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EFFECTS OF REDUCTIVE ALKYLATION ON PEPTIDE RETENTION TIMES

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SUMMARY

Reductive alkylation of primary amino groups is used to introduce nuclear magnetic resonance or radioactive probes into proteins. Because of electrostatic and conformational effects, reductive alkylation is not always complete. We describe herein a rapid high-performance liquid chromatographic method for separating and quantitating mixtures of native and reductively alkylated peptides. Small synthetic peptides were chosen to illustrate the effects of methylation and isopropylation of primary amino groups on chromatographic retention times. Mixtures of unmodified and reductively methylated or isopropylated peptides (Gly-Leu-Tyr, Gly-Gly-Lys-Arg, Arg-Lys-Asp-Val-Tyr and Pro-Gly-Lys-Ala-Arg) could be separated. Chromatography was on a 5-μm, 25 cm $\times$ 0.4 I.D., C_{18} reversed-phase column with 0.1% trifluoroacetic acid in a 10 to 80% linear gradient of acetonitrile in water, a system appropriate for protein digests. The relative concentrations of native, and singly and doubly alkylated peptide were determined as well as the effective retention coefficients for dimethyl and isopropyl groups. The method shows promise for the peptide mapping of partially alkylated proteins.

INTRODUCTION

Free amino groups in proteins may be alkylated under mild conditions by treatment with an aliphatic aldehyde or ketone and sodium borohydride[1] or sodium cyanoborohydride[2]. The availability of isotopically labeled formaldehyde and acetone make reductive alkylation a feasible method for adding a radioactive or nuclear magnetic resonance probe to a protein. The use of acetone as the alkylating agent results in the formation of an α-N-isopropylamino group, and ε-N-isopropyllysyl residues[1]. Reductive methylation, with formaldehyde, produces the corresponding N,N-dimethyl derivatives[1,2]. In either case, the substituent is relatively small, and there is little effect on the physiochemical properties of the protein, thus making it possible to study the "native" state of labeled residues in a protein.

Because of conformational effects and ionic interactions, the reductive alkylation of amino groups is not always complete. The dansylation method of Weiner *et al.*[3] can be used to determine the extent of alkylation of the N-terminal α-amino group. The percent of unmodified lysine can be determined by amino acid analysis[4].

Peptide maps are often useful for locating specific residues in proteins, and in particular, the comparison of peptide maps of the modified and unmodified protein may be most useful in locating modified residues.

Reversed-phase high-performance liquid chromatography (RP-HPLC) with trifluoroacetic acid (TFA) in acetonitrile and water is widely used for the separation of peptides from the enzymatic hydrolysis of proteins[5]. Several sets of coefficients, which may be used to predict retention times for peptides are available[5–9]. These sets of coefficients take into account the contribution of an individual amino acid to the hydrophobic character of the peptide, and the authors generally supply suggestions for adapting the data to chromatographic conditions other than those used to develop the data set. Additionally, mixtures of amino acids and N-methyl amino acids have been separated by RP-HPLC[10,11].

We have prepared isopropyl and dimethyl dervatives of commercially available peptides containing non-terminal lysine residues or no lysine residues, to serve as models for peptides derived by enzymatic hydrolysis of reductively alkylated protein. Using a chromatography system developed for the separation of tryptic and chymotryptic peptides from reductively isopropylated protein[12], we have examined the effects of reductive methylation and isopropylation of one or more amino acid residue on peptide retention time.

EXPERIMENTAL*

Materials

Peptides were obtained commercially; glycyl-glycyl-L-lysyl-L-arginine diacetate (G-G-K-R) from Vega Biochemicals (Tucson, AZ, U.S.A.), L-arginyl-L-lysyl-L-aspartyl-L-valyl-L-tyrosine acetate (R-K-D-V-Y) from Calbiochem–Behring (La Jolla, CA, U.S.A.), glycyl-L-leucyl-L-tyrosine (G-L-Y) from Sigma (St. Louis, MO, U.S.A.) and L-prolyl-glycyl-L-lysyl-L-alanyl-L-arginine · $\frac{1}{2}H_2SO_4$ (P-G-K-A-R) from Serva (Accurate Chemicals, Hicksville, NY, U.S.A.). Reagents for reductive alkylation were the best commercial grades available.

Reductive alkylation

N-isopropyl (Ip) peptides, Ip G-L-Y, Ip G-G-K-R, Ip P-G-K-A-R and Ip R-K-D-V-Y, were prepared by the reductive alkylation method originally described by Means and Feeney[1], and modified by Fretheim *et al.*[13]. The method was further modified for use with micromolar quantities of small peptides as follows. Acetone, or [$^2H_6$]acetone was added to 0.5 ml of 10 μM peptide in 0.2 *M* borate (pH 9.0, 20% *p*-dioxane), to a final concentration of 3.2 m*M*. Small quantities of sodium borohydride were then added with stirring over a 15-min period, to a final concentration of 530 m*M*, the mixture was stirred for an additional 15 min, then titrated to pH 4.5 with 0.5 *M* hydrochloric acid. Excess reagents were removed by gel chromatography on a 50 × 0.9 cm I.D. column of Sephadex G-10 with 0.01 *M* acetic acid. Reductively methylated peptides were similarly prepared, using formaldehyde as the alkylating reagent.

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* Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Amino acid analyses

The compositions of unmodified and alkylated peptides were determined by amino acid analysis. Peptides were hydrolyzed in sealed, evacuated tubes at 110°C for 24 h with 5.7 *M* hydrochloric acid, containing 0.05% phenol. Analyses were performed on a Beckman (Fullerton, CA, U.S.A.) 119 CL amino acid analyzer, using the standard 90-min single-column hydrolyzate protocol[4]. Intact peptides were also analyzed to check for breakdown products and contaminating amino acids.

HPLC retention time

The peptides were analyzed on a Brownlee (Santa Clara, CA, U.S.A.) C_{18} reversed-phase column (25 × 0.4 cm I.D., 5-μm particle size), with a Varian (Sunneyvale, CA, U.S.A.) System 54 liquid chromatograph, operated at room temperature. The mobile phase was 0.1% TFA (Pierce, Rockford, IL, U.S.A.) in water as solvent A and 0.1% TFA in acetonitrile (Burdick & Jackson, Muskegon, MI, U.S.A.) as solvent B. The concentration of acetonitrile was increased linearly from 10 to 80% over a 30-min period at a flow-rate of 1 ml/min. Peptides were monitored at 214 nm with a Varian UV-50 detector.

RESULTS AND DISCUSSION

Unmodified amino acid compositions for both native and reductively alkylated peptides are given in Table I. However, because α-N-alkyl amino acids did not react with ninhydrin, and the ninhydrin color yields for ε-N-alkyl lysine residues were lower than for lysine, reductively alkylated residues were quantitated by difference. RP-HPLC retention times (Table II) for native and alkylated peptides were corrected for t_0, the time needed for unretained material to reach the detector. In 32 experiments, performed at intervals over a period of three years on a general-purpose column, t_0 was 3.2 ± 0.2 min, depending primarily on the condition of the guard column. Both [$^2H_6$]isopropyl and [$^1H_6$]isopropyl peptides were prepared, and no isotope effect was observed. Although monomethyl derivatives are possible, as noted by others[1,2] no significant concentration was detected in the chromatograms.

G-L-Y, which does not contain lysine and thus can be reductively alkylated only at the α-amino group of glycine, presents the simplest case. From the amino acid analyses (Table I) it is seen that reductive isopropylation of the glycine residue was virtually complete, and methylation 80% complete. The cyanoborohydride of Jentoft and Dearborn[2] is more efficient for methylation, but is not effective for isopropylation. Fig. 1 shows the elution order for the three peptides. The presence of tyrosine enabled us to determine the concentrations of these peptides from their adsorbance at 280 nm. A mixture of G-L-Y and its reductively alkylated derivatives was prepared for HPLC from equal volumes of G-L-Y (3.5 m*M*), DiMe G-L-Y (3.5 m*M*) and Ip G-L-Y (1.9 m*M*). From the degree of modification (Table I), the mixture applied to the column consisted of 47% G-L-Y, 29% DiMe G-L-Y and 24% Ip G-L-Y. Similar numbers (45, 35 and 20%) were obtained from the areas of the eluted peaks in Fig. 1.

G-G-K-R, and R-K-D-V-Y could be reductively alkylated at the ε-amino group of the lysine residue as well as at the α-amino group of the N-terminal residue. Fig. 2 shows chromatograms of G-G-K-R, and its derivatives. Native G-G-K-R was

TABLE I

EXTENT OF REDUCTIVE ALKYLATION AS DETERMINED BY AMINO ACID ANALYSIS

The molar ratios of unmodified amino acids in native, isopropyl (Ip), and dimethyl (DiMe) peptides were obtained as nmol per sample, and are reported as molar ratios, and as integer values (in parentheses).

Amino acid	*Peptide*	*Ip peptide*	*DiMe peptide*
G-L-Y			
Gly	0.96 (1)	0.06	0.23
Leu	1.03 (1)	1.04 (1)	1.03 (1)
Tyr	0.98 (1)	0.96 (1)	0.97 (1)
G-G-K-R			
Gly	1.85 (2)	1.13 (1)	1.11 (1)
Lys	1.00 (1)	0.02	0.66
Arg	1.00 (1)	1.00 (1)	1.00 (1)
P-G-K-A-R			
Pro	0.62	0.44	
Gly	1.07 (1)	1.14 (1)	
Lys	0.90 (1)	0.15	
Ala	1.06 (1)	1.06 (1)	
Arg	1.00 (1)	1.00 (1)	
R-K-D-V-Y			
Arg	1.04 (1)	0.70	
Lys	0.96 (1)	0.20	
Asp	1.06 (1)	1.04 (1)	
Val	0.96 (1)	0.96 (1)	
Tyr	1.00 (1)	1.00 (1)	

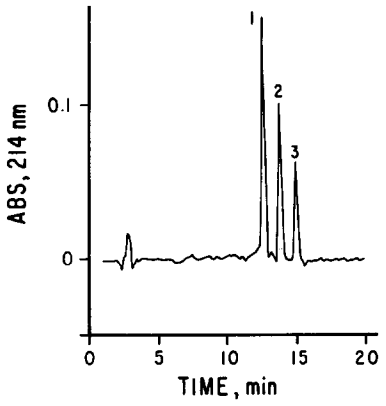
TABLE II

EFFECT OF REDUCTIVE ALKYLATION ON RETENTION TIME

Average retention times, in minutes, for native peptide (t_{R_1}), and peptide reductively alkylated at one (t_{R_2}) and two t_{R_3}) positions were corrected for t_0. Average increases in retention time due to reductive alkylation at one position and the further increase due to the presence of a second alkyl group are listed as (Δt_1) and (Δt_2).

Peptide	t_{R1}	t_{R2}	Δt_1	t_{R3}	Δt_2
G-L-Y	10.0 ± 0.3				
Ip G-L-Y		12.0 ± 0.1	2.1		
DiMe G-L-Y		11.0 ± 0.2	1.0		
G-G-K-R	3.1 ± 0.3				
Ip G-G-K-R		5.2 ± 0.5	2.1	6.6 ± 0.2	1.4
DiMe G-G-K-R		3.8	0.7	4.6	0.8
R-K-D-V-Y	10.7 ± 0.3				
Ip R-K-D-V-Y	11.2 ± 0.2	13.1 ± 0.1	2.4	14.4 ± 0.1	1.3
P-G-K-A-R	(4.8 ± 0.3)*				
	5.3 ± 0.3				
Ip P-G-K-A-R		(7.0 ± 0.2)	(2.2)	(8.2 ± 0.4)	(1.2)
		7.5 ± 0.2	2.2	8.2 ± 0.4	0.65

* Values in parentheses are for the blocked form of P-G-K-A-R.



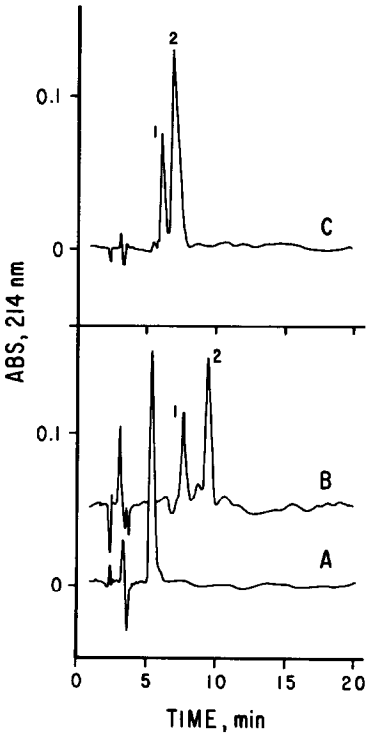


Fig. 1. Separation by RP-HPLC of a mixture of the tripeptide (1) G-L-Y, its reductively methylated (2) and isopropylated (3) forms. Chromatographic conditions are given in the text.

Fig. 2. Chromatograms of (A) G-G-K-R; (B) reductively isopropylated G-G-K-R, (B1) Ip G-G-K-R and (B2) 2Ip G-G-K-R; and (C) reductively methylated G-G-K-R, (C1) DiMe G-G-K-R and (C2) 2DiMe G-G-K-R.

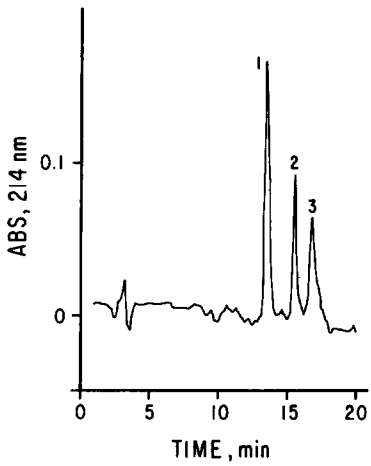
eluted as a single peak (Fig. 2A), early in the development of the gradient, as was expected for a peptide composed only of hydrophilic residues[8]. The reductively alkylated peptides, either the isopropyl (Fig. 2B) or dimethyl (Fig. 2C) derivatives, were retained longer on the column and were eluted as two peaks, representing peptide labeled at either glycine or lysine, or at both. No native peptide was detected in the chromatograms of the modified form. It has previously been reported that the composition of a short peptide is more important in determining its retention coefficient than is its sequence[8]. The data in Fig. 2, where only two peaks are observed for three possible derivatives suggests that the addition of an N-alkyl group is more important than its position. The relative areas of peaks in Fig. 2B represent 20% Ip G-G-K-R and 80% 2Ip G-G-K-R. Estimates of 24% DiMe G-G-K-R and 75% 2DiMe G-G-K-R were obtained from the peak areas in Fig. 2C.

Fig. 3 shows a chromatogram of a mixture of native R-K-D-V-Y, [$^1H_6$]Ip R-K-D-V-Y, and [$^2H_6$]Ip R-K-D-V-Y. In a separate chromatogram (not shown) native R-K-D-V-Y was eluted as a single peak which could be superimposed on the first peak of Fig. 3. In this peptide neither arginine nor lysine was completely isopropylated due to steric effects caused by ionic interactions with the aspartic acid

residue[4]. The chromatograms were consistent with the amino acid analysis. From the areas of the peaks in the chromatogram (Fig. 3) of a mixture of R-K-D-V-Y and Ip R-K-D-V-Y it was determined that 50% of the reductively isopropylated form was labeled at one position, either lysine or arginine, and 30% at both.

Reductively alkylated P-G-K-A-R was expected to represent more closely the type of peptide derived from partial hydrolysis of an alkylated protein, in that there was no primary α-amino group available for alkylation. It proved even more interesting, because the sample we purchased contained 60% P-G-K-A-R (Table I), and a second form having no free N-terminal residue, which shall be called blocked P-G-K-A-R. Earlier analysis[4] showed only proline in the N-terminal position, and no significant contamination with free amino acids. Fig. 4 shows chromatograms of P-G-K-A-R and its isopropyl derivative. The elution pattern (Fig. 4A) of the native peptide showed two major peaks representing about 35 and 55% of the total material, the other 10% was contained in two much smaller peaks following the major ones. Identical increases in retention time were observed (Table II) when lysine residues of the two forms were isopropylated. Proline although less reactive than a residue with a primary α-amino group, was partially converted to a ninhydrin positive product which was eluted near serine on amino acid analysis. Conditions for Schiff's base formation at the proline imino (pK_a = 10.6) and the ε-amino group of lysine (pK_a = 10.5) are similar, suggesting that some isopropylation may be expected. The chromatogram (Fig. 4B) of the products of reductive isopropylation of this mixture showed three distinct peaks, representing an ε-N-isopropyllysyl form of the blocked peptide (20%), Ip P-G-K-A-R (64%), and the modified proline, Ip-lysine product (10%).

These peptides illustrate a variety of the types of peptides obtained by hydrolysis of proteins with trypsin or chymotrypsin. Each of the native and reductively alkylated peptides could be identified by their characteristic retention time in a chro-



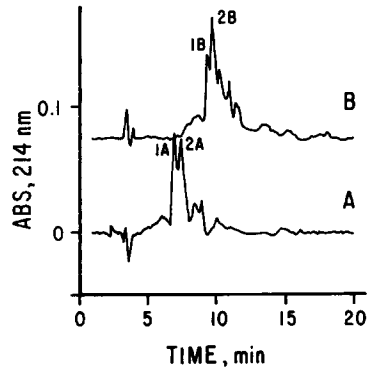


Fig. 3. Chromatogram of a mixture of R-K-D-V-Y, [$^1H_6$]isopropyl R-K-D-V-Y and [$^2H_6$]isopropyl R-K-D-V-Y; (1) R-K-D-V-Y, (2) Ip R-K-D-V-Y and (3) 2Ip R-K-D-V-Y.

Fig. 4. Chromatograms of (A) P-G-K-A-R, and (B) reductively isopropylated P-G-K-A-R. Peaks 1B and 2B are equidistant from 1A and 2A respectively.

matogram of a mixture. Prediction of elution times[5–9] for short peptides is based on the sum of the individual retention coefficients for the amino acids and it is assumed that neither the length of the peptide nor the order of the amino acid residues will have a significant effect. The particular chromatographic column, gradient conditions, and mobile phase modifier (TFA, orthophosphoric acid and perchloric acid) will, of course, affect the retention time of a peptide. Although each of the published sets of retention coefficients[5–9] gave a unique set of predicted elution times for the native peptides, the order of elution was G-G-K-R, P-G-K-A-R, R-K-D-V-Y then G-L-Y, except for the set developed by Browne *et al.*[7], which showed the reverse of the predicted positions of P-G-K-A-R and R-K-D-V-Y. In our system, the most hydrophobic, but shortest peptide G-L-Y was eluted before R-K-D-V-Y, suggesting that some factor based on peptide length should be included.

The effect of reductive alkylation on the retention times of these peptides was remarkably constant (Table II). In the 10 to 80% acetonitrile in water gradient, a single isopropyl amino group increased the retention time of a peptide 2.2 ± 0.2 min. A second isopropyl amino group caused an additional smaller increase of 1.1 ± 0.3 min. A single dimethyl amino group increased the retention time of a peptide by 0.9 ± 0.1 min. The type and number of added alkyl groups, but not the location was important in determining the increase in retention time. Comparable results were obtained with other gradient conditions. In a 10 to 50% acetonitrile in water gradient (0.1% TFA), developed over 15 min, a single isopropyl amino group increased the retention time 2.8 min, and a second group increased it by an additional 1.3 min. In a 5 to 50% (30-min) gradient, the effects were 2.7 min and an additional 1.6 min, respectively, for one or two isopropylamino groups.

In practice, the analysis of a protein digest is simplified, because except for the N-terminal residue of the entire protein, only the lysine residues can be reductively alkylated. Alkylation of the N-terminal residue can be determined separately by dansylation[3]. The tryptic digest of a completely alkylated protein will contain only arginyl peptides, because N,N-dimethyl and N-isopropyl lysine residues resist tryptic cleavage[4,14]. However, these arginyl peptides may contain several alkylated lysine residues and be larger than the optimum for the chromatographic system. Analysis of chymotryptic digests will generally provide more information, since chymotryptic cleavage is not affected by the presence of reductively alkylated residues[15]. Comparison of the chromatograms of chymotryptic digests of native and reductively alkylated protein is useful in the location of reductively alkylated lysine residues in a protein[12]. The results suggest that by including appropriate standards and selecting chemical or enzymatic methods which produce small to moderate sized peptides, one should be able to locate and quantitate specifically alkylated peptides in a partially alkylated protein.

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