCl buffer, pH 10.3, containing 5.0 M-guanidine hydrochloride. Ethyl acetimidate hydrochloride was dissolved in an equivalent amount of NaOH solution and added to the protein solution to maintain a final acetimidate concentration of 0.10–0.15 M (50-fold molar excess of acetimidate to amino groups). When ^{14}C -labelled reagent was used it was diluted 10-fold with unlabelled reagent, neutralized with NaOH, and added to the protein solution. The reaction mixture

was incubated for 1 h at 25°C, dialysed against 0.05 M- NH_4HCO_3 /2.5 M-guanidine hydrochloride, then dialysed against 0.05 M- NH_4HCO_3 and finally lyophilized. The specific activities of the labelled proteins were within 10% of the values calculated for complete amidination.

Titration of amine solutions

The procedure used for the titration of methylamine and NH_4OH solutions was the following. To 1 ml of amine solution was added 20 ml of deionized water and four drops of Bromothymol Blue indicator solution (0.1% in ethanol/water, 1:1 v/v). This solution was then titrated with 1 M-HCl until an endpoint was obtained.

Deamidation of ^{14}C -labelled proteins

The [^{14}C]amidinated proteins (1.2×10^6 – 1.5×10^6 d.p.m./mg) were dissolved in 10 mM-Tris/HCl, pH 8.3, also containing 6.0 M-urea for glyceraldehyde-2-phosphate dehydrogenase, to give 2.0×10^6 – 2.3×10^6 d.p.m./ml. For the deamidation reaction, 100 μl of the protein solution (2×10^5 d.p.m.) was mixed with 100–200 μl of the amine solution which had been adjusted with the appropriate acid to an apparent pH of 11.5. Aliquots (20–25 μl) were removed at intervals and added to 2.0 ml of 12% (w/v) trichloroacetic acid, and two drops of the unlabelled protein solution (10 mg/ml) were then added to the mixture. The precipitate was allowed to stand for 10 min and then was filtered through a Millipore filter (type HA, 0.45 μm). The filter was washed four times with 12% (w/v) trichloroacetic acid and once with water. After washing, the filter was dried (108°C, 5 min) and placed in a scintillation vial with 10 ml of Aquasol. The radioactivity present on the filter was determined with a Searle mark IV liquid-scintillation counter. The counting efficiency for ^{14}C was 94–95% and quench correction was made by an automatic external standard.

Deamidation of proteins for automatic sequence analysis

For the first experiment, 1.44 mg of [^{14}C]acetimidated glyceraldehyde-3-phosphate dehydrogenase (40 nmol, 2.6×10^6 d.p.m.) was dissolved in 4.0 ml of Tris/HCl buffer, pH 8.3, containing 9 M-urea. Methylamine formate buffer (2.2 ml, 9.7 M, pH 11.5) was added to the protein solution, and the mixture was incubated for 4 h at 25°C. Final concentrations of urea and methylamine buffer were 5.8 M and 3.44 M, respectively. The reaction mixture was then dialysed exhaustively against deionized water at 4°C. For the second experiment, 2.7 mg of acetimidated, equine heart myoglobin was dissolved in 1.6 ml of 7.5 M-urea. Methylamine formate buffer (0.9 ml, 9.6 M, pH 11.5)

was added, and the reaction mixture was incubated for 4 h at 25°C. Final concentrations of urea and methylamine buffer were 4.8 M and 3.5 M, respectively. After incubation, the reaction mixture was dialysed exhaustively against deionized water at 4°C.

Amino acid analysis

Samples were hydrolysed *in vacuo* at 106°C for 18–34 h in 6 M-HCl and were analysed on a Durrum D500 amino acid analyzer.

N-Terminal analysis

Automated Edman degradations were performed with an updated Beckman 890B sequencer as previously described (DuBois *et al.*, 1979).

Results and discussion

Deamidation of fully acetimidated proteins with methylamine buffers

Methylamine rapidly and quantitatively displaced the acetimidoyl group from the three fully derivatized proteins studied. As shown in Table 1, the time required for 50% deamidation with 3.44 M-methylamine (+HCl), pH 11.5, was 22–27 min. The rates of deamidation in both methylamine and ammonia buffers for fully acetimidated soya bean trypsin inhibitor under identical conditions are also shown in Table 1. With 3.44 M-ammonia (+HCl), pH 11.5, the half-time of the reaction was 750 min. In contrast, with 3.44 M-methylamine (+HCl), the half time was only 26 min. This result represents a 29-fold increase in the rate of deamidation. In previous reports the reversal step has been effected by conc. NH_4OH /acetic acid (15:1, v/v) at pH 11.5 and 16°C (Reynolds, 1968; Demarchi *et al.*, 1978) with a half-time of approx. 4 h. This rate would translate to a half-time of about 2 h at 25°C, or 4.5

times slower than in the methylamine buffer, which has a much lower concentration.

Several different acids were used to adjust the pH of methylamine solutions, and the rate of deamidation of glyceraldehyde-3-phosphate dehydrogenase using these reagents was studied. As shown in Table 1, deamidation occurred at roughly the same rate in solutions adjusted with HCl or formic acid. When CO_2 was used for adjustment of pH, the half-time was considerably greater (134 min, Table 1), probably as the result of formation of methyl carbamate. When acetic acid was used to adjust the pH, the protein precipitated from the solution within the first 10 min of the reaction. Its use was not investigated further. The rate of deamidation of acetimidated soya bean trypsin inhibitor with the more nucleophilic reagent, dimethylamine, was also examined. Deamidation with dimethylamine was approx. 5–6 times slower than with methylamine under similar conditions of pH and amine concentration. The reagents of choice are, therefore, methylamine/HCl and methylamine/formic acid. The latter buffer offers the advantage that it can be completely removed by dilution and lyophilization.

Amino acid analysis of amidinated and deamidated proteins

There were no major differences in the amino acid composition of native, denatured and acetimidated, and deamidated proteins, except in lysine content. Determination of the lysine and ϵ -acetimidolysine in hydrolysates of acetimidated myoglobin as a function of time gave the expected pseudo-first-order kinetics for the decomposition of ϵ -acetimidolysine. The rate constant for the hydrolysis of ϵ -acetimidolysine at 106°C was $1.37 \times 10^{-2} \text{ h}^{-1}$, and the half life was 51 h. These parameters are similar to those reported by Reynolds (1968) for the hydrolysis of acetimidated ribonuclease at 110°C ($k = 1.5 \times 10^{-2} \text{ h}^{-1}$, $t_{1/2} = 46 \text{ h}$). When the correction was made for

Table 1. Rates of deamidation of three, fully acetimidated proteins at 25°C

Rates of deamidation of [^{14}C]acetimidated proteins were determined as described in the Experimental section. Protein amino groups were fully acetimidated by reaction with a 50-fold excess of ethyl [^{14}C]acetamidate as described in the Experimental section.

Derivatized protein	Amine (3.44 M)	pH adjusted to 11.5 with:	Time of 50% deamidation (min)
Oxidized ribonuclease	Methylamine	HCl	22
Soya bean trypsin inhibitor*	Methylamine	HCl	26
	NH_3	HCl	750
Glyceraldehyde-3-phosphate* dehydrogenase	Methylamine	HCl	22
	Methylamine	HCl	21
	Methylamine	Formic acid	27
	Methylamine	CO_2	134

* These proteins were reduced and alkylated prior to acetimidation.

the decomposition of ϵ -acetimidolysine during acid hydrolysis, the lysine contents of both native and fully acetimidated protein were comparable. Amino acid analysis of deamidated soya bean trypsin inhibitor (4 h, 3.44 M-methylamine/formate, pH 11.5) showed greater than 95% recovery of the initial lysine value of the native protein indicating that the deblocking reaction was essentially complete.

Automated Edman degradation of deamidated protein

Two amidated proteins were studied to ascertain if the methylamine deamidation reaction could alter the primary structure of the proteins, e.g. by conversion of asparagine or glutamine side chains to their methylamide derivatives or by cleavage of the polypeptide chain. The acetimidated proteins were dissolved in 5 M-urea and treated with methylamine/formate buffer (3.5 M, pH 11.5) for 4 h at 25°C; then, their *N*-terminal sequences were determined by the automated Edman technique. The sequences obtained were compared with non-amidated controls, as were the initial and repetitive yields for the degradations. As shown in Fig. 1, the same sequence was obtained for selected cycles with

Fig. 1. Automated Edman degradation of deamidated glyceraldehyde-3-phosphate dehydrogenase

The right panel shows the yields for selected cycles for reduced and alkylated enzyme (40 nmol) that had been acetimidated and then treated with 3.4 M-methylamine/formate, pH 11.5. The left panel shows the yields obtained for selected cycles for reduced and alkylated enzyme (50 nmol). One-letter abbreviations for amino acids follow IUPAC-IUB rules: see *Biochem. J.* (1969) **113**, 1-4.

both control and deamidated glyceraldehyde-3-phosphate dehydrogenase. Backgrounds were no higher with the deacetimidated material, and repetitive yields were comparable. In the second experiment, horse myoglobin (native) was compared to deamidated myoglobin. The yields of glutamine phenylthiohydantoin for cycles 8 and 9 and asparagine phenylthiohydantoin at position 12 were high, indicating no appreciable conversion of glutamine or asparagine to the ω -methylamide derivatives (Fig. 2). Background, repetitive yields, and initial yields were the same within experimental error. These results clearly indicate that, in addition to being rapid and quantitative, the methylaminolysis reaction has no detrimental effects on primary structure as judged by automated sequencing techniques.

Reversible modification of lysine residues and tryptic digestion

Reversible amidation was recently employed in our laboratory to determine the amino acid sequence at the C_H2-C_H3 domain boundary of the mouse IgA 511 immunoglobulin heavy chain (Robinson & Appella, 1980). Initially, a 58-residue tryptic peptide, which included the boundary residues (337 and 338), was isolated from a maleoylated CNBr fragment (CB5). *N*-Terminal analysis established only the

Fig. 2. Automated Edman degradation of deamidated myoglobin

The right panel shows the yields for deacetimidated protein (59 nmol). The conditions for deamidation were the same as in Fig. 1. The left panel shows the yield data for native myoglobin (63 nmol).

sequence of the first 25 residues to Lys-335. Attempts to demaleoylate this peptide, to enable further tryptic digestion, were not successful as judged by C-terminal analyses or gel filtration after tryptic digestion. The corresponding 58-residue peptide was then isolated from acetimidated CB5. Upon deamidation, tryptic digestion now yielded the expected three peptides (Table 2 and Fig. 3). N-Terminal sequence analysis of the peptide which covered residues 335–368 (AM3T3) established the order of residues at the C_H2–C_H3 boundary.

Maleic and citraconic anhydrides have been widely used as acylating agents for the reversible modification of lysine residues (Hartley, 1970; Atassi & Habeeb, 1972; Perham, 1978). Both reagents are insoluble in water and change the charge of the lysine side chains. This change of charge can, of course, be valuable in promoting the solution and disaggregation of large peptides. However, hydroxyl groups of serine, threonine and tyrosine residues can be partially acylated, which can lead to charge heterogeneity of proteins or peptides and decreased yields in Edman degradation unless an additional precautionary step is taken to cleave these ester linkages (King & Perham, 1971; Perham, 1978). Removal of citraconyl groups is rapid (100% deblocking within 3–4 h at pH 4.2 and 40°C; Habeeb & Atassi, 1972; Perham, 1978). However, this rapid deblocking can be a disadvantage in that it may occur to a significant degree during preparation and separation of limited tryptic digests if the pH is allowed to fall below pH 8. This can limit the choice of separation methods. The removal of maleoyl groups is relatively slow [*t*_{1/2} 11 h at pH 3.5 and 37°C (Hartley, 1970)] and, in the case of the maleoylated immunoglobulin heavy-chain peptide, did not occur to any appreciable extent. For

both of the anhydrides, assessment of the extent of reaction and deblocking is made by relatively insensitive spectrophotometric methods. In the case of citraconic anhydride no radiolabelled compound is commercially available.

Table 2. Amino acid composition of the tryptic peptides of deamidated 511H CB5AM3 (CB5DEAM3)

The amidinated peptide (CB5Am3) were prepared and deamidated as described in the Experimental section. DeAM3 (105nmol) was digested with trypsin (1:149, w/w) for 2.5 h at 25°C. The digest was filtered on a column of Sephadex G-75 (Superfine grade). Integral values in parentheses were determined from sequence analysis. Abbreviations: CmCys, carboxymethylcysteine; N.D., not detected.

Amino acid	Composition of peptide:			
	T1	T2	T3	DeAM3
CmCys		0.7 (1)	0.8 (1)	1.5 (2)
Asp	1.2 (1)	0.3	2.0 (2)	3.0 (3)
Thr		3.4 (5)	1.9 (3)	4.1 (8)
Ser	2.0 (2)	1.7 (1)	2.1 (2)	3.9 (5)
Glu	0.3	1.5 (1)	3.8 (4)	4.8 (5)
Pro		1.1 (1)	4.0 (5)	5.5 (6)
Gly	1.9 (1)	2.3 (2)	1.0 (0)	3.2 (3)
Ala	1.2 (1)	1.4 (1)	1.0 (1)	2.4 (3)
Val	0.3	1.1 (1)	2.8 (4)	4.2 (5)
Ile		0.8 (1)	0.3	1.0 (1)
Leu	0.3	1.2 (1)	5.3 (8)	5.1 (9)
Tyr		0.1	0.2	0
Phe	0.7 (1)	0.2	0.7 (1)	0.9 (2)
His		1.0 (1)	0.7 (1)	1.2 (2)
Lys	1.0 (1)	1.0 (1)		1.4 (2)
Arg			0.8 (1)	0.9 (1)
Trp	N.D. (1)			N.D. (1)
Yield	32%	50%	53%	
Residue nos.	311–318	319–335	336–368	331–368

Fig. 3. Gel filtration of the tryptic digest of deamidated 511H CB5AM3 on Sephadex G-75 (Superfine grade) The column (0.8 cm × 200 cm) was equilibrated with 0.1 M-NH₄-CO₃. Fractions (0.9 ml) were collected at a flow rate of 1.9 ml/h.

Derivatization of the amino groups of proteins or peptides by amidination and subsequent removal of amidine groups by methylaminolysis appears to offer a valuable method that complements the use of anhydrides. The amidine linkage is stable over a wide range of experimental conditions, radiolabelled reagent is commercially available, and the extent of blocking and deblocking can be readily assessed by sensitive methods of analysis. Thus, amidination is a valuable alternative to citraconoylation and maleoylation in structural studies.

Uses of reversible amidination

Cleavage of amidine linkages by methylaminolysis can greatly extend the usefulness of amidination reactions. We have been especially interested in the use of radioactively labelled alkylimidates for labelling peptides prior to microsequencing (Perham *et al.*, 1980). Labelling may be carried out on a protein before enzymic or chemical fragmentation (specifically to label lysine-containing peptides) or on the peptide mixture after fragmentation (to label all peptides). The radioactive acetimidoyl groups in either case provide a method for following the isolation and purification of small amounts of derivatized peptides, and they can be readily removed by treatment with methylamine buffers prior to microsequencing.

In other applications of the amidination reaction, such as in amino group modification, structure-function studies and protein semi-synthesis, and in the use of bis-imidates for cross-linking of multimeric proteins and membrane components, methylamine buffers could be used to reversibly cleave amidine linkages and thereby increase the utility of this reaction.

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