Dihydroxypropylation of Amino Groups of Proteins: Use of Glyceraldehyde as a Reversible Agent for Reductive Alkylation†

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ABSTRACT: The mode of derivatization of amino groups of proteins by glyceraldehyde, an aldotriose, depends on the presence or absence of reducing agent. In the presence of sodium cyanoborohydride, the Schiff base adducts of the aldehyde with the amino groups are reduced, and dihydroxypropylation of amino groups takes place (reductive mode). The reductively glycated lysine residue, N^{ϵ}-(2,3-dihydroxypropyl)lysine, is a substituted α-amino alcohol. This α-amino alcoholic function of the derivatized lysine should be susceptible to periodate oxidation, and this oxidation is anticipated to result in the regeneration of the lysine residue. This aspect has been now investigated. Indeed, on mild periodate oxidation (15 mM periodate, 15 min at room temperature) of dihydroxypropylated ribonuclease A, nearly 95% of its N^{ϵ}-(2,3-dihydroxypropyl)lysine residues were regenerated to lysine residues. The removal of the dihydroxypropyl groups by periodate oxidation could be accomplished within a wide pH range with little variation in the recovery of lysines. The possible usefulness of this reversible chemical modification procedure in the primary structural studies of proteins was investigated with a tryptic peptide of dihydroxypropylated streptococcal M5 protein, namely, DHP-T4. This 12-residue tryptic peptide contains one internal N^{ϵ}-(dihydroxypropyl)lysine. The dihydroxypropylated peptide released most of its dihydroxypropyl groups on mild periodate oxidation. Redigestion of the periodate-treated peptide with trypsin generated the two expected peptides, demonstrating the generation of a trypsin-susceptible site. Reductive dihydroxypropylation of amino groups of RNase A resulted in the loss of its enzyme activity, the extent of inactivation increasing with the concentration of the glyceraldehyde used. Periodate oxidation of dihydroxypropylated ribonuclease A regenerated the full enzymic activity of the parent protein. These results demonstrate that dihydroxypropylation of amino groups is a valuable addition to the arsenal of reversible amino group modification procedures.

Derivatization of the amino groups of proteins by reductive alkylation (Means & Feeney, 1968) has become a valuable procedure for chemical modification of the amino groups of proteins (Jentoft & Dearborn, 1983). The ease with which the reductive alkylation could be carried out and also the fact that the alkylated amino group retains its original positive charge have contributed significantly for this versatility. Reductive alkylation, as first introduced by Means and Feeney (1968), involves treatment of the protein with aliphatic carbonyl compounds, aldehydes or ketones, in the presence of sodium borohydride near pH 9.0. More recently, sodium cyanoborohydride has been introduced as a substitute for sodium borohydride in view of its potential for preferential reduction of Schiff base adducts in the presence of free carbonyl compounds (Jentoft & Dearborn, 1979; Dottavio-Martin & Ravel, 1978). Moreover, with sodium cyanoborohydride the reductive alkylation could be carried out in the neutral pH region. However, some caution should be exercised in the use of sodium cyanoborohydride as the reagent in the reductive alkylation experiments, since there is the possible formation of cyanoalkyl derivatives besides the alkyl derivatives (Gidley & Sanders, 1982). Reductive alkylation is also a versatile procedure in that by appropriate choice of the aliphatic aldehyde the desired alkyl group could be introduced on the amino group (Acharya et al., 1985).

We have been studying the reaction of glyceraldehyde and other α-hydroxy aldehydes with proteins (Acharya & Manning, 1980a,b, 1983), specifically that of glyceraldehyde (2,3-dihydroxypropionaldehyde) with hemoglobin S (HbS) in view of its antisickling potential (Manning & Acharya, 1984). The reaction of this aldotriose with Hb in the absence of sodium cyanoborohydride is a prototype of nonenzymic glycation in that the aldotriose forms Schiff base adducts with the protein amino groups and these adducts undergo Amadori rearrangement to form a stable ketoamine adduct, i.e., 2-oxo-3-hydroxypropylation of protein takes place (Acharya & Manning, 1980b) (structure 1, Figure 1). These adducts are relatively stable under physiological conditions but readily regenerate the amino groups on acid hydrolysis. The 2-oxo-3-hydroxypropyl groups on the ϵ-amino groups of proteins are also slowly released from the protein in the alkaline pH region (pH 8.0) in Tris[1]-acetate buffers (Acharya & Sussman, 1984). Therefore, for structural studies of 2-oxo-3-hydroxypropylated HbS, the adducts were reduced with sodium borohydride (Acharya & Manning, 1980a). This reduction converts the 2-oxo-3-hydroxypropyl moieties on the amino groups to acid-stable 2,3-dihydroxypropyl groups (structure 2, Figure 1).

The dihydroxypropylation of the amino groups of proteins could also be accomplished by carrying out the reaction of glyceraldehyde with the protein in the presence of sodium cyanoborohydride ($NaCNBH_3$) (Acharya et al., 1983a,b).

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[1] Abbreviations: DHP, 2,3-dihydroxypropyl; PBS, phosphate-buffered saline; $NaCNBH_3$, sodium cyanoborohydride; Tris, tris(hydroxymethyl)aminomethane; FPLC, fast protein liquid chromatography.

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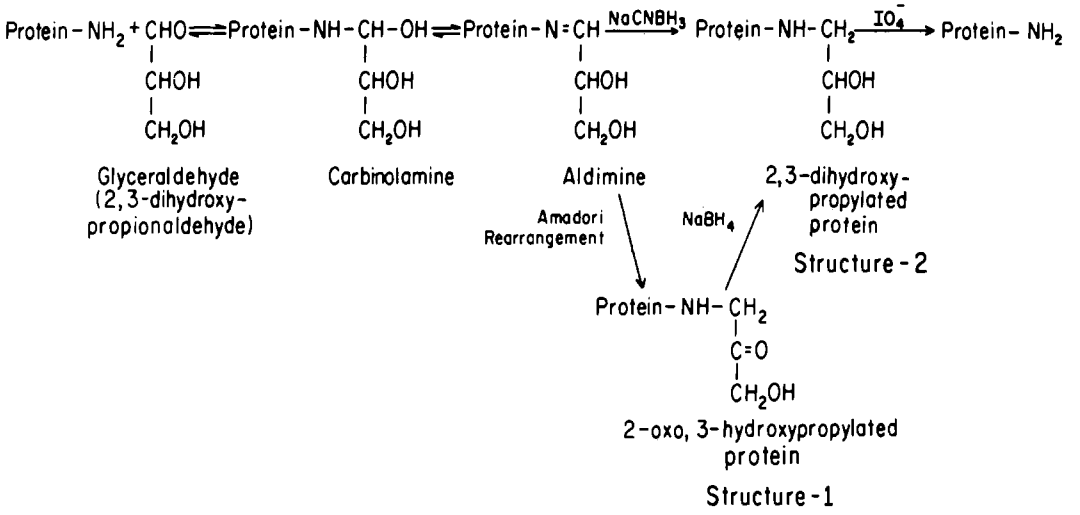

FIGURE 1: Schematic representation of the two modes of derivatization of the amino groups of proteins by glyceraldehyde (aldotriose).

The reductive dihydroxypropylation of amino groups proceeds predominantly to the stage of monoalkylation. The peptide bonds of the derivatized lysine, i.e., N^{ϵ}-(2,3-dihydroxypropyl)lysine (N^{ϵ}-DHP-Lys), are completely resistant to trypsin (Acharya et al., 1984). In the structural studies of streptococcal M proteins, which contain a very high percentage of lysine residues, the reductive dihydroxypropylation of their amino groups proved very valuable to limit tryptic digestion to their arginine residues. Thus, large arginyl peptides could be isolated for automated amino acid sequencing (Manjula et al., 1984, 1986).

The N^{ϵ}-(dihydroxypropyl)lysine residues have a substituted α-amino alcoholic function. Hence, these should be readily attacked by periodate to generate the free α-amino group of lysine residues. This type of reaction, i.e., periodate oxidation of an α-amino alcoholic function [chemical grouping of the type $RNHCH_2C(OH)<$, where R is an alkyl group] was first described by Geoghegan et al. (1979) as the basis for reversible reductive alkylation of protein amino groups. Glycolaldehyde and acetol were used as the alkylating agents to have this type of grouping in the derivative. Glycolaldehyde reacts readily with the protein amino groups to give a high level of modification but has the disadvantage that a second mole of aldehyde can add relatively easily to give a disubstituted derivative. The latter is resistant to periodate oxidation. When sodium borohydride was used as the reducing agent, ~20% of the amino groups modified were present as the disubstituted derivative. Much higher levels of disubstitution were observed when sodium cyanoborohydride was used as the reducing agent. Acetol, a ketone, was less reactive. However, with sodium borohydride as the reducing agent the derivatization resulted in monoalkylation. Acetol gave a much more satisfactory result in the reversible reductive alkylation of turkey ovomucoid than had been obtained with glycolaldehyde. Thus, acetol showed greater specificity for monoalkylation, which in turn lead to a more complete reversal of modification in the periodate step. On the other hand, glycolaldehyde showed higher reactivity toward amino groups but had a lower specificity for monoalkylation, leading to lower levels of reversibility.

Reductive alkylation of proteins with glyceraldehyde at pH 7.4 and 37 °C and with sodium cyanoborohydride as a reducing agent proceeds predominantly to the stage of monoalkylation (Acharya et al., 1984). Thus, reductive alkylation with this aldotriose in the presence of sodium cyanoborohydride appears to show the specificity for monoalkylation similar to that seen on reductive alkylation with acetol and with sodium borohydride as the reducing agent. Besides, in the presence of sodium cyanoborohydride its reactivity toward amino groups is comparable to that of glycolaldehyde (Acharya & Sussman, 1983). Thus, glyceraldehyde appeared to have both the desirable qualities of the two reagents, namely, the selectivity of acetol and the reactivity of glycolaldehyde, to make it a better reagent for reversible reductive alkylation of protein amino groups. Accordingly, we have now investigated the regeneration of the amino groups from dihydroxypropylated peptide as well as from dihydroxypropylated RNase A (DHP-RNase A) on treatment with periodate. The results clearly demonstrate the possible general utility of this reversible chemical modification procedure in the structural studies of proteins.

Materials and Methods

Materials. HPLC-grade acetonitrile was purchased from Burdick and Jackson. Trifluoroacetic acid (TFA) was from Pierce (Rockford, IL). L-(1-Tosylamido)-2-phenylethyl chloromethyl ketone–trypsin (TPCK-trypsin), RNase A, and glyceraldehyde were from Sigma (St. Louis, MO). Sodium cyanoborohydride was from Aldrich Chemical Co. (Milwaukee, WI). Performic acid oxidized RNase A was either obtained from Sigma or prepared by performic acid oxidation of RNase A as described by Hirs (1956).

Reductive Dihydroxypropylation of Proteins. Protein samples (0.25–0.5 mM) in phosphate-buffered saline (PBS), pH 7.4, were incubated with glyceraldehyde in the presence of a 10-fold molar excess (over the aldehyde) of $NaCNBH_3$ at 37 °C for 30 min. After the reaction, the dihydroxypropylated (DHP) protein samples were dialyzed extensively against 0.1 M ammonium bicarbonate, pH 8.0, and the modified protein was isolated by lyophilization. Alternatively, the DHP protein was also isolated by gel filtration of the reaction mixture on a Sephadex G-25 column equilibrated and eluted with 0.1 M acetic acid, followed by lyophilization.

Periodate Oxidation of DHP Protein and DHP Peptides. Routinely, the DHP protein (1–2 mg/mL) or DHP peptide (0.1–0.5 mg/mL) in 10 mM phosphate buffer, pH 7.4, was incubated with 5, 10, or 15 mM sodium metaperiodate for 15 min at room temperature. After the oxidation, the protein was isolated either by dialysis or by gel filtration on a Sephadex G-25 column, equilibrated and eluted with 0.1 M acetic acid. The periodate-treated DHP peptides were isolated by reverse-phase high-performance liquid chromatography

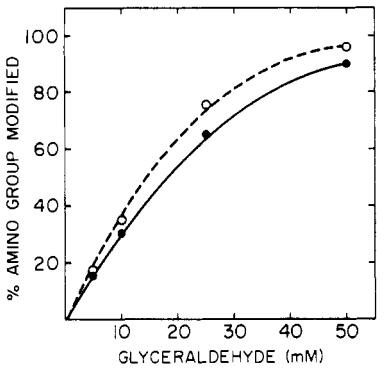

FIGURE 2: Influence of glyceraldehyde concentration on the extent of dihydroxypropylation of (●) native RNase A and (○) performic acid oxidized RNase A. The reductive dihydroxypropylation was carried out in PBS, pH 7.4, and 37 °C for 0.5 h at a protein concentration of 0.5 mM. A 10-fold excess of $NaCNBH_3$ over that of glyceraldehyde was used in all these modification studies. The amino group modification is determined by estimating the decrease in the lysine content after reductive alkylation.

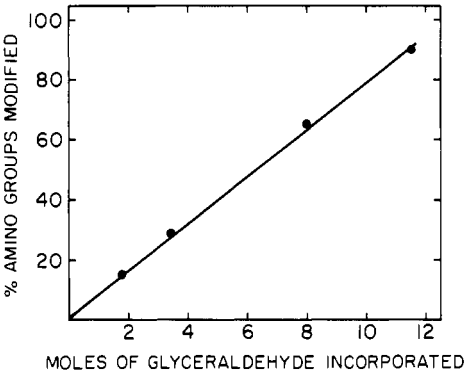

FIGURE 3: Correlation of incorporation of [^{14}C]glyceraldehyde into RNase A with the extent of amino groups modified. The amino group modification is determined by determining the decrease in lysine content after reductive alkylation.

(RPHPLC) (Acharya et al., 1983a,b).

Tryptic Digestion. The protein or peptide samples were taken up in 0.1 M ammonium bicarbonate, pH 8.0, and digested with TPCK-trypsin (Sigma) at an enzyme:protein (w/w) ratio of 1:100. The digested material was isolated by lyophilization.

RNase A Activity Measurements. RNase A activity was followed by the release of acid-soluble nucleotides from RNA by the uranyl acetate–TCA procedure of Klee and Richards (1957) as described earlier (Acharya & Vithayathil, 1975). For calculations of the percentage activity of the protein solutions, the concentration of the protein was determined from the absorbance measurements, a molar extinction coefficient of 9740 at 278 nm being assumed (Saroff & Carroll, 1962).

RESULTS

Mono- and Disubstitution of the Amino Groups during Reductive Alkylation with Glyceraldehyde. The influence of the concentration of glyceraldehyde on the extent of dihydroxypropylation of the amino groups of RNase A was investigated to determine the conditions to obtain a complete derivatization of the lysine residues of the protein (Figure 2). As the concentration of glyceraldehyde was increased from 5 to 50 mM, the extent of modification of lysine residues increased from 15 to 90%. However, the extent of modification of the lysine residues of RNase A was not directly proportional to the concentration of glyceraldehyde. The influence of the cleavage of the disulfide bonds of RNase A on the extent of modification of its lysine residues was also investigated to determine the influence of the native structure of the protein for reductive dihydroxypropylation (Figure 2). It may be seen that at all concentrations of glyceraldehyde the extent of modification seen with performic acid oxidized RNase A (Sigma) is only slightly higher than that with native RNase A.[2] Thus, it is clear that nearly complete modification[3] of the lysine residues of RNase A could be obtained at neutral pH, even without prior cleavage of the disulfide bonds of the protein. This observation is consistent with the results of the earlier studies that all the lysine residues of streptococcal M5 and M6 proteins could be dihydroxypropylated at neutral pH (Manjula et al., 1984, 1986).

In an attempt to determine the complete selectivity of the glyceraldehyde to the amino groups and its propensity to form mono- and disubstituted derivatives of amino groups, the number of moles of glyceraldehyde incorporated into the protein at various concentrations of [^{14}C]glyceraldehyde was determined and correlated with the modification of the amino groups (Figure 3). As can be seen, a linear correspondence was observed between the extent of modification of the amino groups and the moles of glyceraldehyde incorporated. When the average modification of the amino functions of RNase A is 10 groups per mole of protein, nearly 11.5 mol of [^{14}C]-glyceraldehyde was incorporated into the protein. Thus, the modification as seen by the amount of glyceraldehyde incorporated was only about 1.5 mol higher than the number of amino groups derivatized. The results suggest the presence of small amounts of di- and possibly trisubstituted derivatives of the amino groups in the sample of DHP-RNase A. Thus, it is clear that, even when a large excess of glyceraldehyde is used, the dihydroxypropylation proceeds predominantly to the stage of monoalkylation.

Periodate Oxidation of DHP-RNase A. In order to examine the reversibility of the dihydroxypropylation reaction on oxidation with periodate, DHP-RNase A prepared by incubating the protein (0.5 mM) with 10 mM [^{14}C]glyceraldehyde and 100 mM $NaCNBH_3$ was treated with 15 mM periodate in 0.2 M borate buffer, pH 8.6, at 23 °C for 15 min and then subjected to gel filtration on a Sephadex G-25 column. The periodate oxidation resulted in the release of more than 95% of the label bound to the protein, demonstrating the release of dihydroxypropyl groups from the protein.

The extent of reversibility on periodate oxidation was also investigated at lower pHs and was found to be nearly the same when the oxidation was carried out at pH 7.0 or 6.0. A kinetic analysis revealed that the release of the ^{14}C label is nearly complete in about 5 min of the periodate oxidation. However, for most of the oxidation studies reported here the reaction was routinely carried out in PBS, pH 7.4, at room temperature for 15 min.

Regeneration of Lysine Residues on Periodate Oxidation. Mechanistically, periodate oxidation of amino alcohols should generate free amino groups. Thus, in the present case lysine residues should be regenerated on the protein (Figure 1). The regeneration of lysine residues after periodate treatment of DHP-RNase A was confirmed by amino acid analysis. Figure

[2] When reductive glycation is carried out with a limiting concentration of glyceraldehyde (2.5 mM, ca. a 5-fold molar excess over the protein), the amino groups of performic acid oxidized RNase A were derivatized at a significantly faster rate than those of native RNase A.

[3] On complete derivatization of the amino groups, the NH_2-terminal lysine residue of RNase A should be converted to α,α-bis-DHP-Lys. No attempt has been made in this study to determine the position of this derivative of lysine, which is presumably a ninhydrin-negative component.

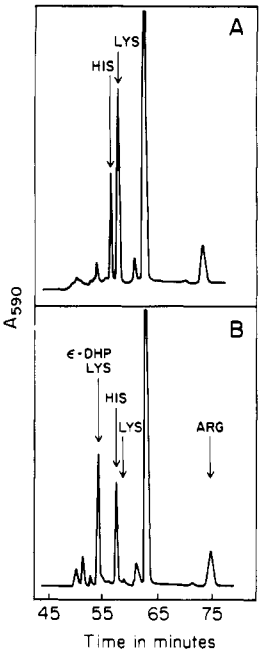

FIGURE 4: Amino acid analysis of periodate-treated DHP-RNase A. A section of the chromatogram from the amino acid analysis of DHP-RNase A (B) and periodate-treated DHP-RNase A (A) is shown. Elution conditions for amino acid analysis were as follows: first buffer 0.2 M citrate, pH 3.25; second buffer 0.2 M citrate, pH 4.21, introduced at 28.8 min; third buffer 1 M sodium citrate, pH 7.2, introduced at 49.3 min. The column temperature was switched from 56 to 64 °C at 28.8 min.

Table I: Regeneration of Lysine Residues on Periodate Oxidation of Dihydroxypropylated RNase A

concn of glyceraldehyde used for dihydroxypropylation (mM)[a]	lysine (mol/mol)	
	before periodate oxidation[b]	after periodate oxidation[b]
5	8.5	10.0
10	7.0	10.0
25	3.5	10.0
50	1.0	9.5

[a] Reductive dihydroxypropylation of RNase A was carried out in PBS, pH 7.4, at 37 °C for 30 min, at a protein concentration of 0.5 mM. The concentration of sodium cyanoborohydride was a 10-fold molar excess over that of glyceraldehyde. [b] Periodate oxidation was carried out in PBS, pH 7.4, at room temperature for 15 min. Protein concentration was 1 mg/mL, and the periodate concentration was 15 mM.

4 shows the elution of the basic amino acids from the Dionex D-500 amino acid analyzer. The N^{ϵ}-DHP-Lys elutes slightly ahead of the histidine peak.[4] As discussed earlier, on reaction of RNase A with 50 mM glyceraldehyde in the presence of 500 mM $NaCNBH_3$, most of the lysine residues of RNase A were modified. On treatment of this modified protein with periodate the N^{ϵ}-DHP-Lys peak disappeared completely with a concomitant appearance of lysine residues. The amount of lysine present in this periodate-treated sample was about 9.5 residues/mol, indicating that the removal of the dihydroxypropyl groups is nearly quantitative. On the basis of the disappearance of N^{ϵ}-DHP-Lys on periodate treatment, the regeneration of lysine from N^{ϵ}-DHP-Lys was of the order of 95% or greater.

[4] The small peaks eluting slightly ahead of N^{ϵ}-DHP-Lys are apparently di- and trisubstituted lysine residues. In DHP-RNase A, prepared with 100 mM aldotriose, the relative amounts of these peaks increased slightly.

88 94 99
Asp Thr Leu Ser Thr Gln Lys Glu Thr Leu Glu Arg

FIGURE 5: Amino acid sequence of segment 88–99 of streptococcal Pep M5 protein. In peptide DHP-T4, the lysine residue at position 94 is present as N^{ϵ}-DHP-Lys.

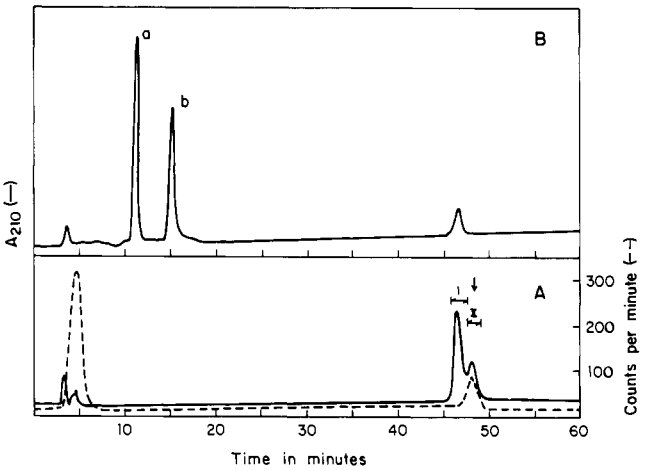

FIGURE 6: HPLC analysis of periodate-treated DHP-T4 and its tryptic digest. RPHPLC was carried out on a Partisil-10 ODS-3 column. The peptides were eluted with a linear gradient of 5–25% acetonitrile in 0.1% TFA over a period of 120 min. The flow rate was 1 mL/min. Peptide elution was monitored at 210 nm. (A) Periodate-treated DHP-T4. Components I and II were isolated and subjected to tryptic digestion. (—) A_{210}; (- -) counts per minute. (B) Tryptic digest of component I of the periodate-treated DHP-T4.

Regeneration of lysine residues from DHP-RNase A on periodate oxidation was also studied as a function of the glyceraldehyde concentration used for the dihydroxypropylation reaction (Table I). It may be seen that the regeneration of lysine residues is good at all the concentrations of (5–50 mM) glyceraldehyde studied.

The influence of the concentration of $NaCNBH_3$ used during the dihydroxypropylation reaction on the extent of reversibility was also investigated. When the relative concentration of $NaCNBH_3$ to glyceraldehyde (20 mM) was increased from 1- to 5- to 10-fold molar excess, the reversibility of dihydroxypropylation on periodate oxidation (as reflected by the release of ^{14}C label) increased from 60 to 70 to 95%, respectively. Thus, use of a 10-fold molar excess of $NaCNBH_3$ over the aldehyde during the reductive dihydroxypropylation seems to provide a dihydroxypropylated protein sample that gives nearly quantitative regeneration of the lysine residues.

Regeneration of Trypsin-Susceptible Site in a Dihydroxypropylated Peptide after Periodate Oxidation. As pointed out earlier, reductive dihydroxypropylation of streptococcal M proteins has proved to be a valuable procedure to limit the tryptic digestion to their arginyl residues (Manjula et al., 1984, 1986). Since periodate oxidation of the dihydroxypropylated protein regenerates lysine residues, this in principle regenerates trypsin-susceptible sites on the protein, and this procedure could thus be useful in identifying overlapping peptides during the course primary structural studies of proteins. This aspect has been investigated with a tryptic peptide obtained from the dihydroxypropylated streptococcal Pep M5 protein, namely, DHP-T4. This is a 12-residue tryptic fragment corresponding to segment 88–99 of the Pep M5 protein and contains a single internal lysine residue (Manjula et al., 1984) (Figure 5). In the dihydroxypropylated protein, the ϵ-amino group of this residue (as well as the other lysines) is dihydroxypropylated, and hence, the peptide bond of this lysine (Lys-94 in the Pep M5 protein) was not hydrolyzed by trypsin. Quantitation of

Table II: Amino Acid Composition of Tryptic Peptides of Periodate-Treated DHP-T4

amino acid	peptide a found[a]	peptide a expected for segment 88–94 of Pep M5	peptide b found[a]	peptide b expected for segment 95–99 of Pep M5
Asp	1.00	1		
Thr	1.65	2	0.85	1
Ser	0.95	1		
Glu	1.05	1	2.10	2
Leu	1.00	1	1.00	1
Lys	0.90	1		
Arg			0.90	1

[a] Calculated assuming the value of Leu as 1.

the N^{ϵ}-DHP-Lys content of dihydroxypropylated streptococcal Pep M5 protein revealed that this reductively alkylated protein may contain about 15% disubstituted lysine residues.

Periodate oxidation of DHP-T4 resulted in the release of the ^{14}C label of glyceraldehyde from the peptide, suggesting the regeneration of the lysine residue. On analysis by RPHPLC, the periodate-treated DHP-T4 showed two components, I and II (Figure 6A). Component II accounted for about 20% of the sample, eluted in nearly the same position as untreated DHP-T4, and was radioactive. The major nonradioactive component I (presumably deprotected DHP-T4) and the radioactive component II (presumably DHP-T4 containing disubstituted lysine) were isolated and subjected to tryptic digestion. RPHPLC of the tryptic digest of the nonradioactive component (Figure 6B) revealed the formation of two new peptides, "a" and "b". On the other hand, no new peptides were formed after incubation of the radioactive component II with trypsin under the same conditions. This clearly demonstrates the generation of a trypsin-susceptible site on periodate oxidation of DHP-T4.

The two new peptides generated on trypsin digestion of the nonradioactive component of periodate-treated DHP-T4 were isolated, and their amino acid compositions were determined (Table II). The amino acid composition of peptide "a" corresponded to segment 88–94 of Pep M5, and that of peptide "b" corresponded to segment 95–99. The content of serine and threonine, the hydroxy amino acids in peptides "a" and "b", was very good, showing that the conditions of periodate oxidation that were employed to remove the dihydroxypropyl groups from the ϵ-amino group are mild and do not result in any significant oxidation of these hydroxy amino acids.

Influence of the Reversible Modification of Amino Groups of RNase A on Its Enzymic Activity. In an attempt to evaluate the general utility of the use of glyceraldehyde as a reversible reagent for the reductive alkylation of the amino groups of proteins, the influence of reductive dihydroxypropylation of RNase A and the subsequent periodate treatment of the derivatized protein on the amino acid composition of the protein and its enzyme activity have been investigated. The amino acid compositions of DHP-RNase A and the periodate-oxidized material are vitrually identical (Table III), except that in the DHP protein the lysines are present as DHP-Lys. This confirms that mild periodate oxidation does not cause any destruction of amino acid residues of the protein other than selectively removing the dihydroxypropyl groups. This result is consistent with the observation of Fields and Dixon (1968) that the selective oxidation of the amino-terminal serine of RNase S protein (residues 21–124 of RNase A) occurs on mild periodate treatment of the protein. This selectivity aspect is further confirmed by the study of the enzymic activity of DHP-RNase A and its periodate-oxidized product.

Table III: Amino Acid Composition of Dihydroxypropylated[a] RNase A and Its Periodate Oxidation[b] Product

amino acid[c]	expected for RNase A	dihydroxypropylated RNase A before oxidation	dihydroxypropylated RNase A after oxidation
Asp	15	14.4	14.6
Thr	10	8.9	9.1
Ser	15	13.8	13.9
Glu	12	11.6	11.5
Pro	4	3.6	3.8
Gly	3	2.8	2.8
Ala	12	12.0	12.0
$^1/_2$-Cys	8	7.1	7.2
Val	9	8.8	8.8
Met	4	3.8	3.7
Iel	3	2.4	2.1
Leu	2	2.1	1.8
Tyr	6	5.6	5.5
Phe	3	2.9	2.8
N^{ϵ}-DHP-Lys		6.4	
His	4	3.8	3.8
Lys	10	3.5	9.8
Arg	4	3.9	3.8

[a] Reductive dihydroxypropylation was carried out at pH 7.4 in PBS buffer for 15 min at a protein concentration of 0.5 mM. Glyceraldehyde concentration was 25 mM, and that of $NaCNBH_3$ was 250 mM. [b] Periodate oxidation was carried out at room temperature, pH 7.4, with a protein concentration of 1 mg/mL and a periodate concentration of 15 mM for 15 min. [c] Calculated with a value of 12 for alanine.

Table IV: Influence of Reductive 2,3-Dihydroxypropylation of RNase A and Its Reversal with Periodate on the Enzymatic Activity of the Protein

concn of glyceraldehyde[a] used in reductive alkylation expt (mM)	% activity[c] after dihydroxypropylation	% activity[c] after periodate treatment
0[b]	100	100
10	70	100
25	35	98
50	15	100

[a] Reductive dihydroxypropylation was carried out in PBS, pH 7.4, at 37 °C for 15 min at a protein concentration of 0.5 mM and a 10-fold molar excess of sodium cyanoborohydride over that of glyceraldehyde. [b] In this control experiment, RNase A (0.5 mM) was incubated in PBS, pH 7.4, with 500 mM sodium cyanoborohydride at 37 °C for 15 min. [c] Percent activity of a derivatized RNase A sample was calculated by comparing its RNA-depolymerizing activity with that of the same amount of unmodified RNase A.

Neither the incubation of RNase A with sodium cyanoborohydride nor the periodate treatment of RNase A had any influence on the enzymic activity of the protein (Table IV). Reductive alkylation with glyceraldehyde resulted in the inactivation of RNase A. The extent of loss in the enzymic activity increased with the increase in the amount of glyceraldehyde used in the reductive dihydroxypropylation. Interestingly, at all concentrations of glyceraldehyde used, periodate treatment of the derivatized protein regenerated nearly complete activity of the protein. This is consistent with the observation that the amino acid composition of periodate-treated DHP-RNase A is nearly identical with that of native RNase A (Table III). Thus, the condition used for either the reductive alkylation or the periodate treatment of the derivatized protein does not result in any irreversible damage to the protein.

Chromatographic behavior of periodate-treated DHP-RNase A on a Mono-S column has also been compared with that of native RNase A and DHP-RNase A with an FPLC system. DHP-RNase A (prepared using 25 mM glyceraldehyde and 250 mM sodium cyanoborohydride) chromatographed ahead of the RNase A peak position as a broad peak, and it did not contain any unmodified RNase A. On periodate

oxidation of the DHP-RNase A, it disappeared completely with the concomitant appearance of material eluting at the RNase A peak position. Thus, this FPLC analysis of the regenerated protein confirms that glyceraldehyde could be used as a good reversible agent for reductive alkylation of the amino groups of proteins.

Discussion

The derivatization of the amino groups of proteins or peptides by dihydroxypropylation and subsequent removal of the dihydroxypropyl groups when desired by mild periodate oxidation complement the other reversible chemical modification procedures for amino groups (Lundblad & Noyes, 1984). Of the various reagents suitable for reversible chemical modification, some modify the positively charged amino groups to a negatively charged form, for example, maleic anhydride (Hartly, 1980) and citraconic anhydride (Atassi & Habeeb, 1972). Others like ethyl thiotrifluoroacetate convert the positively charged amino group of the protein to an uncharged form (Goldberger & Anfinsen, 1962). On the other hand, the alkyl imidates retain the positive charge of the original amino groups (Hunter & Ludwig, 1972). Dihydroxypropylation, which results in the reductive alkylation of amino groups, is expected to cause only small changes in the pK_a of the ϵ-amino groups. Thus, under physiological conditions, the derivatized amino group is still positively charged (Means & Feeney, 1968). The dihydroxypropyl moieties on the amino groups are stable over a wide range of experimental conditions. The extent of blocking and deblocking could be readily estimated by amino acid analysis (Acharya et al., 1984). Dihydroxypropylation also has an advantage over amidation, in that glyceraldehyde is stable under physiological conditions whereas alkyl imidates undergo hydrolysis around neutral pH, and hence, the protein modification reaction with alkyl imidates is a competition with hydrolysis of the reagent (Inman et al., 1980). This is also the case in the use of anhydrides. Thus, dihydroxypropylation appears to be a valuable alternative to amidination, citraconylation, and maleoylation in the structural studies of proteins (Manjula et al., 1984, 1986).

Periodate oxidation has been used extensively in structural protein chemistry (Yamasaki et al., 1982; Fields & Dixon, 1968; Powers & Whitaker, 1977). It has been used as a general reagent for the oxidation of carbohydrates in the studies of glycoprotein structure (Hughes & Jeanloz, 1966; Yasuda et al., 1971). Limited oxidation of the vicinal glycols of the carbohydrate chains of glycoproteins to an "aldehydic" function followed by reduction with tritiated sodium borohydride has been used as a procedure for preparing radiolabeled proteins. Vicinal diol linkages have been introduced into bifunctional reagents as a means of having a cleavable linkage in the reagent (Coggins et al., 1976; Smith et al., 1978). The mild conditions under which the vicinal diols could be cleaved by periodate prompted the present studies. The regeneration of the lysine residues from the DHP proteins should further enhance the utility of the dihydroxypropylation reaction in the structural studies of proteins.

This study confirms the general utility of α-hydroxy carbonyl compounds as reversible reagents for the reductive alkylation of the amino groups of proteins. Geoghegan et al. (1979) have described the use of periodate for the removal of α-hydroxyalkyl groups (hydroxyethyl and hydroxyisopropyl) on the amino groups of proteins. The hydroxyethyl group can be introduced onto the protein amino groups by reductive alkylation with an α-hydroxyacetaldehyde, i.e., glycolaldehyde (Geoghegan et al., 1976; Acharya & Sussman, 1983). However, this reagent appears to have a disadvantage in that on reductive alkylation a second mole of the aldehyde can add relatively easily to the amino group, giving a tertiary amine (just as in the case of reductive methylation). This appears to be particularly the case if one wishes to use $NaCNBH_3$ as the reducing reagent. The substituted derivative is resistant to periodate oxidation, thereby decreasing the extent of reversibility of the modified lysines. The α-hydroxyisopropyl group is introduced by reductive alkylation with acetol (α-hydroxyacetone); this reagent shows higher selectivity for monoalkylation. Therefore, the extent of reversibility is higher if acetol is used for reductive alkylation. More recently, the reversibility of reductive glycation on periodate oxidation has also been investigated (Wong et al., 1985). However, reductive glycation using hexoses is a slow process and takes a few days to obtain a 60–80% modification of the amino groups of proteins. Nevertheless, the extent of reversibility obtained has been high, with 80–95% of amino groups modified by reductive glycation being regenerated with periodate in less than 10 min.

The reductive dihydroxypropylation of amino groups of proteins appears to proceed around neutral pH predominantly to the stage of monoalkylation even when $NaCNBH_3$ is used as the reducing agent. Moreover, the reductive dihydroxypropylation is complete in about 15–30 min. Since the disubstitution of the type seen with glycolaldehyde occurs only to a low level ($\sim$10%), the reversibility on periodate oxidation is high ($\sim$90%). Thus, reductive dihydroxypropylation provides the advantages of reductive hydroxyethylation in being a relatively fast reaction and also of reductive glycation in terms of high yield of lysine residues from the derivatized residue on periodate oxidation. The results of this study show that reductive dihydroxypropylation of amino groups of proteins and their regeneration by mild periodate oxidation is a very useful procedure in the structural studies of proteins and should be a valuable addition to the existing reversible chemical modification procedures.

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Registry No. RNase A, 9001-99-4; L-Lys, 56-87-1; $HOCH_2CH(OH)CHO$, 367-47-5; streptococcal Pep M5 protein (segment 88–99), 108214-27-3.

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Oxidation of Tryptophans in an Interhelical Hydrophobic Cluster of Myoglobin Alters the Thermodynamics of the Denaturation Transition†

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ABSTRACT: Model folding studies of sperm whale myoglobin have illustrated the presence of hydrophobic interfacial regions between elements of secondary structure. The specific oxidation of two tryptophan residues, in the A-H helix contact of sperm whale myoglobin, to the less hydrophobic oxindolylalanine residues is utilized to probe the contribution of hydrophobic packing density in this contact region. The acid denaturation of the modified protein is no longer a simple two-state process exhibiting the presence of stable intermediates. The relative stability of the intermediate is shown to be +5.3 kcal/mol less stable than native myoglobin. This value is consistent with the predicted relative stability, based upon electrostatic model calculations, of the docking of the A helix with a des-A helix myoglobin. The presence of stable intermediate structures in the denaturation pathway of the modified protein is consistent with the proposed role of hydrophobic interactions in damping structural fluctuations and statistical mechanical models of noncooperative protein unfolding. These results demonstrate the relationship between large-scale fluctuations and the frictional forces governing small-scale motions within the protein core.

In their description of the packing of α-helices in myoglobin, Richmond and Richards (1978) examined the loss of solvent contact area upon the association of secondary structures. Defining the site of interhelix contact regions in the protein by a perpendicular line segment to the axes of the two compared helices (the contact normal), calculation of the loss of solvent accessibility upon association of the isolated helices revealed six major contact pairs. The large decreases in accessibility imply strong hydrophobic interactions in these interhelical contacts (Chothia, 1974).

One of these interhelical contacts is between the A helix and the H helix and is centered about the valine at position 10 in the myoglobin sequence. Two of the six surrounding residues in this complex are tryptophan residues at positions 7 and 14 (Figure 1A). Tryptophan residues are relatively large, and their contribution to the hydrophobic cluster may be only partial as they are not completely buried, yet tryptophan residues have potentially one of the largest decreases in contact area upon folding (Richmond & Richards, 1978).

Comparing the locations of these interhelical contact region as defined by Richmond and Richards (1978) with the $\langle\chi^2\rangle$ values (root mean square deviations in atomic coordinates) of the backbone atoms of myoglobin obtained by X-ray dif-

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