Journal of Chromatography, 297 (1984) 37–48
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMSYMP. 348

APPLICATION OF REDUCTIVE DIHYDROXYPROPYLATION OF AMINO GROUPS OF PROTEINS IN PRIMARY STRUCTURAL STUDIES: IDENTIFICATION OF PHENYLTHIOHYDANTOIN DERIVATIVE OF ε-DIHYDROXYPROPYL-LYSINE RESIDUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

A. SEETHARAMA ACHARYA*, LESLIE G. SUSSMAN and BELUR N. MANJULA
The Rockefeller University, 1230 York Avenue, New York, NY 10021 (U.S.A.)

SUMMARY

The general utility of reductive alkylation of amino groups of proteins with glyceraldehyde (2,3-dihydroxypropionaldehyde) in the presence of sodium cyanoborohydride, *i.e.* dihydroxypropylation, as an aid in generating arginine peptides of proteins by tryptic digestion has been investigated. The dihydroxypropylation of the amino groups of ribonuclease A and the streptococcal Pep M5 protein proceeds predominantly to the stage of monoalkylation. The derivatized lysine namely, ε-dihydroxypropyl-lysine is stable to acid hydrolysis, and is eluted slightly ahead of histidine in the amino acid analyzer. The peptide bonds of ε-dihydroxypropyl-lysine residues are resistant to tryptic digestion. The arginine peptides of dihydroxypropylated ribonuclease A, and dihydroxypropylated streptococcal Pep M5 protein have been isolated by reversed-phase high-performance liquid chromatography (HPLC) of the tryptic digest of the derivatized proteins. The phenylthiohydantoin (PTH) derivative of ε-dihydroxypropyl-lysine has been prepared. It is eluted at a position intermediate to that of the PTH derivatives of proline and tryptophan in reversed-phase HPLC on DuPont Zorbax ODS columns. Thus the PTH-ε-dihydroxypropyl-lysine could be identified during the sequence studies of the dihydroxypropylated peptides. The presence of dihydroxypropyl groups on the ε-amino groups of lysine residues in the dihydroxypropylated peptides does not interfere with the Edman degradation studies. The ease of the dihydroxypropylation reaction, the resistance of the peptide bonds of ε-dihydroxypropyl-lysine residues to trypsin, and the identification of the PTH derivative of ε-dihydroxypropyl-lysine residues by reversed-phase HPLC makes the dihydroxypropylation procedure a valuable addition to the arsenal of procedures for limiting the tryptic digestion to the arginine residues of proteins and peptides.

INTRODUCTION

During the course of our studies on the reaction of glyceraldehyde (2,3-dihydroxypropionaldehyde) with hemoglobin S, we observed that the derivatization of

0021-9673/84/$03.00

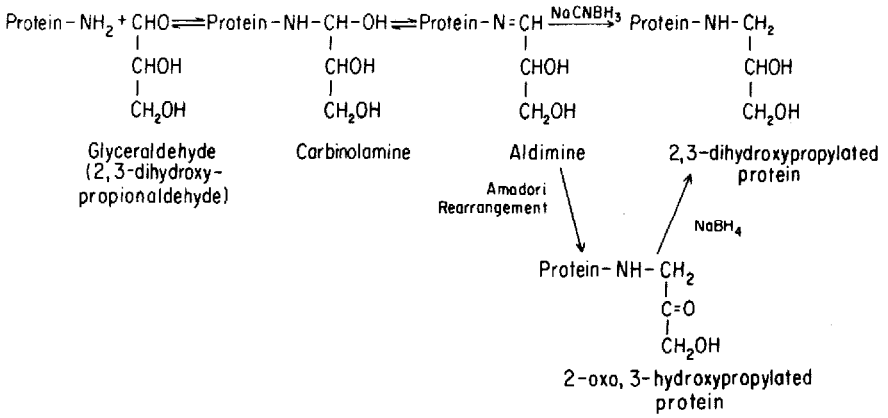

Fig. 1. Schematic representation of the reaction of glyceraldehyde with the amino groups of proteins in the presence or absence of reducing agents.

ε-amino groups of lysine residues of the protein by dihydroxypropylation rendered the peptide bonds of these lysine residues resistant to tryptic digestion. In the initial studies, the protein was incubated with glyceraldehyde in the absence of any reducing agent. The reversible Schiff base adducts of glyceraldehyde formed by the condensation of the carbonyl function of the aldehyde with the amino groups of protein (Fig. 1) undergo an intramolecular rearrangement reaction, referred to as Amadori rearrangement, to form more stable ketoamine adducts, *i.e.* 2-oxo-3-hydroxypropylation of protein occurs[1]. Sodium borohydride reduction of the ketoamine adducts, converts the 2-oxo-3-hydroxypropyl groups to acid stable 2,3-dihydroxypropyl (DHP) groups[1,2]. Alternatively, the 2,3-dihydroxypropylation of the amino groups of protein could also be achieved by incubating the protein with glyceraldehyde in the presence of sodium cyanoborohydride at pH 7.4[3]. In this case, the direct dihydroxypropylation of the protein occurs without the intermediate formation of 2-oxo-3-hydroxypropyl adduct (Fig. 1). In this respect, dihydroxypropylation is analogous to reductive methylation[4,5]. The ease with which the dihydroxypropylation can be carried out and the resistance of the peptide bonds of ε-DHP-lysine residues to tryptic hydrolysis, has prompted us to investigate the general utility of the reductive dihydroxypropylation procedure in generating arginine peptides of protein by tryptic digestion. In the present study, we have carried out the complete dihydroxypropylation of the amino groups of RNase A and streptococcal M5 protein[6], quantitation of ε-DHP-lysine, Edman degradation of the dihydroxypropylated arginine peptides, and the identification of the phenylthiohydantoin (PTH) derivative of ε-DHP-lysine by reversed-phase high-performance liquid chromatography (RP-HPLC). These studies have clearly demonstrated that the reductive dihydroxypropylation of amino groups of proteins is a valuable addition to the arsenal of procedures for limiting the tryptic digestion to the arginine residues of proteins and peptides.

MATERIALS AND METHODS

HPLC grade acetonitrile and methanol were purchased from Burdick & Jack-

son. Trifluoroacetic acid (TFA), and PTH-amino acid standard were from Pierce (Rockford, IL, U.S.A.). L-(1-Tosylamido-2-phenyl) ethyl-chloromethylketone trypsin (TPCK-trypsin), RNase A and glyceraldehyde were from Sigma (St. Louis, MO, U.S.A.). Sodium cyanoborohydride was from Aldrich (Milwaukee, WI, U.S.A.).

Reductive dihydroxypropylation of proteins

Protein at a concentration of 0.25 to 0.5 m*M* in phosphate-buffered saline, pH 7.4 was incubated with glyceraldehyde in the presence of ten-fold molar excess (over the aldehyde) of sodium cyanoborohydride, at 37°C for 30 min. After the reaction, the protein samples were dialyzed extensively against 0.1 *M* ammonium bicarbonate pH 8.0, and the modified protein was isolated by lyophilization.

Amino acid analysis

This was carried out on a Durrum D-500 amino acid analyzer using a three buffer sodium citrate elution system based on the method of Spackman *et al.*[7]. Protein and peptide samples were hydrolyzed in 6 *M* hydrochloric acid at 110°C for 22 h.

Tryptic digestion of DHP-proteins

The protein samples were taken in 0.1 *M* ammonium bicarbonate, pH 8.0 and digested with TPCK-trypsin at 37°C at an enzyme to protein ratio of 1:100. The digested material was isolated by lyophilization.

HPLC of DHP-peptides of RNase A

RP-HPLC fractionation of the peptides of DHP-RNase A was carried out on a 250 × 4.6 mm Whatman ODS-3 (Partisil-10) column, using an HPLC system assembled in the laboratory from commercially available components[8]. A linear gradient of 5–50% acetonitrile–0.1% TFA was used for elution.

HPLC of DHP-peptides of streptococcal Pep M5 protein

Pep M5 protein, prepared as described by Manjula and Fischetti[6], was dihydroxypropylated and subsequently digested with trypsin. The RP-HPLC fractionation of tryptic peptides of DHP-Pep M5 was carried out with a Waters HPLC system on Waters μBondapak C_{18} column, using an acetonitrile–0.5% TFA gradient for elution of peptides.

Identification of PTH-ε-DHP-lysine by HPLC

ε-DHP-lysine was prepared as described by Nigen and Manning[10]. The PTH derivative of ε-DHP-lysine was prepared in the spinning cup of the Beckman sequencer. Conversion of the anilinothiazolinone (ATZ)-amino acid to the PTH derivative was carried out in 1 *M* hydrochloric acid at 80°C for 10 min. The resulting PTH-ε-DHP-lysine was analyzed by HPLC on a 250 × 4.6 mm DuPont Zorbax ODS column using a Hewlett–Packard Model 1084B instrument. The column was operated at 50–60°C and developed with a gradient consisting of acetonitrile and sodium acetate. Solvent A was sodium acetate, pH 5.3–5.5 and Solvent B was 100% acetonitrile.

Amino acid sequence analysis

This was carried out in the Beckman updated 890 B automated sequencer as previously described[9].

RESULTS

Chromatography of ε-DHP-lysine on the amino acid analyzer

In our initial studies on the reaction of glyceraldehyde with HbA, the formation of ε-DHP-lysine was estimated by analyzing an acid hydrolysate of the dihydroxypropylated protein on the short column of a Moore-Stein amino acid analyzer[1,10]. The ε-DHP-lysine was eluted slightly ahead of lysine. In order to increase the versatility of the reductive dihydroxypropylation reaction as a general procedure for the modification of amino groups in proteins, we have now standardized the conditions for the separation of ε-DHP-lysine in the single column system using a Durrum D-500 amino acid analyzer (Fig. 2). The ε-DHP-lysine elution position was sensitive to the pH, and depending on the pH of the second buffer and the time at which the

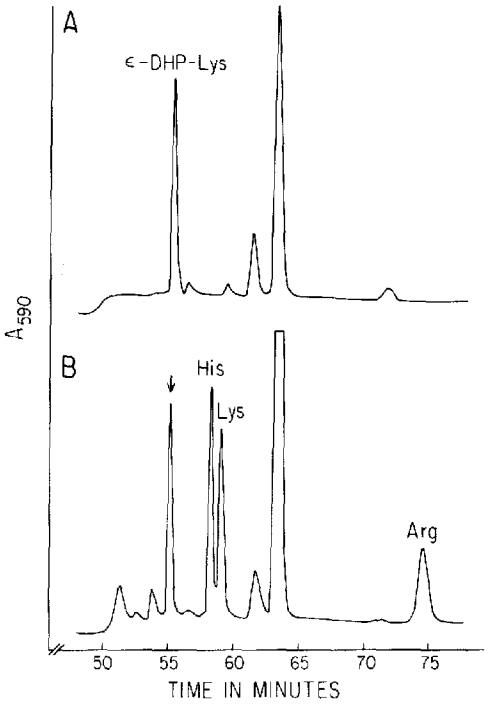

Fig. 2. A section of the chromatogram from the amino acid analysis of: (A) ε-DHP-lysine and (B) RNase A modified by reductive alkylation with glyceraldehyde. RNase A (0.05 m*M*) was incubated at pH 7.4 and 37°C with 20 m*M* glyceraldehyde in the presence of 200 m*M* sodium cyanoborohydride for 30 min and then desalted on a Sephadex G-25 column, equilibrated and eluted with 0.1 *M* acetic acid. The modified protein was isolated by lyophilization and hydrolyzed with 6 *M* hydrochloric acid at 110°C for 22 h. Elution conditions for amino acid analysis were: first buffer was 0.2 *M* sodium citrate, pH 3.25; the second buffer, 0.2 *M* sodium citrate, pH 4.21 was introduced at 28.8 min; and the third buffer, 1 *M* sodium citrate, pH 7.2 was introduced at 49.3 min. The column temperature was switched from 56°C to 64°C at 28.8 min.

elution with the second buffer was initiated, ε-DHP-lysine was either eluted together with histidine or ahead of histidine. For quantitation, the color value of histidine was used. Under the conditions shown in Fig. 2, the ε-DHP-lysine was eluted at 55 min, histidine at 58 min, and lysine at 59 min.

Reductive dihydroxypropylation of the amino groups of RNase A

Chromatography of the acid hydrolysate of RNase A, reductively dihydroxypropylated using 20 m*M* glyceraldehyde in the presence of 200 m*M* sodium cyanoborohydride, is shown in Fig. 2. There was a decrease in the lysine content of RNase A with a concomitant formation of a new chromatographically distinct component, eluted at the position of ε-DHP-lysine. Thus this chromatographic system will be very useful for monitoring the extent of dihydroxypropylation of lysine residues on reductive alkylation with glyceraldehyde. Quantitation of the ε-DHP-lysine peak as well as the decrease in the lysine content revealed that on an average six of the total of ten lysine residues of RNase A were modified.

In an attempt to determine whether complete dihydroxypropylation of the amino groups of RNase A could be carried out without using denaturants, RNase A was dihydroxypropylated using 50 and 100 m*M* glyceraldehyde in the presence of 0.5 *M* and 1.0 *M* sodium cyanoborohydride, respectively. On increasing the concentration of glyceraldehyde from 20 m*M* to 50 and 100 m*M*, the extent of modification of the lysine residues of RNase A increased to 90 and 98%, respectively (Table I). Thus, nearly complete modification of the lysine residues of RNase A could be obtained in the absence of denaturing agents.

Amino acid analysis of RNase A dihydroxypropylated using 50 and 100 m*M* glyceraldehyde showed that most of the modified lysine is present as ε-DHP-lysine (peak eluted at 55 min). Besides the ε-DHP-lysine, small amounts of another new component, eluted around 52.5 min, were present in these two chromatograms, which were essentially absent in the RNase A that was dihydroxypropylated using 20 m*M* glyceraldehyde. This component accounted for about 8% (using the color value of histidine) of the ε-DHP-lysine in these derivatized RNase A samples. In view of the fact that disubstitution of the amino groups occurs during reductive alkylation of proteins with formaldehyde[4] and glycolaldehyde[12], the new component generated when a large excess of the carbonyl component is used is assumed to be ε-N,N-bis-dihydroxypropyl-lysine. These results suggest that the reductive dihydroxypropyla-

TABLE I

DIHYDROXYPROPYLATION OF RIBONUCLEASE A

All reactions were carried out at pH 7.4 and 37°C for 30 min in the presence of 10-fold molar excess of sodium cyanoborohydride over the aldehyde.

*Glyceraldehyde concentration (m*M*)*	*Lysines modified (%)*
10	40
20	60
50	90
100	98

tion of the amino groups of RNase A proceeds predominantly to the stage of monoalkylation.

Reductive dihydroxypropylation of streptococcal Pep M5 protein

The streptococcal Pep M5 protein, a biologically active 197-residue fragment of the type 5 *M* protein, contains 35 lysine and 6 arginine residues[6,13]. The protein does not contain methionine or tryptophan residues. Thus arginine residues are the site of choice for possible generation of peptides for sequence studies[13].

The amino groups of the Pep M5 protein were modified by dihydroxypropylation using 100 m*M* glyceraldehyde in the presence of 1 *M* sodium cyanoborohydride. All the 35 lysine residues of Pep M5 protein were modified by dihydroxypropylation, even in the absence of denaturant. Again, as in the case of RNase A, the modification proceeded mostly to the stage of monoalkylation. The amount of the component eluted at 52.5 min, presumably ε-N,N-bis-dihydroxypropyl-lysine, was about 20% of the total dihydroxypropylated lysines. In addition to the ε-amino groups of the lysines, the α-amino group of Pep M5, namely threonine, also appeared to be dihydroxypropylated[13]. In the case of hemoglobin A, it has been previously shown that the α-amino groups of the α- and β-chains exhibit a higher reactivity than the ε-amino groups[3].

Resistance of peptide bonds of ε-DHP-lysine residues to tryptic hydrolysis

The general utility of reductive dihydroxypropylation of the ε-amino groups of lysine residues for generating arginine peptides of proteins by tryptic digestion depends on the complete resistance of the peptide bonds of ε-DHP-lysine to tryptic digestion. The RP-HPLC map of the tryptic digest of a performic acid-oxidized sample of completely dihydroxypropylated RNase A is shown in Fig. 3. The tryptic peptide map shows only five chromatographically distinct components. The tryptic peptide map of unmodified performic acid-oxidized RNase A shows about 14 chro-

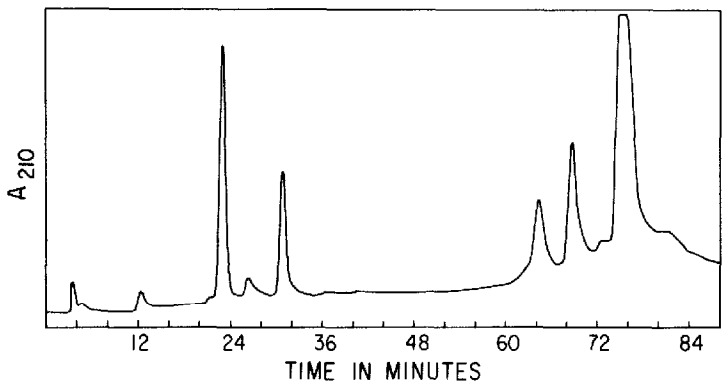

Fig. 3. Chromatography of tryptic peptides of dihydroxypropylated RNase A. RNase A was dihydroxypropylated with 100 m*M* glyceraldehyde in the presence of 1 *M* sodium cyanoborohydride. This results in the complete modification of all ten lysine residues of the protein. The modified protein was performic acid-oxidized and digested with TPCK-trypsin. The tryptic digest was fractionated by RP-HPLC on a Partisil-10 ODS-3 column. The peptides were eluted with a linear gradient of 5–50% acetonitrile–0.1% TFA over a period of 130 min. Flow-rate: 1 ml/min. Peptide elution was monitored at 210 nm.

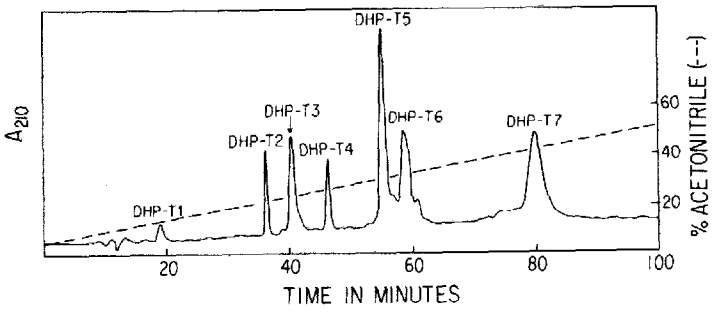

Fig. 4. Fractionation of the tryptic peptides of DHP-Pep M5 by HPLC on a Waters μBondapak C_{18} reversed-phase column. The peptides were eluted with a linear gradient of 5–70% acetonitrile–0.05% TFA over a period of 140 min. Flow-rate: 1 ml/min. The effluent was monitored at 210 nm using an ISCO variable-wavelength detector. The seven peptides were designated DHP-T1 through DHP-T7, in the order of their elution.

matographically distinct components in this RP-HPLC system. Thus, the results clearly demonstrate that the dihydroxypropylation has significantly reduced the number of trypsin-susceptible peptide bonds in oxidized RNase A.

The tryptic peptide map of dihydroxypropylated streptococcal Pep M5 protein is shown in Fig. 4. Pep M5 protein contains six arginine residues[6,13]. It can be seen from Fig. 4 that the digest contains seven major tryptic peptides, designated DHP-T1 through DHP-T7, and this number is consistent with the number of arginine residues in this protein. All but one peptide, namely DHP-T7, contained arginine, (one mole/mole of peptide). The latter peptide is apparently the carboxy-terminal tryptic peptide of the streptococcal Pep M5 protein. The amino acid composition of these seven peptides together accounted for all of the 197 residues of this protein.

The peptide map shown in Fig. 4 is for a 3-h tryptic digest of DHP-Pep M5. The peptide map of a 24-h tryptic digest of the DHP-Pep M5 protein was the same as that of the 3-h digest, thus demonstrating the complete resistance of the peptide bonds of ε-DHP-lysine residues to tryptic digestion.

HPLC of PTH derivative of ε-DHP-lysine

In an attempt to determine the possibility of using the DHP-tryptic peptides for sequence studies, and to identify the PTH-ε-DHP-lysine that may be generated during the Edman degradation studies, ε-DHP-lysine was reacted with phenylisothiocyanate, the ATZ derivative was converted to the PTH derivative and analyzed by RP-HPLC on a DuPont Zorbax ODS column[14]. The chromatography of the PTH-ε-DHP-lysine is shown in Fig. 5B, and the chromatographic pattern of the PTH amino acid standard is shown in Fig. 5A. It may be seen that the PTH derivative of ε-DHP-lysine is eluted at *ca.* 12.5 min, a position close to that of PTH-valine. The absorption spectra of the PTH derivative of ε-DHP-lysine was very similar to that of the PTH derivatives of the other amino acids. Thus it is apparent that the presence of the DHP function on the ε-amino group of lysine does not interfere with the cyclization of the ATZ derivative and that the resulting PTH derivative of ε-DHP-lysine could be identified by RP-HPLC.

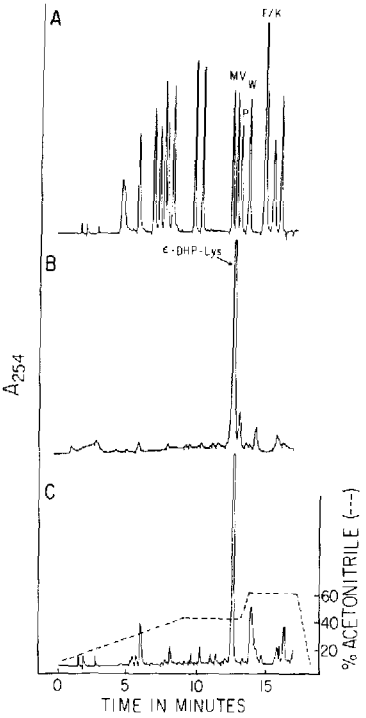

Fig. 5. RP-HPLC of PTH derivatives of (A) standard amino acid mixture, (B) ε-DHP-lysine and (C) first Edman degradation cycle of peptide DHP-T3 of Pep-M5 protein on DuPont Zorbax ODS column. The column was operated at 60°C. Flow-rate: 1.5 ml/min. Solvent A: 0.05 *M* sodium acetate, pH 5.3; solvent B: acetonitrile. The acetonitrile gradient employed is indicated in (C). The PTH derivatives of amino acids are identified by single letter notation. M = Methionine; P = proline; W = tryptophan; F = phenylalanine; and K = lysine.

Automated Edman degradation of the tryptic peptide DHP-T3 of streptococcal Pep M5 protein

In an attempt to establish whether the presence of a dihydroxypropyl group on the ε-amino groups of lysine has any influence on the automated Edman degradation, the tryptic peptide DHP-T3 (see Fig. 4) of streptococcal Pep M5 protein was chosen for a detailed study. This is a 23-residue peptide, and from the amino acid composition it was clear that this peptide corresponds to the segment 65 to 87 of streptococcal Pep M5 protein[13]. The amino acid sequence of this region is shown in Fig. 6. This peptide contains five lysine residues, one of these being the amino-terminal of this peptide, the other four lysine residues being internal. In DHP-T3, all the lysines are present as the ε-dihydroxypropyl derivative. The chromatogram of the PTH derivative obtained after the first cycle of Edman degradation is shown in Fig. 5C. As can be seen from Fig. 5 this PTH derivative was eluted at the position corresponding to that of PTH-valine/ε-DHP-lysine. Since this peptide does not contain any valine, the PTH derivative formed in the first cycle should be of that of ε-DHP-ly-

70 80
Lys-Thr-Ala-Glu-Leu-Thr-Ser-Glu-Lys-Lys-Glu-His-Glu-Ala-Glu-Asn-Asp-Lys-Leu-Lys-Gln-Gln-Arg

Fig. 6. Amino acid sequence of segment 65–87 of Pep M5 protein. The amino acid composition of DHP-T3 corresponds to this region of the molecule.

sine. DHP-T3 was sequenced through its carboxy terminal arginine residue. At all of the positions corresponding to the lysines in the sequence, the PTH derivative of ε-DHP-lysine was identified. Thus, it is clear that the presence of dihydroxypropyl groups on the ε-amino groups of lysine residues in DHP-T3 did not influence the cyclization and/or the cleavage reaction during the sequence studies of DHP-peptides. Besides, the absolute and the repetitive yields for the Edman degradation of DHP-T3 were nearly the same as that of the peptides not containing the dihydroxypropyl function on their ε-amino groups. This also demonstrates the stability of the dihydroxypropyl substituent on the ε-amino groups of lysine under the conditions of cleavage and the formation of PTH derivative.

Separation of PTH derivative of ε-DPH-lysine from the PTH derivative of valine

A better resolution of the PTH derivatives of valine and ε-DHP-lysine is desirable for a more general use of the dihydroxypropylation reaction in the primary structural studies. Accordingly, attempts were made to resolve these two derivatives by using a shallower gradient of acetonitrile. The results are presented in Fig. 7. The PTH derivative of ε-DHP-lysine is now eluted at *ca.* 16 min. (Fig. 7B), *i.e.* at a position intermediate to that of the PTH derivatives of proline and tryptophan (Fig. 7A). When the PTH derivative of ε-DHP-lysine is chromatographed along with the standard PTH amino acids, it is eluted at a distinct position after PTH-proline (Fig. 7A). This chromatographic resolution makes it possible to identify the PTH-ε-DHP-lysine, even when a DHP-peptide or protein contains valine residues. Thus it is clear that the DHP-peptides could be subjected to normal automated Edman degradation studies, and the PTH derivative of ε-DHP-lysine could be easily identified by RP-HPLC, just as the PTH derivatives of other amino acid residues.

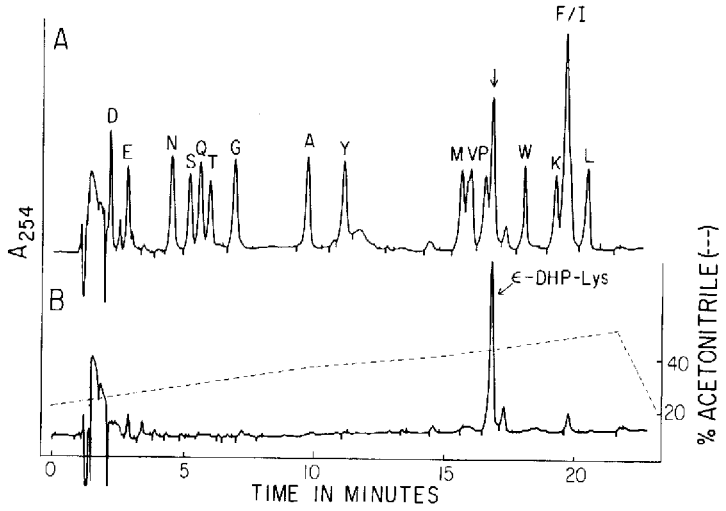

Fig. 7. Separation of PTH-ε-DHP-lysine from PTH-valine by HPLC on DuPont Zorbax ODS column. (A) PTH-amino acid standard, containing PTH-ε-DHP-lysine and (B) PTH-ε-DHP-lysine alone. Column temperature: 50°C. Flow-rate: 1.2 ml/min. Solvent A: 0.1 *M* sodium acetate, pH 5.5; solvent B: acetonitrile. The solvent gradient is indicated in (B). D = Aspartic acid; E = glutamic acid; N = asparagine; S = serine; Q = glutamine; T = threonine; G = glycine; A = alanine; Y = tyrosine; M = methionine; V = valine; P = proline; W = tryptophan; K = lysine; F = phenylalanine; I = isoleucine; and L = leucine.

DISCUSSION

Recent advances in the methodology have made it possible to sequence long stretches of polypeptide chains[15]. This has sparked considerable interest among protein chemists to develop new methods, as well as to improve the existing methods, for generating large fragments of proteins (50–75 residues) for primary structural studies[16,17]. The interest in this area has been furthered by the advances in the methodology for purification of the larger polypeptide fragments by ion-pair reversed-phase HPLC of denatured polypeptides[18,19].

Of the various endopeptidases, trypsin is by far the most specific enzyme, being specific for the peptide bonds formed by the carboxyl groups of basic amino acid residues, namely lysine and arginine. There has been a number of attempts to make use of this high specificity of trypsin to limit the cleavage of the protein either to its arginine residues[20,21] by selectively modifying the ε-amino groups of lysine residues, or to the lysine residues by selectively modifying the guanidino groups of the arginines[22,23]. A number of reagents have been introduced for the modification of the ε-amino groups of lysine residues, for example, acylation using maleic[21] or citraconic anhydride[20]. The insolubility of these reagents, and the possibility of the reaction of these anhydrides at the hydroxy groups of serine, threonine and tyrosine residues are some of the limitations of the procedure[24]. On the other hand, these blocking groups are readily removed at low pH[20,21]. Though the aspect of reversibility is advantageous for regenerating trypsin-susceptible sites when needed, the rapid deblocking of the amino groups could be a disadvantage if a limited cleavage at one or more of the arginine residues of the modified protein is attempted at pH values below 7.0. At these pH values, a significant deblocking of the citraconyl groups could occur to regenerate new trypsin-susceptible sites. Thus a higher stability of the blocking groups appears to be desirable for such studies. This could be achieved by amidination of the ε-amino groups[25]. Preparation of the fully amidinated protein requires a large excess of the reagent (generally fifty-fold) over the ε-amino groups, and the recommended pH for the reaction is *ca.* 10.5[25].

Unlike trypsin, the enzyme clostripain has been shown to have a high specificity for the peptide bonds of arginine residues[26,27]. However, this specificity is not absolute. For example, with RNase A, besides the cleavage of arginyl peptide bonds, this enzyme cleaved the molecule at a lysine residue as well[27]. A recent study by Manjula *et al.*[9], revealed, in addition to the arginyl bond cleavage, the possible cleavage of some of the lysyl peptide bonds of the streptococcal Pep M5 protein by clostripain. The submaxillaris protease is another enzyme that shows selectivity for the peptide bonds of arginine residues[28]. However, the studies with model proteins and peptides have indicated incomplete cleavage of some of the arginyl peptide bonds. Thus, there appears to be a need for further improvement in the methodology for limiting the tryptic cleavage exclusively to arginine residues by modification of the ε-amino groups.

In the present study, we have investigated the feasibility of using reductive alkylation of the ε-amino groups of lysine residues of protein around pH 7, to render their peptide bonds resistant to tryptic digestion. The reductive methylation of proteins with formaldehyde generally yields mono- as well as dimethyl-lysine[4]. Though trypsin does not catalyze the hydrolysis of peptide bonds adjacent to ε-N,N-dime-

thyl-lysyl residues in peptides or in model substrates[29], monomethyl derivatives do appear to be recognized by trypsin although the hydrolysis-rates are slower. Thus incomplete methylation could lead to confusing results. Therefore, the latter aspect need to be taken into consideration when reductive methylation is used to block the sites of tryptic cleavage in proteins.

The results presented in this study clearly demonstrate that the ε-amino groups of RNase A and streptococcal Pep M5 protein could be derivatized nearly completely under physiological conditions by glyceraldehyde in the presence of sodium cyanoborohydride. The reagent, *i.e.* glyceraldehyde, has a good solubility, and the derivatization proceeds predominantly to the stage of monoalkylation. The derivatized lysine, *i.e.* ε-DHP-lysine, is stable under the conditions of acid hydrolysis, and is eluted as a distinct peak just ahead of histidine. Hence, it could be easily quantitated. The PTH derivative of ε-DHP-lysine is eluted just after the PTH derivative of proline. The presence of dihydroxypropyl groups on the ε-amino groups of lysine residues does not appear to interfere with the Edman degradation of DHP-peptides, and the PTH derivative of ε-DHP-lysine formed could be easily identified by RP-HPLC. Thus dihydroxypropylation of the amino groups has several advantages as a general procedure for modification of amino groups.

The dihydroxypropyl groups on the ε-amino groups are very stable even at pH values below 7.0, *i.e.* the conditions for obtaining limited cleavages at one or more arginine residues by digestion of DHP-proteins with trypsin. The DHP-groups are also stable under the acidic conditions generally used for the ion-pair reversed-phase HPLC of peptides and proteins. The hydrophilicity of the dihydroxypropyl groups on the ε-amino groups should help to reduce the hydrophobicity of the larger polypeptide fragments generated by tryptic digestion of the DHP-protein, and hence should make the peptides more soluble in the solvent systems to be used in the purification studies. The dihydroxypropylation also appears to have the additional advantage of reversibility in that the dihydroxypropyl groups on the ε-amino groups could be removed by mild periodate treatment when desired[30]. Thus reductive dihydroxypropylation of amino groups of proteins appears to be a valuable alternative to amidination, citraconylation and maleylation in the primary structural studies of proteins.

ACKNOWLEDGEMENTS

This research was supported in part by the National Institutes of Health Grant HL-27183 (to A.S.A.) and a Grant-in-Aid (AHA 83-1102, to B.N.M.) from the American Heart Association with parts of the funds contributed by the New York Heart Association. A.S.A. is an Established Fellow of the New York Heart Association, and B.N.M. in an Established Investigator of the American Heart Association. The interest of Drs. James M. Manning and Vincent A. Fischetti is gratefully acknowledged. The assistance of Ms. Sheenah M. Mische and Ms. Donna Atherton is very much appreciated.

REFERENCES

1 A. S. Acharya and J. M. Manning, *J. Biol. Chem.*, 255 (1980) 7218–7224.
2 A. S. Acharya and J. M. Manning, *J. Biol. Chem.*, 255 (1980) 1406–1412.
3 A. S. Acharya, L. G. Sussman and J. M. Manning, *J. Biol. Chem.*, 258 (1983) 2296–2302.
4 G. E. Means and R. E. Feeney, *Biochemistry*, 7 (1968) 2192–2201.
5 N. Jentoft and D. G. Dearborn, *J. Biol. Chem.*, 254 (1979) 4359–4365.
6 B. N. Manjula and V. A. Fischetti, *J. Immunol.*, 124 (1980) 261–267.
7 D. H. Spackman, S. Moore and W. H. Stein, *J. Biol. Chem.*, 200 (1953) 493–506.
8 A. S. Acharya, A. DiDonato, B. N. Manjula, V. A. Fischetti and J. M. Manning, *Int. J. Pept. Protein Res.*, 22 (1980) 78–82.
9 B. N. Manjula, S. M. Mische and V. A. Fischetti *Proc. Nat. Acad. Sci. U.S.*, 80 (1983) 5475–5479.
10 A. Nigen and J. M. Manning, *Proc. Nat. Acad. Sci. U.S.*, 74 (1977) 367–371.
11 F. M. Richards and H. W. Wycokoff, *Enzymes*, 4 (1971) 647–806.
12 K. F. Geoghegan, D. M. Ybarra and R. E. Feeney, *Biochemistry*, 18 (1980) 5392–5399.
13 B. N. Manjula, A. S. Acharya, S. M. Mische, T. Fairwell and V. A. Fischetti, *J. Biol. Chem.*, 259 (1984) 3686–3693.
14 C. L. Zimmerman, E. Appella and J. J. Pisano, *Anal. Biochem.*, 75 (1976) 77.
15 M. W. Hunkapiller, R. M. Hewick, W. J. Dreyer and L. E. Hood, *Methods Enzymol.*, 91 (1983) 399–413.
16 A. Fontana, D. Dalzoppo, C. Grandi and M. Zambonin, *Methods Enzymol.*, 91 (1983) 311–318.
17 H. V. Huang, M. W. Bond, M. W. Hunkapiller and L. E. Hood, *Methods Enzymol.*, 91 (1983) 318–324.
18 F. Regnier, *Methods Enzymol.*, 91 (1983) 137–190.
19 M. Hermodson and C. Mahoney, *Methods Enzymol.*, 91 (1983) 352–367.
20 M. Z. Atassi and F. S. A. Habeeb, *Methods Enzymol.*, 25 (1972) 546–553.
21 B. S. Hartley, *Biochem. J.*, 119 (1970) 805–822.
22 E. Patthy and E. L. Smith, *J. Biol. Chem.*, 250 (1975) 557–564.
23 K. Toi, E. Bynum, E. Norris and H. A. Itano, *J. Biol. Chem.*, 242 (1967) 1036–1043.
24 R. N. Perham, in H. L. Kornberg, J. C. Metcalf, D. H. North, C. J. Pogson and K. F. Tipton, *Techniques in Protein and Enzyme Biochemistry*, Vol. B110, Elsevier/North Holland, Amsterdam, 1978, pp. 1–39.
25 J. K. Inman, R. N. Perham, G. C. Dubois and E. Appella, *Methods Enzymol.*, 91 (1980) 559–569.
26 M. W. Mitchell, *Methods Enzymol.*, 47 (1977) 165–174.
27 M. W. Mitchell and W. F. Harrington, *J. Biol. Chem.*, 243 (1968) 4863.
28 I. Schenkein, M. Levy, E. C. Franklin and B. Frangione, *Arch. Biochem. Biophys.*, 182 (1977) 64–70.
29 L. Ponez and D. G. Dearborn, *J. Biol. Chem.*, 258 (1983) 1844–1850.
30 A. S. Acharya, L. G. Sussman and B. N. Manjula, *Fed. Proc., Fed. Amer. Soc. Exp. Biol.*, (1984) in press.