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Note

Preparation of a positively charged maleimide and its application to the high-performance liquid chromatographic separation of the tryptic peptides of lysozyme

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The high-performance liquid chromatographic (HPLC) separation of the tryptic peptides of reduced, alkylated hen egg white lysozyme (HEWL) has been reported previously[1]. Alkylation was achieved with the conventional reagents N-ethylmaleimide (NEM) and iodoacetic acid. In this paper we extend these studies by use of a new alkylating reagent 1-(3-dimethylaminopropyl)-1H-pyrrole-2,5-dione hydrochloride (acronym PAM for *p*ropyl*a*mino*m*aleimide). This reagent was developed in order to have the rapid reaction rate of a maleimide and the increased solubility of a charged product. At pH 5.0–5.5, the conditions employed in this study, the amino group of the reagent is undoubtedly protonated; thus both the reactant and the product carry a positive charge. Since the alkylation reaction can take place under mildly acidic conditions, the risk of disulfide–thiol interchange during alkylation can be avoided. This is an important concern in our investigation of the refolding pathway of reduced HEWL and other dynamic disulfide forming reactions. In our studies we have encountered solubility problems with NEM derivatives at both the protein and tryptic peptide levels. The low solubility of some of these derivatives has required that manipulations be carried out at inconveniently high dilutions. Lyophilization and subsequent solution, particularly of very small samples, gives no assurance that solubility fractionation has not occurred.

It is the purpose of this paper to demonstrate the usefulness of PAM as an S-alkylating reagent in protein and peptide research.

EXPERIMENTAL

Preparation of PAM

Crushed ice, 1500 g, and 408 g 3-dimethylaminopropylamine (Aldrich, Milwaukee, WI, U.S.A.) were charged into a large beaker with mechanical stirring. Maleic anhydride, 400 g, was added in portions and the temperature was maintained at 0–5°C by the addition of ice. When most of the anhydride was dissolved, the mixture was allowed to warm until solution was complete. The water was then removed with a rotary evaporator, in an 80°C water bath, at first under aspirator pressure and finally with an oil pump. A very viscous syrup resulted. Acetic anhy-

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dride, 650 ml, was slowly added with cooling and stirring, the temperature being maintained below 60°C. It was then allowed to stand overnight. Ethanol, 1000 ml, was added and the mixture was evaporated with a rotary evaporator to remove excess acetic anhydride and acetic acid. The residue was dissolved in 1500 ml ethanol and gaseous hydrogen chloride was passed into the mixture until it was very strongly acidic. Cooling and scratching induced crystallization and the mixture was refrigerated overnight. The solids were collected and recrystallized twice from ethanol, the second time after treatment with activated charcoal and hot filtration. The resulting white crystals, 180 g, showed a melting point of 195°C (uncorr.). The NMR spectrum (^2H_2O) was recorded on a R-12 spectrometer (Perkin-Elmer, Norwalk, CT, U.S.A.). The spectrum was consistent with the desired structure, showing, in addition to other signals, δ = 2.92 ppm (s) (6 H), representing the N-methyl groups, and δ = 6.92 ppm (s) (2 H) representing the vinyl protons of the maleimide ring. A sample was submitted for elemental analysis. For $C_9H_{14}N_2O_2 \cdot HCl$ calculated (found), C = 49.40 (49.23); H = 6.92 (6.89); N = 12.80 (12.62). (Micro-Analysis, Wilmington, DE, U.S.A.).

Preparation of PAM-alkylated lysozyme and its tryptic peptides

HEWL (Miles Labs., South Africa) was reduced with dithiothreitol at pH 8.6 (ref. 2). The lyophilized reduced enzyme (23 mg) was dissolved in 10 ml of 8 *M* urea in 0.10 *M* acetic acid. To this, 39 mg of PAM was added and the pH maintained at 5.0–5.5 for 15 min. The solution was acidified to pH 3.0 with glacial acetic acid and applied to a Sephadex G-25 M column (100 × 2.5 cm I.D.) (Pharmacia, Uppsala, Sweden). The PAM protein was eluted using 0.10 *M* acetic acid and lyophilized. Tryptic digestion was carried out at pH 6, for 22 h at room temperature. Subsequent preliminary separation of the peptides on a Sephadex G-25 M column was accomplished as described previously for the preparation of tryptic peptides from NEM and iodoacetic acid-blocked lysozyme[1]. Peptide pools A, B, C and D from the Sephadex column were dissolved in 0.10 *M* acetic acid (3 ml) and further separated by HPLC (usually 25-μl injections).

HPLC

HPLC separations were performed with a Varian Model 5000 liquid chromatograph (Varian, Palo Alto, CA, U.S.A.). A Varian reversed-phase MicroPak MCH-10 column (30 cm × 4 mm I.D.) was employed with a guard column of Vydac RP resin. Separation of the tryptic peptides was achieved using a gradient of 0.10 *M* ammonium chloride, pH 4.1 (reservoir A) and acetonitrile (reservoir B). The program was 0–10 min, 5–22% B; 10–12 min, 22–24% B, 12–14 min, 24% B, 14–19 min, 24–28% B; 19–25 min, 28–36% B[1]. Detection was at 205 nm or 280 nm.

Peptides collected from HPLC were lyophilized, hydrolyzed with 6 *M* hydrochloric acid for 48 h at 105°C, and analyzed on a Beckman Model 119C amino acid analyzer (Beckman, Palo Alto, CA, U.S.A.) as previously described[1]. PAM-modified cysteine residues were determined as S-succinyl cysteine. Standard S-succinyl cysteine was prepared as the dicyclohexylamine salt according to Calam and Waley[3].

Electrophoresis

Electrophoresis of NEM-lysozyme and PAM-lysozyme was performed in an

acid–urea polyacrylamide gel slab. The gel, which was inspired by Panyim and Chalkley[4], was significantly modified to give a final concentration of 4 *M* urea (ultrapure-grade Schwarz-Mann, Spring Valley, NY, U.S.A.), 12% acrylamide (Bio-Rad Labs., Richmond, CA, U.S.A.), 0.2% N,N'-methylene-bis-acrylamide, 1% N,N,N',N'-tetramethylenediamine (TEMED), 0.3% riboflavin-5'-phosphate, 0.8% ammonium persulfate and 5% glacial acetic acid. Polymerization of the gel was carried out at 4°C with continuous illumination by a fluorescent lamp. The buffer solution was 5% acetic acid and electrophoresis was carried out for 4 h at 4°C with a potential gradient of 50 V/cm (see ref. 4).

RESULTS AND DISCUSSION

For many purposes it is necessary that the thiol reagent reacts rapidly with the thiol groups. PAM is very soluble in water and dissolves quickly. The speed of reaction of PAM with thiol groups is demonstrated by the following simple experiment: 1 millimole each of PAM and reduced glutathione were placed in a test tube, 1 ml of ^2H_2O was added. The materials quickly dissolved and the mixture transferred to an NMR tube. The tube was placed in the probe of the spectrometer and the area near 7 ppm scanned. Although less than a minute had elapsed since the addition of the solvent, no signal was observed in this vicinity, indicating that the reaction was complete. The signals expected for the product were present. The pH, after the reaction, was observed to be near 3.0. The ring nitrogen of PAM is separated from its charged group by three methylene groups, which surely insulate the ring system from the electronic effects of the dimethylamino nitrogen. We can be confident that the intrinsic reactivity of PAM with thiols differs very little from that of NEM[5].

The HPLC program developed for the separation of NEM-blocked and iodoacetic acid-blocked tryptic peptides of lysozyme was adequate for the separation of the PAM-blocked peptides as well. In Table I the elution conditions for the alkylated peptides are compared. The dipeptide Cys^{115}–Lys^{116} is not reported since it elutes

TABLE I

COMPARISON OF THE ELUTION PROPERTIES OF TRYPTIC PEPTIDES FROM HEN EGG WHITE LYSOZYME, ALKYLATED WITH DIFFERENT REAGENTS

Peptides were alkylated with iodoacetic acid (IAA)*; N-ethylmaleimide (NEM)*; or 1-(3 dimethylaminopropyl)-1H-pyrrole-2,5-dione (PAM). The first number gives the percentage of acetonitrile at which the peptide eluted. The number in parentheses is the retention time in minutes. The entire elution program is given in the text. N.E. = not eluted.

Alkylated peptide	*G-25 pool containing peptide*	*Alkylating agent*		
		IAA	*NEM*	*PAM*
Cys^6–Lys^{13}	B, C	24 (12)	24.5 (14)	24 (13)
Gly^{22}–Lys^{33}	B, C	27 (18)	31.5 (22)	27 (18)
Trp^{62}–Arg^{68}	D	24.5 (14)	26.5 (17)	25 (15)
Asn^{74}–Lys^{96}	A	N.E.	N.E.	35.5 (24)
Gly^{126}–Leu^{129}	C	< 10 (< 3)	< 10 (< 3)	22 (10)

* See ref. 1.

with the solvent front. The remaining seven cysteinyl residues in lysozyme are accounted for. The following advantages of the PAM alkylation are seen: PAM-Gly^{22}–Lys^{33} eluted earlier than the corresponding NEM peptide, which coeluted with Ileu^{98}–Arg^{112}; the Asn^{74}–Lys^{96} peptide, containing three PAM cysteinyl residues, was eluted easily while the corresponding NEM peptide was not eluted with the 0.10 *M* salt–acetonitrile gradient; the PAM-blocked peptide Gly^{126}–Cys^{127}–Arg^{128}–Leu^{129} (the bond between arginine and leucine is resistant to trypsin digestion) is retained and well separated from the solvent front.

The peptide Cys^{6}–Lys^{13} eluted in two positions, by itself and with Asn^{46}–Arg^{61}. The association of the two peptides was strong; variations in the program did not separate them. This close association with Asn^{46}–Arg^{61} was previously observed with the NEM-blocked Cys^{6}–Lys^{13} peptide[1]. PAM-blocked Trp^{62}–Arg^{68} peptide eluted more quickly than the corresponding NEM-blocked peptide, eluting at almost the same organic solvent concentration as Asn^{46}–Arg^{61}; however these peptides are

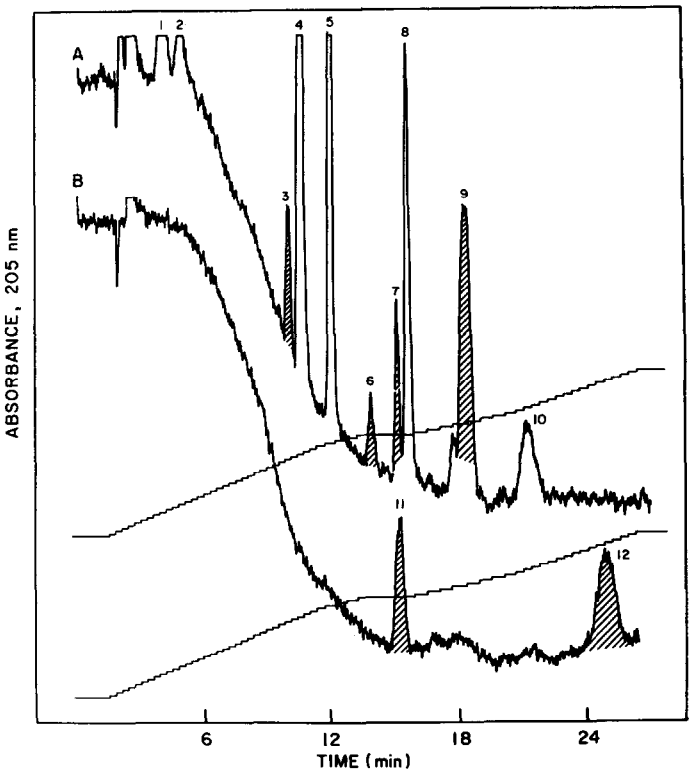

Fig. 1. A composite chromatogram of the HPLC sepparation of the PAM-lysozyme tryptic peptides. The upper curve (A) shows the peptides from Sephadex G-25 column pools B and C. The lower curve (B) shows the two peptides obtained in pure form from the G-25 column; Trp^{62}–Arg^{68} (pool D) and Asp^{74}–Lys^{96} (pool A). Shaded peaks are those peptides containing PAM-cysteinyl residues. A gradient of 0.10 *M* ammonium chloride and acetonitrile was used as described in the text. Detection was at 205 nm. The peak numbers correspond to the following peptides: 1 = Val^{2}–Arg^{5}; 2 = Thr^{69}–Arg^{73}; 3 = Gly^{126}–Leu^{129}; 4 = His^{15}–Arg^{21}; 5 = Phe^{34}–Arg^{45}; 6 = Cys^{6}–Lys^{13}; 7 = Cys^{6}–Lys^{13} and Asn^{46}–Arg^{61}; 8 = Gly^{117}–Arg^{125}; 9 = Gly^{22}–Lys^{33}; 10 = Ileu^{98}–Arg^{112}; 11 = Trp^{62}–Arg^{68}; 12 = Asn^{74}–Lys^{96}.

readily separated beforehand on the G-25 Sephadex column. The entire tryptic peptide map including the PAM-peptides is shown in Fig. 1. An improved separation of the cysteinyl containing peptides from the other peptides in lysozyme was obtained when the peptides were blocked with PAM rather than NEM. Furthermore, there were no obvious solubility problems with any of the peptide pools.

It was expected that the acid hydrolysis product of PAM-cysteine would be S-succinyl-cysteine as is the case with NEM-blocked cysteine[6]. Amino acid analysis of PAM-glutathione, studied as a model compound, and the PAM peptides from lysozyme confirmed the formation of this product. Recoveries of S-succinyl-cysteine were in the 70–80% range, when the samples were adequately de-gassed[6], and the optimal hydrolysis times established. The amino acid analyses of the PAM peptides are given in Table II.

The introduction of a charged group at derivatized thiols can be expected to alter the electrophoretic and chromatographic behavior of peptides and proteins compared with the NEM derivatives. This was confirmed in our laboratory by polyacrylamide gel electrophoresis of NEM-alkylated, reduced lysozyme and PAM-alkylated, reduced lysozyme; the respective R_F values relative to native HEWL were 0.46 and 0.53. Because of its positive charge in acid solution, PAM would be expected to have the potential for enhanced reactivity with thiols in the neighborhood of negatively charged groups.

In summary, a new malemide has been prepared and characterized. On reversed-phase HPLC the tryptic peptides from PAM lysozyme were readily separated.

TABLE II

AMINO ACID ANALYSES OF PAM-PEPTIDES FROM HEWL

The data are given in residues/mole; the numbers in parentheses refer to the number of residues expected from the known sequence of lysozyme. All samples were hydrolyzed for 48 h in 6 *M* hydrochloric acid at 105°C. Peptides were collected from HPLC using the ammonium chloride–acetonitrile gradient given in the text, except that Gly^{22} Lys^{33} was collected from ammonium acetate–acetonitrile.

Amino acids	*Gly^{126}–Leu^{129}*	*Cys^{6}–Lys^{13}*	*Gly^{22}–Lys^{33}*	*Asn^{74}–Lys^{96}*	*Trp^{62}–Arg^{68}*
Aspartic acid			0.9 (1)	5.4 (4)	1.9 (2)
Threonine				1.0 (1)	
Serine		0.7	0.8 (1)	3.6 (4)	0.2
Glutamic acid		1.5 (1)			
Glycine	0.9 (1)	0.9	1.8 (2)		1.3 (1)
Alanine		3.0 (3)	1.7 (2)	2.9 (3)	
Valine			0.8 (1)	1.3 (1)	
Methionine		0.8 (1)			
Isoleucine				1.8 (2)	
Leucine	0.8 (1)	1.2 (1)	1.0 (1)	2.9 (3)	
Tyrosine			0.9 (1)		
Lysine		0.4 (1)	1.0 (1)	1.1 (1)	
Tryptophan*			+ (1)		+ (2)
Arginine	1.0 (1)				1.0 (1)
Cysteine, as:	(1)	(1)	(1)	(3)	(1)
Cysteic acid	1.2				
succinyl-cysteine		1.4	0.8	2.2	0.6

* The (+) indicates that tryptophan is present but not determined.

After acid hydrolysis cysteine is determined as S-succinyl cysteine. These properties are comparable to those of NEM derivatives, but aqueous solubility was substantially increased. We believe that this thiol blocking agent will be useful in other protein and peptide investigations.

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