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Reversible Reductive Alkylation of Amino Groups in Proteins†

Kieran F. Geoghegan, Donna M. Ybarra, and Robert E. Feeney*

ABSTRACT: Amino groups of proteins can be alkylated by treatment with a carbonyl compound and a reducing agent [Means, G. E., & Feeney, R. E. (1968) *Biochemistry 7*, 2192]. We are now reporting on a reversible method of reductive alkylation in which the amino groups are first alkylated by treatment with an α-hydroxy aldehyde or ketone in the presence of sodium borohydride. Since the chemical grouping $RNHCH_2C(OH)<$ (R = alkyl) is readily attacked by periodate to give the primary amine (RNH_2), this modification is effectively reversed by treatment with periodate. Reversal of the modification is effected by treating the modified protein with relatively low levels of $NaIO_4$ (10–20 mM) for ~30 min and is accompanied by a full or partial recovery of the activity lost as a result of modification. The best results were obtained with glycolaldehyde (HOH_2CCHO) and with acetol (HOH_2CCOCH_3). Glycolaldehyde reacts readily with amino groups to give modification at a high level but has the disadvantage that a second mole of the aldehyde can add relatively easily to the amino groups, giving a tertiary amine which is resistant to attack by periodate. With sodium borohydride as the reducing agent, ~20% of the amino groups modified by extensive treatment are converted to this irreversibly modified form. Higher levels of dialkylation are observed when sodium cyanoborohydride is used. Acetol, a ketone, is less reactive; with sodium borohydride as the reducing agent, only one molecule reacts with each amino group. Again, sodium cyanoborohydride can cause significant irreversible modification by favoring dialkylation of amino groups. The method was applied to four selected proteins: lysozyme, which is known to be rapidly inactivated by periodate; turkey ovomucoid, which is a trypsin inhibitor with an essential lysine residue and contains 25% carbohydrate; chicken ovomucoid, which is very similar in general structure to the turkey ovomucoid, but which has an essential arginine residue instead of a lysine; ribonuclease, in which the amino groups are essential for activity.

Reductive alkylation is a selective and versatile technique for the chemical modification of amino groups in proteins (Means & Feeney, 1968). It is accomplished by treating the protein with low concentrations of a simple aliphatic aldehyde or ketone and a reducing agent, usually sodium borohydride or sodium cyanoborohydride. The positive charge of the amino groups is retained, and the amounts of reducing agent used appear to be insufficient to cause reductive cleavage of disulfide bonds (Means, 1977).

We are now extending the earlier work in our laboratory

† From the Department of Food Science and Technology, University of California, Davis, Davis, California 95616. *Received June 29, 1979; revised manuscript received September 6, 1979.* This work was supported in part by National Institutes of Health Grants GM 23817 and AM 13686. Portions of this work were presented at the Pacific Slope Biochemical Conference, June 25–27, 1979, Reno, NV.

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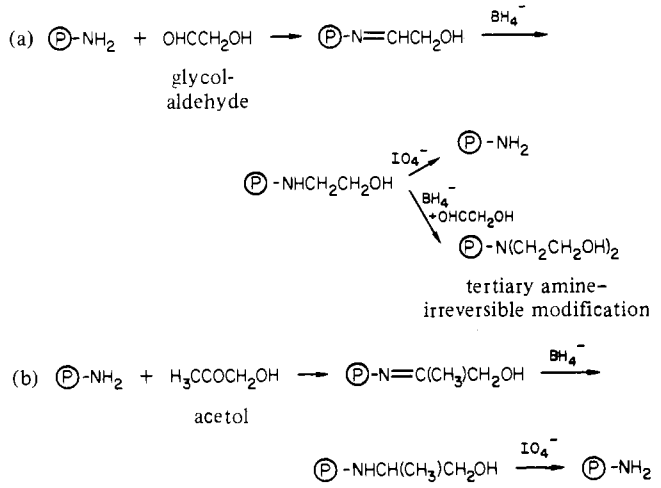
Scheme I



to include a reversible method of reductive alkylation. The modification of an amino group by reductive alkylation with 1 equiv of an α-hydroxy aldehyde or ketone gives the structure RNHCHR'C(OH)< (R' = H or alkyl). Since this grouping is readily attacked by periodate at pH values 7–9 (Fleury et al., 1949), the modification is reversible and the primary amino group can be regenerated. (For discussions of the oxidation, see Sklarz (1967) and Skoog & West (1976).) Periodate is a relatively harsh reagent, but it can be used successfully with proteins if the conditions are carefully controlled and one is aware of the possible side reactions. Thiol groups are quickly oxidized (Gan et al., 1968), as are NH_2-terminal serine, threonine, and cysteine residues (Fields & Dixon, 1968), but we find that the great lability of the α-amino alcohol grouping allows its oxidation to proceed under conditions much milder than those required for destruction of other amino acid side chains (Maekawa & Kushibe, 1954; Clamp & Hough, 1965; Krysteva & Dobrev, 1977).

We have experimented with a number of α-hydroxycarbonyl compounds as modifying reagents and have had the best results with glycolaldehyde, HOH_2CCHO, and acetol, H_3CCOCH_2OH. This paper describes our preliminary work with model compounds and four proteins, during which we have tried to assess the potential value of the technique as an addition to the currently available methods for reversible modification of amino groups.

Reductive alkylation generally proceeds more readily with aldehydes than with ketones (Means & Feeney, 1968). Formaldehyde is the most reactive material for the reaction with amino groups: it adds quickly to monomethylamino groups to give the dimethyl derivative. Means & Feeney (1968), using sodium borohydride exclusively as the reducing agent, found that dialkylation is much slower with acetaldehyde and that acetone forms only the monoalkylated product.

Reductive alkylation of an amino group with glycolaldehyde results initially in the production of the 2-hydroxyethyl derivative, a periodate-labile secondary amine (Scheme Ia). This can react with a second mole of the aldehyde, however, to give a tertiary amine. Tertiary amines are resistant to attack by periodate (Fleury et al., 1949). It is clearly important, therefore, that reagents and conditions be selected to give the maximum ratio of monoalkylated to dialkylated lysines, since dialkylation is an irreversible modification. One important factor is the choice of reducing agent. Means & Feeney (1968) used sodium borohydride with satisfactory results, but sodium cyanoborohydride (Borch et al., 1971) has increasingly been used for reductive alkylations of proteins because of its extra stability and selectivity of action (Dottavio-Martin & Ravel, 1978; Jentoft & Dearborn, 1979).

Experimental Procedures

Reductive Alkylation of α-N-Acetyl-L-lysine. The reductive 2-hydroxyethylation of α-*N*-acetyl-L-lysine was performed at a range of pH values by using both $NaBH_4$ and $NaBH_3CN$ as the reducing agent. The α-amino group was unblocked by acid hydrolysis and the distribution of products determined by amino acid analysis (see next section).

In the experiment with borohydride, 28 mg (0.15 mmol) of α-*N*-acetyl-L-lysine (Sigma) was dissolved in 7.5 mL of each of a series of buffers at 0 °C. Phosphate buffers at pH 6, 7, and 7.5 were used. Borate buffers were used for the experiments at pH 8, 9, and 10. To each tube was added 80 mg (1.3 mmol) of glycolaldehyde (Aldrich). The pH of each tube was checked and, if necessary, adjusted, after which each tube was treated with 25 mg of sodium borohydride (Aldrich). After 90 min, the addition of glycolaldehyde and borohydride was repeated in the same quantities. After a further 90-min period at 0 °C, the excess borohydride was destroyed by acidification. The α-amino groups of the α-*N*-acetyllysines were unblocked by treatment with 3 N HCl at 110 °C for 12 h, and the mixture of products was characterized on the amino acid analyzer.

The experiment with sodium cyanoborohydride was done in the same way as that for $NaBH_4$, except that 50 mg of $NaBH_3CN$ was added to the buffered reaction solutions in a single step and the reaction was allowed to proceed at 0 °C for 4 h. Acid hydrolysis was again used to unblock the α-amino groups of the products.

The distribution of products resulting from reductive alkylation of α-*N*-acetyl-L-lysine with acetol was examined in similar experiments with $NaBH_4$ and $NaBH_3CN$.

Amino Acid Analysis. Acid hydrolysates of proteins reductively alkylated with glycolaldehyde or acetol were routinely analyzed on a Technicon amino acid analyzer employing a pH 5.1 buffer. No gradient was required to obtain clear separation from lysine of the ϵ-*N*-(2-hydroxyethyl)lysine and ϵ-*N*,*N*-bis-(2-hydroxyethyl)lysine produced by modification with glycolaldehyde and of the ϵ-*N*-(hydroxyisopropyl)lysine resulting from reductive alkylation with acetol. All the modified lysines emerged from the column before lysine itself (Figure 1).

Quantitation of Chemical Modification of Amino Groups. Means & Feeney (1968) and Means et al. (1972) showed that TNBS[1] (Habeeb, 1966) reacts hundreds of times faster with primary amines than with secondary amines. The TNBS procedure of Fields (1972) was used to estimate the extent of chemical modification of a protein's amino groups by comparison of the color yield from the modified material with that from the native protein. The method is rapid and simple, and an estimate of the extent of modification can be obtained soon after the alkylated protein becomes available. (Amino acid analysis, on the other hand, cannot be undertaken until the protein has been hydrolyzed.) Caution is necessary, however, if a precise determination of the extent of alkylation is required. It has been shown that amino groups in a single protein can have different relative reactivities toward TNBS for reasons to do with their environment in the protein structure [for an example, see Haynes et al. (1967)]. Over the usual short reaction time (5 min) at room temperature, only about one-

[1] Abbreviation used: TNBS, trinitrobenzenesulfonic acid.

third of the amino groups in turkey ovomucoid were trinitrophenylated [estimate based on the extinction coefficient from Fields (1972)]. Comparison of the color yields from native and modified proteins allowed the extent of alkylation in the modified sample to be estimated to within 10–15% of the result given by amino acid analysis. This was routinely taken as a useful interim result which could later be replaced by the value from the slower but more exact amino acid analysis. Wang et al. (1976) have described conditions under which all the amino groups of ribonuclease reacted with TNBS after 30 min at 37 °C, but the difficulty remains that no distinction can be made between mono- and dialkylated amino groups by this method.

Amino acid analysis provides an estimate of the extent of modification among the ϵ-amino groups of a protein that is independent of their relative reactivities to chemical reagents and allows the yields of mono- and dialkylation to be distinguished. Slobodian et al. (1962) reported that over 90% of the molar color yield of ninhydrin with free lysine is attributable to the α-amino group. Means & Feeney (1968) estimated that dimethylation of the ϵ-amino group caused a drop in molar color yield of 18% in our analysis system. Assuming that this corresponds to a complete abolition of reactivity to ninhydrin, we assigned the same value to the drop in color yield caused by dialkylation of the ϵ-amino group with glycolaldehyde or acetol.

The drops in molar color yield resulting from monoalkylation with glycolaldehyde and acetol were estimated by calculating the peak area for the monoalkylated derivative that corresponds to a given decrease in the peak area for lysine. For both alkylations, the calculated drop was 10% of the total. Accordingly, peak areas representing ϵ-*N*,*N*-dialkyllysines were corrected by multiplication with a factor of 1.22, while peaks representing ϵ-*N*-monoalkyllysines were multiplied by a factor of 1.11. The extent of modification was then calculated as a percentage of total lysine by comparison of the peak areas for lysine and its alkylated derivatives.

Reductive 2-Hydroxyethylation of Proteins. In a typical experiment which resulted in the modification of 60% of the amino groups of lysozyme, 30 mg of lysozyme (Sigma) was dissolved in 0.2 M sodium borate buffer, pH 9.0, at a concentration of 5 mg/mL and treated with 60 mg of glycolaldehyde and 10 mg of solid sodium borohydride. The reaction was performed at room temperature. After 30 min, the solution was acidified to pH 5 with acetic acid and dialyzed against water. The protein was lyophilized and the extent of modification of its amino groups determined by the TNBS method (Fields, 1972) and/or amino acid analysis (see below).

With some experience in modifying a given protein, the concentrations of reagents can be varied to obtain desired levels of modification. Heavy modification is most conveniently achieved by repeating additions of glycolaldehyde and borohydride to the protein solution.

Reductive Hydroxyisopropylation of Proteins. The reaction pathway for this modification is shown in Scheme Ib. The following treatment resulted in the modification of 55% of the amino groups of turkey ovomucoid. Ovomucoid (20 mg) was dissolved at a concentration of 10 mg/mL in 0.2 M sodium borate buffer, pH 9.0, containing 10% acetol. The solution was treated with a total of 30 mg of sodium borohydride added in small portions. Ten minutes after the final addition of reducing agent, the solution was carefully adjusted to pH 5 with acetic acid to destroy excess borohydride without causing rapid foaming and possible denaturation of the protein. The ovomucoid was isolated after gel filtration and dialysis, and the extent of modification was estimated by the TNBS method and/or amino acid analysis.

Enzyme and Inhibitor Assays. The activity of modified and periodate-treated lysozyme was assayed by the method of Shugar (1952). The inhibitory activity of avian ovomucoids against trypsin and chymotrypsin was assayed by the method described previously (Haynes & Feeney, 1968). The activity of ribonuclease was assayed by the method of Kalnitsky et al. (1959).

Periodate Treatment of Modified Proteins. Fleury et al. (1949) gave an account of the oxidation of α-amino alcohols by periodate in aqueous solution. The optimum pH for the oxidation varies between 7.5 and 9.3 with minor variations in the structure of the amino alcohol, but the authors report rapid oxidation of ethanolamine, 2-amino-2-methylpropan-1-ol, serine, and diethanolamine at 4 °C. We routinely treated 2-hydroxyethylated proteins (2 mg/mL) with 15 mM sodium periodate in 0.1 M sodium phosphate buffer, pH 7, and hydroxyisopropylated proteins with 15 mM sodium periodate in 0.2 M sodium borate buffer, pH 8.6. In each case, the treatment was done at room temperature for a period of 20–30 min. Excess periodate was destroyed by adding glucose or ethylene glycol to the solution.[2] (A convenient check on the presence of periodate before and after this step is to add a small drop of the reaction solution to a few drops of potassium iodide solution. If periodate is present, the iodide is oxidized to iodine and the solution turns reddish brown.) The protein was finally isolated by dialysis or gel filtration, lyophilized, and characterized by the same methods used after the initial modification.

Selection of Buffer for Periodate Oxidations. The cleavage by periodate of vicinal diols is a well-known reaction in organic chemistry. The neutral sugar components of the carbohydrate chains in glycoproteins contain such groups, and their oxidative cleavage is an inevitable side reaction in the reversal of the present modifications unless special conditions are selected to obstruct it. We have noticed that avian ovomucoids, which contain ~25% carbohydrate by weight, undergo a change in texture of the lyophilized protein, a loss of their ready solubility in water, and conversion to a slightly off-white color when they are treated with periodate in phosphate buffer at pH 7. These changes do not occur when the protein is treated with periodate in borate buffer at pH 8.6.

The destruction of the neutral sugar in the carbohydrate portions of the glycoprotein under different conditions was estimated in the following experiment. Turkey ovomucoid (2 mg/mL) was treated with 15 mM sodium periodate in the following buffers: (a) 0.1 M sodium phosphate, pH 7; (b) 0.2

[2] In the present work, periodate oxidations were routinely terminated by adding an excess of a 1,2-diol compound. This had the advantage of allowing oxidations to be stopped at an exactly known time, which is convenient for time-point experiments. Under these conditions, however, the protein is briefly exposed to formaldehyde resulting from the oxidation of the 1,2-diol. Although only small extents of irreversible reaction with formaldehyde should occur under these conditions (Galembeck et al., 1977), controls in which the unmodified protein is treated with periodate and 1,2-diols should be included to exclude changes caused by either the periodate or the formaldehyde. When it is not important to terminate the reaction at an exactly known time, alternative procedures for removing the periodate can be used. A rapid and simple technique, which we have used successfully with turkey ovomucoid, ribonuclease, and ovotransferrin, is to mix the solution containing periodate and the protein with a slurry in water of a few grams of the anion-exchange resin AG 1-X8 (acetate form) (Bio-Rad). Periodate is bound strongly to the resin, which is then removed from the protein solution by filtration; separation of the last traces of protein from the resin is completed by washing briefly with water. A further alternative could be simply to remove periodate by dialysis. Gel filtration on Sephadex media does not appear advisable in view of the possible effects of periodate on dextrans.

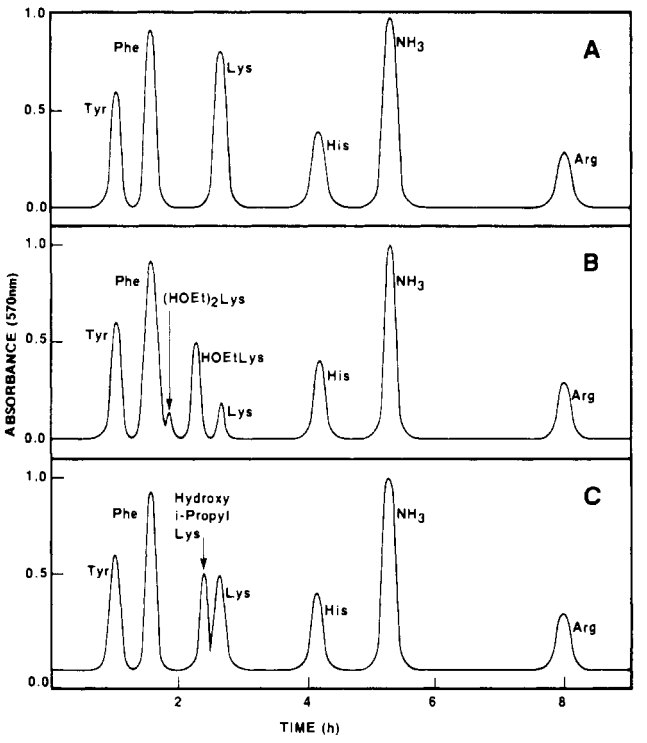


FIGURE 1: Portion of chart from amino acid analysis of turkey ovomucoid showing (A) native protein, (B) protein modified by reductive alkylation with glycolaldehyde, and (C) protein modified by reductive alkylation with acetol. Absorbance (570 nm) refers to the color produced by reaction of the amino acids with ninhydrin.

M boric acid–sodium borate, pH 8.6; (c) 0.1 M sodium phosphate, pH 7, containing 0.2 M boric acid; (d) 0.1 M sodium pyrophosphate, pH 8.6. The protein was exposed to periodate for 20 min, after which excess periodate was destroyed with ethylene glycol. After dialysis and freeze-drying, the isolated proteins were dissolved in 0.2 M borate buffer, pH 9, and treated with small amounts of solid sodium borohydride to reduce any carbonyl groups resulting from the action of periodate on the sugars. The solutions were then made 0.05 M in H_2SO_4 and heated to 80 °C for 1 h to hydrolyze the acetal bonds (Smith & Unrau, 1959). Finally, the acid solutions were adjusted to pH 7 with 1 N NaOH, and the protein was isolated and dried. The products of the periodate treatment under the different conditions were tested for their neutral sugar content by the phenol–sulfuric acid test (Dubois et al., 1956) and for their polypeptide content by the Lowry method (Lowry et al., 1951). Oxidation of the sugars followed by reduction and hydrolytic removal of the products should result in a change in the hexose/polypeptide weight ratio in the protein.

Results

Effects of pH and Reducing Agent on Reductive Alkylation of α-N-Acetyl-L-lysine. The effect of pH on the composition of reaction products from reductive alkylation of α-*N*-acetyl-L-lysine with glycolaldehyde is shown in Figure 2A, with sodium borohydride as reducing agent, and in Figure 2B, with sodium cyanoborohydride as reducing agent. The difference between the actions of the two hydride donors is clear. $NaBH_3CN$, being much more stable than $NaBH_4$ at the lower pH values, gives appreciable reductive alkylation at the more acidic end of the range, under conditions where borohydride is rapidly hydrolyzed. Borohydride is most effective in the region of pH 9–10. In contrast, cyanoborohydride gives a lower yield of alkylation at the higher pH values. A possible reason for this

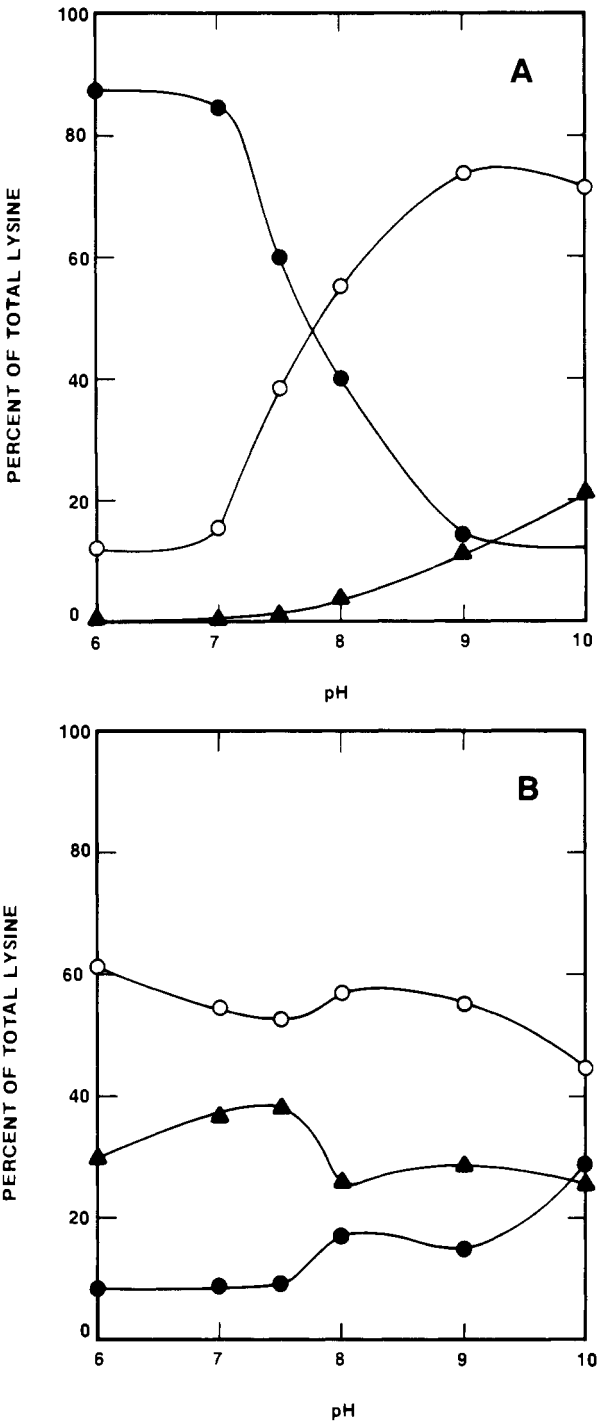


FIGURE 2: Composition of products of reductive alkylation with glycolaldehyde of α-*N*-acetyl-L-lysine using (A) sodium borohydride and (B) sodium cyanoborohydride as reducing agent. The composition of the reaction products was determined by amino acid analysis after removal of the acetyl group from the α-amino group by acid hydrolysis. The products shown are lysine (●), ε-*N*-(2-hydroxyethyl)lysine (○), and ε-*N*,*N*-bis(2-hydroxyethyl)lysine (▲).

is that cyanoborohydride requires protonation of the imine intermediate to enable it to effect reductive alkylation (Borch et al., 1971). Similar results were obtained with acetol in place of glycolaldehyde.

The most important observation is the difference that exists in the distribution of products obtained with the two hydride donors. Cyanoborohydride, possibly because of its longer survival in aqueous solution, gave a much higher proportion of dialkylation than was obtained with borohydride. Since the product of dialkylation is resistant to attack by periodate and

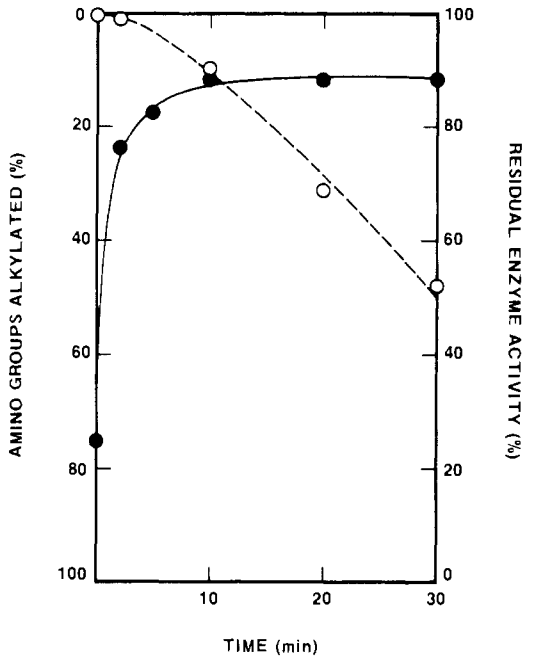


FIGURE 3: Periodate treatment of lysozyme modified by reductive alkylation with glycolaldehyde, showing the percentage of amino groups remaining modified (●) as determined by amino acid analysis and activity (○).

the modification therefore becomes irreversible, reductive alkylation of a protein using cyanoborohydride is likely to result in the irreversible loss of much of the protein's activity. For this reason, we selected sodium borohydride as the reducing agent for use in the experiments with proteins.

Reversible Modification of Lysozyme. Lysozyme was chosen as the protein for the first experiments in modification with glycolaldehyde, followed by its reversal with periodate, mainly because its amino groups are not intimately involved in the catalytic process, and there has been a report of its inactivation by periodate (Maekawa & Kushibe, 1955). The inactivation was related to the destruction of a tryptophan residue in the enzyme, and its extent was linearly related to a decrease in the absorbance at 280 nm.

Reductive 2-hydroxyethylation of lysozyme was performed on 30-mg samples of the protein (5 mg/mL) dissolved in 0.2 M sodium borate buffer, pH 9.0. Glycolaldehyde (20 mg in one sample and 60 mg in a second sample) was dissolved in the protein solutions, which were then treated with 10 mg each of solid sodium borohydride. After 30 min the solutions were acidified to pH 5 and the protein was isolated by the method described under Experimental Procedures. Amino acid analysis showed that 41 and 75%, respectively, of the ϵ-amino groups in the two lysozyme samples had been modified. Only a trace of dialkylation was detected in the less modified sample; in the more heavily modified sample, 12% of the amino groups were dialkylated and 63% of the amino groups were monoalkylated. Both modified products had 100% of the activity of a control sample.

A more detailed study of the unblocking step was undertaken because of the known possibility of activity losses resulting from the action of periodate on groups other than the modified lysines. The protein with 75% of its amino groups modified was treated with 18 mM sodium periodate in 0.2 M sodium phosphate buffer, pH 7, at room temperature. Samples were withdrawn at intervals and tested for activity and extent of the remaining chemical modification (Figure 3). Although a discernible loss of activity occurred during this treatment, reversal of the monoalkylation was completed in 10 min with

Table I: Reductive 2-Hydroxyethylation of Turkey Ovomucoid and Its Reversal with Periodate

	after modifn step		after periodate step	
expt	amino groups modified (%)	act. vs. trypsin (%)	amino groups modified (%)	act. vs. trypsin (%)
1	27	59	4	95
2	30	44	12	84
3	80	0	29	56

90% of the enzyme's activity still intact (it is always hard to know, in a study of chemical modification of a protein, whether a loss in biological activity of a preparation is due to complete inactivation of some of the molecules or to partial inactivation of all of the molecules). The result suggests that even proteins which are unusually labile to periodate may be able to withstand the brief exposure to the oxidizing agent required for the unblocking of 2-hydroxyethylated amino groups. It also shows that the occurrence of some dialkylation makes it unlikely that complete reversal of modification of all the amino groups in a protein can be achieved with glycolaldehyde.

Reversible Modification of Avian Ovomucoids with Glycolaldehyde. When turkey ovomucoid was reductively alkylated with glycolaldehyde, its inhibitory activity against trypsin decreased sharply. The removal of the modifying groups and the regeneration of activity were only partly successful (Table I). Modified and periodate-treated ovomucoid samples had full inhibitory activity against chymotrypsin, showing that the molecule was not denatured by either step. Modification of the amino groups of chicken ovomucoid and its reversal by periodate had no effect on its trypsin-inhibitory activity.

Reversible Modification of Avian Ovomucoids with Acetol. Reductive isopropylation (reductive alkylation with acetone) of amino groups yields the dialkyl derivative in only a very small proportion of the product when sodium borohydride is the reducing agent (Means & Feeney, 1968; Fretheim et al., 1979). We anticipated that the same would be true for modification with acetol and investigated the possibility of using it as the basis of a reversible reductive alkylation.

Turkey and chicken ovomucoids were reductively alkylated with acetol, the products isolated, and their activity and extent of modification determined. The modification was reversed by periodate treatment (Figure 4). In turkey ovomucoid, the activity lost on modification was recovered at a rate that was rapid relative to the rate of unblocking of amino groups, suggesting that the modified essential lysine is more reactive to periodate oxidation than other modified residues in the protein. Activity of the chicken ovomucoid was unaffected by the modification of its lysines and its reversal.

The reversal of modification with acetol took place in 0.2 M sodium borate buffer, pH 8.6. The pH was selected initially on the basis of the work of Fleury et al. (1949) on the pH dependence of the periodate oxidation of different α-amino alcohols, but the use of borate buffer may be advantageous with this glycoprotein (see below).

Reductive Hydroxyisopropylation of Ribonuclease. Means & Feeney (1968) reported a loss of the activity of ribonuclease accompanying the reductive methylation or isopropylation of its amino groups. As expected, treatment of the enzyme with acetol and sodium borohydride caused a loss of activity in the same way. In two experiments on modification with acetol and sodium borohydride, samples of ribonuclease had 56 and 59% of their amino groups modified and each lost all (>95%) of their activity. Treatment with periodate (15 mM $NaIO_4$ in borate buffer, pH 8.6, for 25 min at 20 °C) restored 70 and

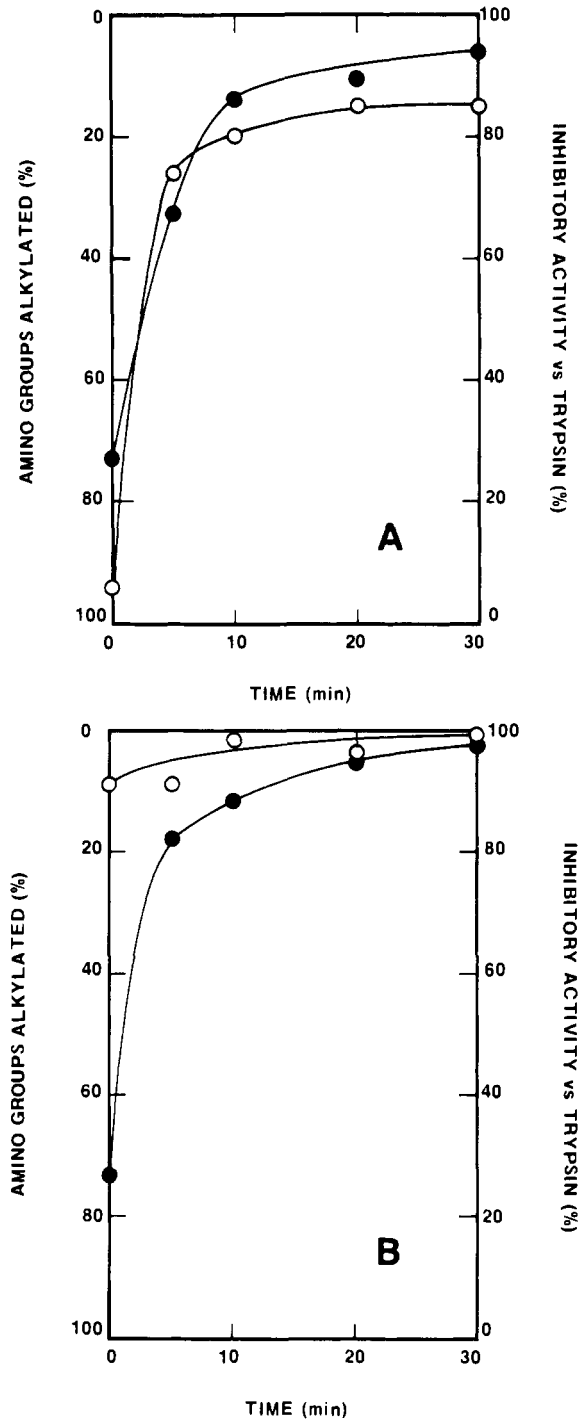


FIGURE 4: Periodate treatment of (A) turkey ovomucoid and (B) chicken ovomucoid modified by reductive alkylation with acetol, showing the percentage of amino groups remaining modified (●) as determined by amino acid analysis and inhibitory activity vs. trypsin (○).

75%, respectively, of full activity to these samples.

We have not established the reason for the failure to recover full activity in these experiments with ribonuclease. Goebel et al. (1946) reported that the enzyme is inactivated by treatment with periodate but did not identify the reaction responsible. In the present case, successive losses of ~15% of activity resulted from treatment of the enzyme with sodium borohydride alone followed, later, by sodium periodate alone. Control experiments are very important when proteins are being exposed to such reagents.

Effect of Periodate Treatment on Carbohydrates of Ovomucoid. It was found (Table II) that turkey ovomucoid which had been treated with periodate in borate or pyrophosphate buffer at pH 8.6 suffered only slight changes in its ratio of neutral sugar to polypeptide. In contrast, ovomucoid treated with the oxidizing agent in phosphate buffer at pH 7 in the presence or absence of boric acid showed a marked decrease in its sugar to polypeptide weight ratio, suggesting that neutral sugar was being cleaved by periodate. Although borate is well-known to form complexes with neutral sugars at pH values close to 9, we found no clear evidence of a special protective effect of borate buffers.

Table II: Effect of pH and Buffer Ion on Periodate Damage to Carbohydrates in Turkey Ovomucoid

periodate treatment in[a]	g of hexose/ g of polypeptide	loss of neutral sugar (%)
none (control)	0.117	0
borate, 0.2 M, pH 8.6	0.115	2
pyrophosphate, 0.2 M, pH 8.6	0.111	5
phosphate, 0.1 M, pH 7	0.093	21
phosphate, 0.1 M, pH 7 (containing 0.2 M boric acid)	0.088	25

[a] Samples were treated with 15 mM sodium periodate at room temperature for 20 min, after which excess periodate was decomposed with ethylene glycol. The protein was isolated, treated with sodium borohydride to reduce carbonyl groups, and then subjected to mild acid hydrolysis (see text).

Discussion

Studies on the reductive alkylation with glycolaldehyde and acetol of α-*N*-acetyl-L-lysine showed that a higher proportion of dialkylation was achieved when sodium cyanoborohydride was the reducing agent than when the same number of amino groups was modified using sodium borohydride. We attribute this to the low reactivity of sodium cyanoborohydride toward carbonyl compounds at pH >5 (Borch et al., 1971) which allows glycolaldehyde to survive in the presence of the reducing agent and thus to be available for imine formation with the monoalkylamino groups.

The ovomucoids make up 10% of the protein in egg white from many types of birds and are inhibitors of proteolytic enzymes with specificities that differ between species (Feeney & Allison, 1969). The ovomucoids of turkey and chicken are both proteins of molecular weight close to 28 000 with very similar amino acid compositions and sequences. Each contains a large fraction of carbohydrate (hence their name); neither contains tryptophan. Chicken ovomucoid is an inhibitor of trypsin, with a single inhibitory site containing one essential arginine. Turkey ovomucoid is a "double-headed" inhibitor of trypsin (one site) and chymotrypsin (second site). A single lysine residue is essential for the activity against trypsin. M. Laskowski and his colleagues have reported extensively on the amino acid sequences of ovomucoids from many species and have shown that a very high degree of homology exists between them (Kato et al., 1978).

Chicken and turkey ovomucoids are thus very similar in their general structures but are affected in different ways by chemical modification of their amino groups (Haynes et al., 1967). The trypsin-inhibitory activity of chicken ovomucoid was virtually unaffected by trinitrophenylation of its amino groups. The same treatment caused a sharp loss of activity against trypsin in the turkey ovomucoid but left its activity against chymotrypsin intact. It seemed useful to attempt the reversible chemical modification of the amino groups in these two ovomucoids because activity losses caused by the modification of amino groups were expected only in the turkey protein, while

activity losses caused by deleterious side reactions should be seen in both proteins.

The occurrence of dialkylation can be a troublesome problem in reductive alkylation experiments on proteins when glycolaldehyde is the alkylating compound and sodium borohydride the reducing agent. In experiments with turkey ovomucoid, we repeatedly experienced difficulty in recovering full activity after reductive alkylation with glycolaldehyde. Haynes et al. (1967) identified "fast"- and "slow"-reacting amino groups in turkey ovomucoid; the rate constant for modification of the fast amino groups with TNBS was equal to that for the associated loss of activity against trypsin. We believe that the persistent irreversible losses of 10–15% of the activity of turkey ovomucoid (more when modification was heavy) that we have experienced in modification experiments with glycolaldehyde are due to the occurrence of irreversible dialkylation of the fast-reacting essential lysine in a certain percentage of the molecules. Molecules in which this occurs are irreversibly inactivated. Only a small percentage of the amino groups in a sample of the ovomucoid need be dialkylated in order to effect substantial permanent activity losses. Turkey ovomucoid contains 11 lysines. Dialkylation of a single fast-reacting amino group in 10% of the molecules in a sample represents the irreversible modification of <1% of the lysine residues in that sample. Low levels of ϵ-*N*,*N*-bis(2-hydroxyethyl)lysine (of the order of 1–3% of the total lysines) were detected in hydrolysates of the modified and periodate-treated turkey ovomucoid fractions from these experiments, sufficient to explain the irreversible loss of 10–15% of the activity against trypsin. Higher levels of dialkylation were associated with larger permanent losses of activity.

In view of this difficulty with glycolaldehyde, we turned to acetol as a possibly superior alkylating agent for the purpose of a reversible modification. Means & Feeney (1968) showed that reductive alkylation of amino groups with acetone resulted only in monoalkylation (the reducing agent was sodium borohydride). The isopropylamino derivative itself is not, of course, subject to attack by periodate, but reductive alkylation with acetol gives a modified amino group with the required structure for lability to periodate. Acetol gave much more satisfactory results in the reversible reductive alkylation of turkey ovomucoid than had been obtained with glycolaldehyde, suggesting that its specificity for monoalkylation may make it the best reagent for use in the present method. Further experience with the method will reveal whether this greater specificity, which leads to a more complete reversal of modification in the periodate step, or the greater reactivity with amino groups of the aldehyde is the more desirable quality. Possibly both compounds will be useful, with the choice between them depending on the particular problem.

When a glycoprotein is the subject of experiments in reversible reductive alkylation, there is a strong case to be made for using a pH close to 9 for the reversal step. Oxidative damage to the carbohydrate moieties is minimized under these conditions.

Several reversible reagents already exist for amino groups (Means & Feeney, 1971). Among these are reagents which modify the positively charged amino groups to a negatively charged form (maleic anhydride and citraconic anhydride), reagents which convert the amino groups to an uncharged form (ethyl thiotrifluoroacetate), and reagents which leave a positive charge on the modified groups (alkyl acetimidates). Reductive alkylation falls into this last category. Alkylation causes a small change in the pK_a of amino groups (Means & Feeney, 1968) and steric blocking to a degree related to the size of the added group. Modification impairs the ability of essential amino groups to take part in the formation of enzyme–substrate intermediates and, in the case of turkey ovomucoid at least, to play a part in the formation of an enzyme–inhibitor complex. Reversal of the modification requires exposure of the protein to periodate, a relatively harsh reagent, but the exposure is brief, the conditions of temperature and pH are mild, and low concentrations of the oxidizing agent are used.

Reversible reductive alkylation has some features in common with the technique of amidination. In this method, the amino groups of a protein are selectively converted to an amidine derivative by reaction with an alkyl acetimidate. The method has been described and discussed by Hunter & Ludwig (1972). As with alkylation, amidination results in minimal disturbance of the protein structure. The amidino group is more strongly basic than the amino group which it replaces, and charge–charge interactions in the protein and between protein molecules can be disturbed with a resulting alteration in functional properties (Hunter & Ludwig, 1972). Reversal of the modification demands treatment of the protein with concentrated ammonia–glacial acetic acid (15:1 v/v), pH 11.3, over several hours at room temperature. These harsh conditions have been reported to cause side reactions (Ludwig & Hunter, 1967). Reversible reductive alkylation and amidination should be complementary rather than redundant techniques in future studies of the structural and functional roles of amino groups in proteins.

The widespread functional importance of amino groups in proteins and their relatively high reactivity have made them the frequent subject of chemical modification studies. It is always important in experiments of this sort to show that changes in the behavior of the protein are a primary result of the modification of certain groups. For this reason, there are advantages to having techniques of modification that are reversible, so that it can be shown that removal of the modifying group leads to a return of the protein's original properties.

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Amino Acid Sequence Studies on the α Chain of Human Fibrinogen. Complete Sequence of the Largest Cyanogen Bromide Fragment†

D. D. Strong, K. W. K. Watt,‡ B. A. Cottrell, and R. F. Doolittle*

ABSTRACT: The largest fragment produced by complete cyanogen bromide digestion of the α chain of human fibrinogen contains 236 residues and has a calculated molecular weight of 23 949. The complete amino acid sequence of the fragment was determined by the isolation of peptides generated by plasmin, trypsin (including digestion of citraconylated material), staphylococcal protease, and chymotrypsin. In addition, some key subfragmentation was achieved by selective chemical cleavage at tryptophan residues. The fragment has an unusual amino acid composition, more than half of its residues being glycine, serine, threonine, and proline. There are very few nonpolar residues, although 7 of the α-chain's 10 tryptophans occur in this fragment. The fragment contains 2 cysteine residues located 30 residues apart which are connected by an intrachain disulfide bond in the native molecule. The tryptophans occur with a definite periodicity that highlights a series of 13-residue homology repeats. The fragment also contains the two principal α-chain cross-linking sites.

Vertebrate fibrinogen molecules are composed of three pairs of nonidentical polypeptide chains ($\alpha_2\beta_2\gamma_2$). Invariably, the largest of these are the α chains, which range from 60 000 to 80 000 in molecular weight, depending on the particular species (Doolittle, 1973). In the case of human fibrinogen, the α chains have molecular weights of about 65 000 (McKee et al., 1966, 1970). The chains contain 10 methionines and give rise to 11 unique fragments upon digestion with cyanogen bromide (Doolittle et al., 1977a). Of these, complete sequences have been reported for the 10 smallest (Blombäck et al., 1972; Takagi & Doolittle, 1975; Cottrell & Doolittle, 1976, 1978; Doolittle et al., 1977a,b; Lottspeich & Henschen, 1978a,b; Doolittle et al., 1979a). Only the largest fragment has remained undetermined. In this article we report the complete amino acid sequence of that fragment, designated HαCNI. It contains 236 residues and has a calculated molecular weight of 23 949. The sequence determination was complicated by a number of unusual features of this part of the α chain. It has a very distinctive amino acid composition, more than half of the residues being serine, glycine, threonine, and proline. As a result, many of the enzymatically derived peptides have similar properties and compositions, and the purification and proof of uniqueness were a formidable challenge. Moreover, the task was further complicated by a number of anomalous cleavages, including the partial tryptic cleavages of two different arginyl–proline bonds, chymotryptic action adjacent to certain threonyl residues, and staphylococcal protease cleavage at a particular seryl residue.

Experimental Section

Materials and Methods. Almost all the materials and methods used in this study have been described in detail in

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‡ Present address: Department of Medicine, Harvard Medical School, Boston, MA.

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