Enhanced Reactivity of Lysozyme with Formaldehyde during Cold Denaturation

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The reactivity of lysozyme with formaldehyde at low temperatures (0 to −20 °C) was compared with lysozyme reactivity at 37 °C. Lysozyme reaction with formaldehyde, in the presence and absence of sodium cyanoborohydride, was also compared. The progress of reactions was evaluated by measurement of protein primary amino groups, lysozyme enzymatic activity, and occurrence of cross-linkage as evidenced by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Results obtained indicated that the chemical modification of lysozyme by formaldehyde was significantly enhanced at cold freezing temperatures (−6 and −10 °C) when compared with 37 °C. Cold-temperature protein modification with formaldehyde gave rise to inactive enzyme which was partially cross-linked. Reductive methylation at 37 °C did not appreciably affect enzyme activity or cause protein cross-linkage. Taken together, these results gave a strong indication that the observed enhanced reactivity of lysozyme with formaldehyde at low temperature was likely due to cold denaturation of the protein.

Keywords: *Lysozyme; formaldehyde; denaturation*

INTRODUCTION

Fish muscle proteins undergo denaturation and aggregation during freezing and frozen storage (Shenouda, 1980; Haard, 1992). This denaturation leads to changes in texture making fish flesh stored for long periods of time rubbery and eventually unacceptable for human consumption (Sikorski *et al.*, 1976; Mackie *et al.*, 1986). In formaldehyde-producing fish, e.g., some species of the gadoid family, the deterioration of fish flesh during frozen storage is faster compared with that of non-formaldehyde-producing fish (Gill *et al.*, 1979). Ang and Hultin (1989) reported that formaldehyde can hasten the denaturation of muscle protein during frozen storage. Both formaldehyde and dimethylamine are produced during frozen storage by the enzyme trimethylamine oxidase, located in viscera and in the microsomal fraction of muscle from fish (Castell *et al.*, 1970; Rehbein and Schreiber, 1984; Parkin and Hultin, 1982).

The mechanism of action of formaldehyde in protein denaturation as it relates to the deterioration of frozen fish is not clearly understood. It is striking that at the beginning of frozen storage, when little formaldehyde is produced, denatured and aggregated fish muscle proteins can be readily solubilized with buffers containing 1% sodium dodecyl sulfate (SDS) or 1% SDS–2-mercaptoethanol (2-ME) and that these proteins become increasingly more insoluble in these detergent buffers as storage progresses (Rehbein and Karl, 1985; Laird and Mackie, 1981; Matthews *et al.*, 1980; Ragnarsson and Regenstein, 1989; LeBlanc and LeBlanc, 1989). Owusu-Ansah and Hultin (1987) showed that much of the denaturation and aggregation of the muscle proteins was due to hydrophobic interactions between protein molecules and that covalent cross-linking can occur if formaldehyde is produced during frozen storage (2% of total muscle proteins). Ang and Hultin (1989) suggested that the reaction of fish myosin with formaldehyde might alter the protein structure so that it denatures more readily during frozen storage when compared to conditions in which no formaldehyde was present. Sotelo and Mackie (1993) showed that reaction conditions of temperature, ionic strength, and concentration of formaldehyde can influence the cold-temperature denaturation and aggregation of bovine serum albumin (BSA) and that formaldehyde caused aggregation of BSA in frozen solution. They also showed that some cryoprotectants can prevent formaldehyde denaturation during frozen storage. These results raised questions concerning the influence of low temperature on the reactivity of formaldehyde with proteins in solution.

The reaction of formaldehyde with proteins is well-known (Means and Feeney, 1968; Means, 1977). Three types of formaldehyde reactions with proteins have been described at 37 °C and pH 7.0. These include reversible, acid labile, and acid resistant reactions. Lysyl ϵ-NH_2 and α-NH_2 groups are involved in all three types of reactions. Formaldehyde reactivity with arginine, asparagine, glutamine, histidine, tryptophan, and tyrosine side groups is reported to be involved in both acid labile and acid resistant reactions (Tome *et al.*, 1985). Furthermore, reaction of proteins with formaldehyde can cause methylene bridges to occur between amino acid side chains (Galembeck *et al.*, 1977). When formaldehyde is reacted with proteins in the presence of a reductive alkylating agent such as sodium cyanoborohydride, primary amino groups are readily modified to methyl and dimethyl derivatives (Jentoft and Dearborn, 1979).

In this study, we describe the reaction of formaldehyde at low temperature using a well-characterized model globular protein and compare the reactivity with previously reported results conducted at higher temperatures (Galembeck *et al.*, 1977; Jentoft and Dear-

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born, 1979). Lysozyme was chosen because of its stability and easily measurable enzymatic activity; furthermore, its tertiary structure has been defined (Blake *et al.*, 1965). Our objective was to gain some general insight concerning the intrinsic reactivity of formaldehyde with proteins in foods undergoing low-temperature storage and to investigate if cold denaturation was an important factor in protein reactivity (Privalov *et al.*, 1986; Privalov, 1990).

MATERIALS AND METHODS

Materials. Sodium cyanoborohydride and chicken egg white lysozyme (grade I) were from Sigma Chemical Co. Washed and lyophilized *Micrococcus lysodeikticus* cells were from Worthington Biochemical Co. Formaldehyde (37% w/w) was purchased from Fisher Scientific. 2,4,6-Trinitrobenzenesulfonic acid (TNBS) and trifluoroacetic acid [high-performance liquid chromatography (HPLC) grade] were from Pierce Chemical Co. All electrophoresis reagents were from Bio-Rad. Molecular weight markers were from Integrated Separation Systems.

High-Performance Liquid Chromatography. Lysozyme was HPLC purified using a Vydac C_{18} reversed-phase HPLC semipreparative column similarly as previously reported (Kurosky *et al.*, 1993). A linear gradient of solvent A (0.1% trifluoroacetic acid) and solvent B (0.1% trifluoroacetic acid in acetonitrile) was used at a flow rate of 1.75 mL/min. The gradient was 29–95% solvent B over 65 min. The column eluate was monitored at 215 nm, and 1 min fractions were collected. The major lysozyme-containing peak, identified by amino acid analysis, was pooled and lyophilized.

Conditions for Reaction of Lysozyme with Formaldehyde. Reactions were conducted in 2 mL Eppendorf polypropylene tubes tightly sealed with O-ring-containing screw caps (Cel Associates, Inc.). HPLC-purified lysozyme was dissolved in phosphate-buffered saline (PBS) (8.95 mM KH_2PO_4, 41.05 mM Na_2HPO_4, and 32 mM NaCl), pH 7.0 and μ 0.18, at a concentration of 1 mg/mL. Reaction mixtures contained 0–500 μg of formaldehyde (0–34 mol of formaldehyde/mol of primary amino group). Reactions were allowed to proceed for 24 h at temperatures of 37, 0, −6, −10, and −20 °C. Temperatures from 0 to −20 °C were maintained by a Haake refrigerated water bath using ethanol as coolant. After reaction, the protein mixtures were dialyzed and available amino group concentrations and enzymatic activity determined. Reaction mixtures were dialyzed prior to determination of available amino groups, i.e., formaldehyde unreacted amino groups.

In a separate series of experiments reductive methylations were carried out using $NaCNBH_3$ essentially as described by Jentoft and Dearborn (1979). Reactions were conducted in 1 mL reaction volumes containing 1 mg/mL of lysozyme, 3.3 mM formaldehyde, 20 mM $NaCNBH_3$, and 100 mM $NaHCO_3$, pH 9.0, or PBS, pH 7.0. Reactions were stopped by the addition of HCl.

Lysozyme Enzymatic Activity. Lysozyme activity was assayed at 25 °C according to the method of Gorin *et al.* (1971) employing *M. lysodeikticus* as substrate. Lysozyme activity was determined by the rate of decrease in absorbance at 570 nm of a mixture of enzyme plus substrate (units/mg = A_{570}/1000 mg of protein) (Gorin *et al.*, 1971). Lysozyme concentrations were calculated according to the method of Aune and Tandford (1969) using $E^{1\%}_{280} = 26$.

Free Amino Group Determination. Free amino group concentrations were measured by the TNBS assay described by Lin *et al.* (1969). The assays were performed at 25 °C.

Amino Acid Compositional Analysis. On occasion, protein solutions were subjected to amino acid analysis using an Applied Biosystems 420H (derivatizer/hydrolyzer) amino acid analyzer with on-line acid hydrolysis and precolumn phenylthiocarbamyl derivatization as previously described (Smith *et al.*, 1991).

Polyacrylamide Gel Electrophoresis. Samples for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) were prepared by adding 100 μL of Laemmli sample buffer (1% dithiothreitol was used in place of 2-ME) to 50 μg of lysozyme and heating for 4 min at 100 °C (Laemmli, 1970). An aliquot (20 μL) of this mixture was applied to each well. Gels were prepared according to the procedure of Laemmli (1970) (100 × 750 × 0.75 mm) and were subjected to electrophoresis at 100 V using a Mini-Protean II Cell (Bio-Rad). Following electrophoresis, the gels were fixed for 10 min in 12.5% trichloroacetic acid. Staining and destaining were performed using the rapid Coomassie Blue staining method (Research Products International Corp.).

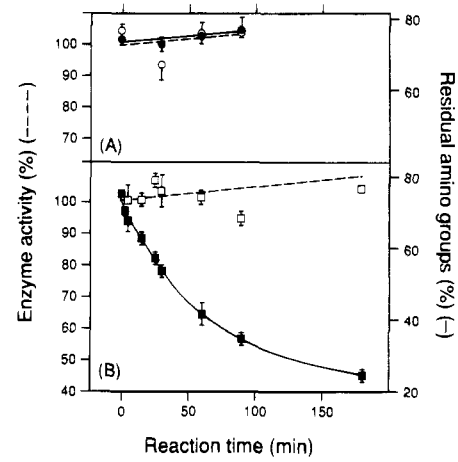

Figure 1. Time course of reaction of lysozyme with formaldehyde in the absence (panel A) and presence (panel B) of $NaCNBH_3$ at 37 °C. Each point represents the mean of duplicate samples. Lysozyme, 1 mg/mL; formaldehyde, molar excess of 7-fold over primary amino groups; $NaCNBH_3$, 20 mM.

RESULTS AND DISCUSSION

The reactivity of formaldehyde with lysozyme was evaluated under different reaction conditions including pH and temperature and in the presence or absence of the reductive alkylating agent, sodium cyanoborohydride. We were especially interested in elucidating differences in formaldehyde reactivity at low temperatures when compared with 37 °C. Formaldehyde-reacted lysozyme was evaluated for enzymatic activity to inquire about changes in the native structure of the protein. In addition, amino group reactivity was followed during the time course of the chemical modification reaction.

Reactions of lysozyme with formaldehyde in the presence and absence of sodium cyanoborohydride were compared at 37 °C and pH 9.0 as shown in Figure 1. Both reactions are known to proceed optimally at pH 9.0 (Galembeck *et al.*, 1977; Jentoft and Dearborn, 1979). In the absence of the reductive alkylating agent, the enzymatic activity and amino group reactivity were not significantly affected over the time course of the reaction, although a slight increase (~10%) was noted (Figure 1A). However, in the presence of the reductive alkylating agent there was a significant reduction in the available amino groups (~60%), whereas the enzymatic activity was essentially not affected or was slightly elevated (Figure 1B). Thus, at 37 °C and pH 9.0 reductive methylation of about 60% of the available amino groups (six ϵ-NH_2 plus 1 α-NH_2) did not have an appreciable effect on the activity of the enzyme (Figure 1B). These results were consistent with the relative locations of the α- and ϵ-amino groups of lysozyme which occur largely on the surface of the molecule and are not part of the active site region (Blake *et al.*, 1965; Wilson *et al.*, 1992). In addition, Galembeck *et al.* (1977)

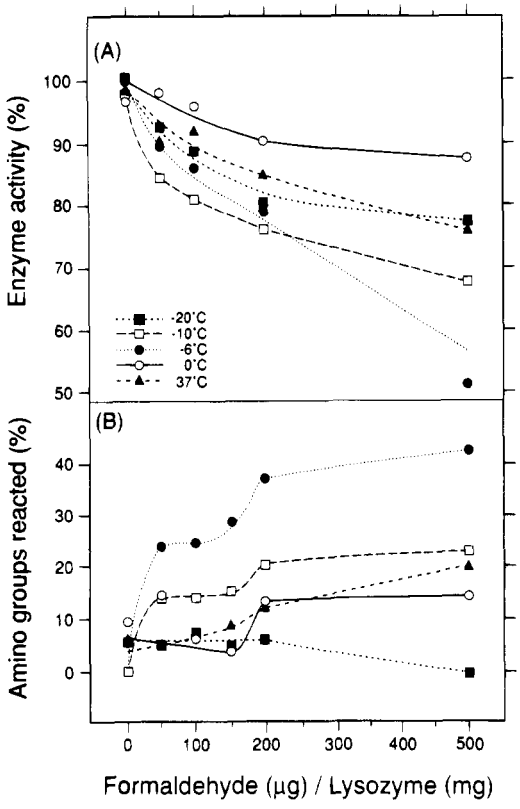

Figure 2. Reaction of lysozyme with increasing concentrations of formaldehyde at different temperatures. Each point represents the mean of duplicate samples. Enzyme activity is shown in panel A. Amino group reactivity with TNBS is given in panel B: 37 °C, ■; 0 °C, ○; −6 °C, ▲; −10 °C, □; −20 °C, ●. Lysozyme, 1 mg/mL; formaldehyde, 0−34 mol/mol primary amino group; reaction time, 24 h.

showed that incorporation of 1−2 equiv of ^{14}C-labeled formaldehyde into lysozyme did not affect enzyme activity.

The reaction of lysozyme with formaldehyde at various temperatures at pH 7.0 in the absence of sodium cyanoborohydride is shown in Figure 2. Under the reaction conditions described, loss of lysozyme activity correlated with increasing amounts of added formaldehyde. Strikingly, loss of activity was appreciable at low temperatures when compared to 37 °C, especially at −6 and −10 °C (Figure 2A). There was increased amino group reactivity except for −20 °C, which showed relatively little reactivity (Figure 2B); however, each of the curves in Figure 2B showed a somewhat sigmoidal correlation with increasing concentration of formaldehyde. This was consistent with the increase in the molar ratio of formaldehyde to lysozyme from 3.4-fold to 34-fold. As the concentration of formaldehyde increased to 34-fold molar excess over primary amino groups the kinetics of the reaction approached pseudo first order. The observed 20% loss of enzyme activity at −20 °C (Figure 2A) when compared to virtually no amino group reactivity (Figure 2B) is presently an open question and probably relates to factors other than that observed at higher temperatures. For example, this may have been due to modification of groups other than amino groups. Amino group reactivity did not directly correlate with loss of enzyme activity (Figures 1 and 2). Since the catalytic site of lysozyme involves glutamic acid and aspartic acid residues, lysine modification would not be expected to influence activity (Blake *et al.*, 1965; Wilson *et al.*, 1992).

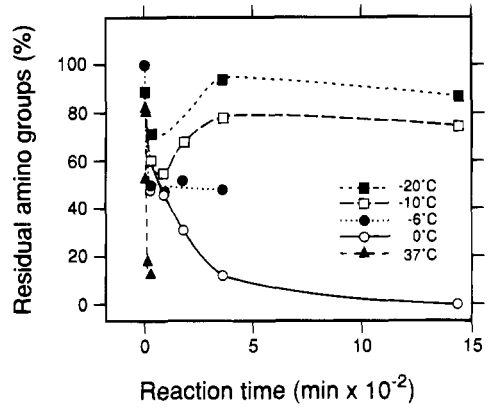

Figure 3. Time course of reaction of lysozyme with formaldehyde at pH 7.0 in the presence of $NaCNBH_3$ at different temperatures. Lysozyme, 1 mg/mL; formaldehyde, 3.3 mM or 7-fold molar excess over primary amino groups; $NaCNBH_3$, 20 mM.

The reaction mixtures described above were examined for the occurrence of cross-linkage or covalent aggregation using SDS−PAGE. The highest amount of cross-linkage was evidenced in the reactions conducted at −6 °C using 500 μg of formaldehyde/mg of lysozyme (results not shown). Protein bands at 29 and 39 kDa were evident that likely corresponded to dimer and trimer forms of lysozyme (mass = 14 300 kDa). A 29 kDa band was also evident in reactions conducted at 37 and −10 °C, although it was relatively fainter than at −6 °C. Aggregation was not indicated in lysozyme samples incubated at −20 or 0 °C. Galembeck *et al.* (1977) also reported dimer and trimer formation after reaction of lysozyme with formaldehyde at room temperature.

The reaction of lysozyme amino groups with formaldehyde was considerably different in the presence of sodium cyanoborohydride as shown in Figure 3. The rate of reaction consistently correlated with increasing temperature. At temperatures below 0 °C (−6, −10, and −20 °C) the reaction rates were slowed after 30 min, as evidenced by the upturn in the curves as the reactions progressed (Figure 3). The observed differential and slow rates of reactivity were likely due to solution freezing effects. The reductive methylation of all seven amino groups of lysozyme at 37 and 0 °C was essentially complete after 250 and 1000 min, respectively. However, as shown in Figure 1B the enzymatic activity at 37 °C was virtually unaffected even when conducted at pH 9.0. Reductive methylation reactions should proceed faster at pH 9.0 than at pH 7.0. Reductive methylation reactions did not show any indication of protein aggregation (results not shown), consistent with results reported by others (Galembeck *et al.*, 1977; Jentoft and Dearborn, 1979).

Initially, our interest in comparing formaldehye reactivity in the presence and absence of sodium cyanoborohydride was to inquire whether or not use of the reductive alkylating agent would accelerate formaldehyde reactivity and facilitate our studies by shortening reaction times. In addition, we wanted to investigate whether or not cold denaturation effects were indicated by the kinetics of reductive methylation. Clearly the reactions of formaldehyde with lysozyme in the presence of sodium cyanoborohydride involved a substantially different mechanism(s) from those without sodium cyanoborohydride. In the absence of sodium cyanoborohydride the reactions were slower and, furthermore, likely involved modification of side chains other than primary amino groups since loss of enzyme

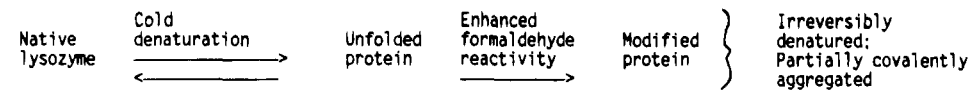

Figure 4. Proposed scheme for reaction of formaldehyde with lysozyme at cold temperatures. Cold denatured (unfolded) lysozyme has enhanced, irreversible reactivity with formaldehyde when compared with native protein.

activity was observed. Tome *et al.* (1985) have shown that formaldehyde can modify other residues in addition to lysine and N-terminal α-NH_2 groups; other modified side chains included histidine, arginine, tryptophan, and tyrosine.

Low-temperature reaction of lysozyme with formaldehyde (Figure 2) suggested that reactions performed at −6 and −10 °C had enhanced reactivity when compared with other temperatures studied. This could be explained by protein unfolding or denaturation at those temperatures. Ang and Hultin (1989) have reported enhanced denaturation as evidenced by loss in solubility, decrease in ATPase activity, and increase in surface hydrophobicity of cod myosin during freezing at −25 and −80 °C and reaction with formaldehyde. However, they did not evaluate the extent of denaturation over a range of temperatures. Also, Sotelo and Mackie (1993) showed that formaldehyde caused aggregation of BSA when stored frozen at −15 and −30 °C but not at 40, 26, 6, and −73 °C. Interestingly, more aggregation was observed at −15 °C than at −30 °C. However, although aggregation is suggestive of denaturation, it does not necessarily imply protein unfolding. Our lysozyme results, involving measurement of enzyme activity, amino group reactivity, and protein aggregation, strongly suggested that protein unfolding was maximally promoted by freezing at −6 and −10 °C. Furthermore, in a separate preliminary study using high concentrations of formaldehyde (170-fold molar excess over primary amino groups in lysozyme) in supercooled solution reaction conditions conducted at −6 °C, we did not observe significant formaldehyde reactivity, suggesting that under these reaction conditions the protein was not denatured (results not shown). Under supercooled conditions the hydrogen-bonded structure of water and the entropy of the aqueous solution would not be as significantly affected as in the frozen state.

The reaction of formaldehyde with lysozyme amino groups shown in Figure 2B indicated that protein unfolding also increased amino group reactivity. Although the amino groups of lysozyme are all on the surface of the molecule (Blake *et al.*, 1965), their accessiblity to formaldehyde must have been more restricted in the native protein when compared with the unfolded protein. These results further supported the occurrence of unfolding at low temperature. Jentoft and Dearborn (1979) presented evidence that reductive methylation of BSA increased in 8 M urea.

Our results, as well as those of Sotelo and Mackie (1993), indicated that, at least in the case of lysozyme and BSA, low-temperature unfolding may be maximal between −6 and −15 °C. Further studies will be required to define more precisely the optimal low temperature for unfolding of these proteins. Physical studies of myoglobin have provided strong evidence for the occurrence of cold denaturation of proteins (Privalov *et al.*, 1986; Privalov, 1990). Ang and Hultin (1989) have proposed an interesting model to explain denaturation of cod myosin during freezing and modification with formaldehyde. Their model suggests that the chemical modification of myosin hastens the denaturation of the protein, implying a cause and effect relationship between modification and denaturation. Our results suggest that protein denaturation (reversible) is an effect of cold temperature and that the extent of modification is dependent on the concentrations of formaldehyde and protein reactive groups present as well as the conformational state of the polypeptide chain. During cold denaturation, in the presence of formaldehyde, irreversibly denatured modified protein forms, some of which may be insoluble and/or covalently aggregated as a result of cross-linking reactions. Figure 4 illustrates a proposed scheme of reactions which is most consistent with our results. This reaction scheme is similar to that reported for formaldehyde denaturation of BSA during frozen storage (Sotelo and Mackie, 1993).

The observed reaction rates of lysozyme with formaldehyde were likely influenced by the freezing process and phase changes that occurred. During freezing some concentration of protein and salts would be expected. The level of formaldehyde employed was already in high molar excess in the upper range (psuedo first order), and further concentration would have had little enhancing effect on reactivity. However, the increase in ionic strength and the freeze concentration of protein may have had some influence on reaction rates. Nevertheless, despite these caveats, concentration effects probably do not detract significantly from the conclusions presented herein, especially since expected formaldehyde reaction rates at −6 °C would be about 20−80-fold slower than at 37 °C (Kittsley, 1964). However, concentration effects could be a significant factor in more dilute formaldehyde reaction conditions that are prevalent during frozen storage of fish.

CONCLUSION

We have obtained evidence to strongly indicate that formaldehyde reactivity with lysozyme was enhanced by cold denaturation of the protein and occurred maximally at about −6 to −10 °C within a range of temperatures studied of 37 to −20 °C. These results have general implications regarding the impact of chemical modification reactions occurring during frozen storage of food proteins.

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