

# Chemical Modification of Lyophilized Proteins in Nonaqueous Environments

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Lyophilized proteins were reacted *in vacuo* with a volatile reagent or dispersed in octane and reacted with dissolved reagent. Three novel derivatives were formed with iodomethane: (a) quaternized trimethyl amino groups, (b) N<sup>1</sup>,N<sup>3</sup>-dimethylimidazolium cation, and (c) phenolic O-methyl ether. Acid anhydrides acylated amino groups and formed mixed anhydrides with side-chain carboxyl groups. Under nonaqueous conditions it was observed that: (i) The same derivatives are formed as under aqueous conditions. (ii) Hydrolytic breakdown of protein is prevented. (iii) Less reagent is required. (iv) Unreacted reagent can be recovered. (v) Water-labile derivatives can be isolated as stable intermediates. (vi) The yield of a derivatized functional group was directly related to its pK<sub>a</sub>, its surface exposure, and the pH of the solution from which the protein was lyophilized. (vii) The physicochemical factors governing the reactivity of protein functional groups in nonaqueous environments appear to reflect the protein solution structure prior to lyophilization.

**KEY WORDS:** Protein modification; iodomethane; acetic anhydride; trimethylamino; dimethylhistidine.

## 1. INTRODUCTION

Chemical modification of native proteins was one of the first methods employed to investigate structure-function relationships. Comprehensive descriptions of the techniques, reagents, and strategies for the modification of proteins in solution are available in general reviews of the field (Means and Feeney, 1971; Glazer *et al.*, 1976; Lundblad and Noyes, 1984; Imoto and Yamada, 1989; Lundblad, 1995). The requirement for an aqueous environment restricts the choice and effectiveness of chemical modifying reagents because many are insoluble in water, react rapidly with water, or form water-unstable derivatives with the side-chain functional groups of the protein.

It is now well established that the catalytic properties of enzymes remain intact in organic solvents (Klibanov, 1989; Chen and Sih, 1989; Westcott and Klibanov, 1994). These findings imply that proteins retain their native structure when lyophilized and dispersed in organic solvents. Evidence has been obtained that crystallized proteins have essentially the same structure in water and organic solvent (Fitzpatrick *et al.*, 1993; 1994). In the lyophilized state proteins are also in a nonaqueous environment; however, physicochemical studies on whether lyophilized proteins retain their solution structure have not been conclusive. Some investigations indicate that the structure in the lyophilized state is the same as in solution (Careri *et al.*, 1980; Schinkel *et al.*, 1985; Rupley and Careri, 1991), while others indicate some limited but reversible conformational change (Poole and Finney, 1983; Prestrelski *et al.*, 1993; Desai *et al.*, 1994). There are likely to be

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differences among proteins in this regard, but the results of the extensive studies with enzymes in organic solvents (Klibanov, 1989; Chen and Sih, 1989; Wescott and Klibanov, 1994) provide strong evidence that most proteins in the lyophilized state at least retain the essential elements of their native structure in solution. If this is true, the reactivity of functional groups in lyophilized proteins should reflect the reactivity of functional groups of the protein in solution and provide information on the solution structure and its properties. The objective of the present investigation was to demonstrate the feasibility and utility of nonaqueous chemical modification of proteins.

## 2. MATERIALS AND METHODS

### 2.1. Proteins

Bovine insulin,  $\alpha$ -chymotrypsin, ribonuclease, and serum albumin were purchased from Sigma Chemical Company. Acetylated bovine serum albumin was prepared using acetic anhydride under aqueous conditions (Means and Feeney, 1971). Inactive DIP- $\alpha$ -chymotrypsin was prepared by incubation with diisopropylfluorophosphate (Darbre, 1986, p. 141).

### 2.2. Chemicals and Solvents

$H_2N$ -Met( $^+SMe$ ) $_2$  and [ $^{13}C$ ]iodomethane were purchased from Sigma Chemical Company. [Acetic-1- $^{14}C$ ]anhydride (9.20 mCi/mmol) was obtained from NEN Research Products and [ $^3H$ ]acetic anhydride (6.94 Ci/mmol) was obtained from Amersham Canada Ltd. All other chemicals, reagents, and solvents were high-purity preparations obtained from commercial sources.

### 2.3. Preparation of $^{13}C$ -Methylated Amino Acids

*Ac-NH-Tyr(OMe)-NH<sub>2</sub>*. N-Acetyl-L-tyrosine amide (100 mg) was dissolved in 0.5 M sodium metaborate buffer (10 ml), pH 11. A 1:1 (v/v) solution (300  $\mu$ l) of [ $^{13}C$ ]iodomethane in acetonitrile was added, the reaction vessel sealed, and the biphasic mixture shaken in a water bath at 37°C for 24 hr. The solution was cooled in an ice bath and the methylated acetyltirosine amide was extracted

with chloroform (3  $\times$  10 ml). The combined extracts were dried under a stream of nitrogen.

*H<sub>2</sub>N-Lys( $\epsilon$ - $^+NMe_3$ )*. Polylysine HBr (30 mg) was dissolved in 5 mM sodium deuteroxide (3 ml) and a 1:1 (v/v) solution (20  $\mu$ l) of [ $^{13}C$ ]iodomethane in acetonitrile was added. The pH was adjusted to 10.5 without buffering and the sealed vial was shaken at 37°C for 4.5 hr. The polylysine derivative was hydrolyzed 18 hr in 6 N HCl to liberate the N $_{\epsilon}$ -trimethylated free amino acid.

*Me<sub>3</sub>N $^+$ -Gly-Leu*, *Me<sub>3</sub>N $^+$ -Ile-NH<sub>2</sub>*, *Me<sub>3</sub>N $^+$ -Ala-Ala*, and *Me<sub>3</sub>N $^+$ -Phe-Gly-Gly*. The unmethylated peptides (13 mg) were placed in a glass vial and dissolved in 5 mM sodium deuteroxide (1 ml). A 1:1 (v/v) solution (20  $\mu$ l) of [ $^{13}C$ ]iodomethane in acetonitrile was added and the sealed reaction was shaken at 37°C for 9 hr in a water bath.

*Ac-NH-His(Im $^+Me_2$ )-NH<sub>2</sub>*. Histidine amide (40 mg) was dissolved in dH<sub>2</sub>O (5 ml) and acetylated with acetic anhydride (5  $\times$  20  $\mu$ l) while maintaining the pH at 9 by the addition of 5 N NaOH. Sodium metaborate was added to a final concentration of 200 mM and the pH was adjusted to 11 by the addition of 1 M NaOH. The solution was transferred to a scintillation vial and a 1:1 (v/v) solution (200  $\mu$ l) of [ $^{13}C$ ]iodomethane in acetonitrile was added. The capped biphasic mixture was shaken for 72 hr at 37°C and the aqueous layer was separated. Acetate and other anions were removed prior to analysis by ion exchange using a Dowex-1 anion exchanger.

*Me<sub>3</sub>N $^+$ -Cys(NH<sub>2</sub>)-S-S-Cys(NH<sub>2</sub>)N $^+Me_3$* . Cystine dimethylester dihydrochloride (10 mg) was dissolved in dH<sub>2</sub>O (4 ml) and the pH was adjusted and maintained at 9.5 by the addition of concentrated ammonia. After 4 hr the solvent was evaporated under reduced pressure. The sample was suspended in D<sub>2</sub>O (1 ml) and the supernatant containing the cystine diamide was reacted at 37°C for 8 hr with [ $^{13}C$ ]iodomethane (20  $\mu$ l). The "pH" was maintained at 10 using 1 M sodium carbonate buffer (100  $\mu$ l).

### 2.4. N-1-[5-Dimethylamino-1-naphthalene-sulfonyl]-2-hydroxy-1,3-diaminopropane

Dansylchloride (500 mg) and 1,3-diamino-2-hydroxypropane (500 mg) were dissolved in pyridine

(8 ml) and 1:1 (v/v) pyridine/*N,N*-dimethylformamide (10 ml), respectively. The diamine solution was stirred vigorously at room temperature and the solution of dansylchloride was added slowly below the surface with a syringe. After 15 min, the reaction was diluted 1:1 (v/v) with  $\text{dH}_2\text{O}$  and the crude mixture stored at  $-20^\circ\text{C}$  until required (yield  $\sim 90\%$ ). Aliquots of the solution were subjected to high-voltage paper electrophoresis in *pH* 6.5 buffer (Kaplan, 1972) for 25 min at a voltage gradient of 40 V/cm. The fluorescent amine ( $\mu_{\text{dansylsulfonic acid}} = -0.8$ ) was eluted from the paper with  $\text{dH}_2\text{O}$ , adsorbed to a Dowex 50X8-200 cation exchanger, and converted to the free amine by elution with 1 M ammonia. The fractions containing the amine were collected and lyophilized.

### 2.5. Aqueous Methylation of Proteins at *pH* 7.5 and *pH* 10

Insulin (20 mg), DIP- $\alpha$ -chymotrypsin (20 mg),  $\alpha$ -chymotrypsin (20 mg), and ribonuclease (20 mg) were placed in screw-capped vials and dissolved in 200 mM sodium phosphate buffer (10 ml), *pH* 7.5, or 200 mM sodium metaborate buffer (10 ml), *pH* 10. A 1:1 (v/v) solution (250  $\mu\text{l}$ ) of [ $^{13}\text{C}$ ]iodomethane in acetonitrile was added below the surface of the solution. The vial was sealed tightly and the biphasic mixture shaken at  $37^\circ\text{C}$  for 24 hr in a temperature-controlled water bath.

### 2.6. Methylation of Proteins at *LpH* 7.5 and *LpH* 10 in Octane

Ribonuclease and  $\alpha$ -chymotrypsin were lyophilized directly in reaction vessels. Only in the case of insulin at *LpH* 7.5 (where *LpH* denotes the *pH* of lyophilization) was it necessary to lyophilize the protein from a larger volume and transfer it to the reaction vessel. Insulin (20 mg) was lyophilized from a solution of 1 mM sodium phosphate buffer (40 ml), *pH* 7.5, and 40 mM sodium metaborate buffer (1 ml), *pH* 10. Ribonuclease and  $\alpha$ -chymotrypsin were lyophilized from a solution of 40 mM sodium phosphate buffer (1 ml), *pH* 7.5, and 40 mM sodium metaborate buffer (1 ml), *pH* 10. Anhydrous octane (2 ml) was added to the protein and the medium was sonicated until the protein was finely dispersed and [ $^{13}\text{C}$ ]iodomethane (100  $\mu\text{l}$ ) was added. To prevent the loss of iodomethane, the

vessels were sealed. The protein dispersion was stirred 12 hr in an oven at  $75^\circ\text{C}$  for the *LpH* 10 reactions and 24 hr for the *LpH* 7.5 reactions. The tube was opened, the derivatized protein was centrifuged with two washes of octane, and the residual octane was removed under vacuum.

### 2.7. *In Vacuo* Methylation of Proteins at *LpH* 7.5 and *LpH* 10

The proteins were prepared as described above for the octane reaction but were lyophilized directly, as in the case of ribonuclease and  $\alpha$ -chymotrypsin, or transferred subsequently, as in the case of insulin, into one compartment of a two-compartment glass reaction vessel furnishing a threaded opening. [ $^{13}\text{C}$ ]iodomethane (10  $\mu\text{l}$ ) was delivered to the second compartment using a plastic tube attached to the end of a micropipette. The top of the reaction vessel was closed with a screw cap and the reagent chamber was submerged in liquid nitrogen. The screw cap was removed, the end of the tube was fitted with a vacuum hose, and the vessel was sealed *in vacuo*. The reaction vessel was incubated in an oven as described above. At the end of the reaction, the reagent chamber was placed in liquid nitrogen, the vacuum seal was broken, and the modified protein was removed.

### 2.8. *In Vacuo* Methylation of $\alpha$ -Chymotrypsin at *LpH* 8.0 in Presence of Inhibitors

$\alpha$ -Chymotrypsin (100  $\mu\text{g}$ ) was lyophilized directly in the two-chambered reaction vessel from an unbuffered solution (1 ml) at *pH* 8.0, and containing 10 mM indole, 10 mM *N*-acetyl-L-tryptophan, or no inhibitor. Iodomethane (25  $\mu\text{l}$ ) was added, and the vessel was sealed under vacuum and placed in an oven at  $75^\circ\text{C}$  for 24 hr.

### 2.9. Quantification of the Activity of *In Vacuo* Methylated $\alpha$ -Chymotrypsin

The protein was dissolved in 100 mM sodium formate buffer, *pH* 4.0 (1 ml). An aliquot containing 10  $\mu\text{g}$  of enzyme was taken for determination of activity. Rate measurements were carried out at *pH* 8.0 and  $20^\circ\text{C}$  on a *pH*-stat apparatus using

10 mM N-acetyl-L-tyrosine ethyl ester in 0.1 M KCl and 5% acetonitrile as substrate.

### 2.10. *In Vacuo* Acetylation of $\alpha$ -Chymotrypsin at *LpH* 9.0 with [ $^3\text{H}$ ]Acetic Anhydride

A solution of  $\alpha$ -chymotrypsin (2.5 mg/ml) was adjusted to *pH* 9.0 with 1 N NaOH. Aliquots (1 ml) were lyophilized in the protein compartments of five reaction vessels. [ $^3\text{H}$ ]Acetic anhydride (10  $\mu\text{l}$ , 1 mCi/mmol) was added to the reaction compartment of four reaction vessels. The fifth was used as a control to which no reagent was added. The reaction vessels were sealed under vacuum as described above and placed in an oven at 75°C. Reaction vessels were then removed at various time points and the reactions terminated and the protein isolated as described above.

### 2.11. Quantification of $^3\text{H}$ Incorporation into Amino Groups

The quantification procedure employed was that used in the competitive labeling technique (Young and Kaplan, 1989) with [ $^{14}\text{C}$ ]acetic anhydride (25  $\mu\text{Ci}/\text{mmol}$ ) used to prepare the  $^{14}\text{C}$ -labeled protein. Peptides containing the  $^3\text{H}/^{14}\text{C}$ -acetylated  $\alpha$ -amino groups and  $\epsilon$ -amino groups were separated by high-voltage paper electrophoresis (Kaplan, 1972). Aliquots of  $^3\text{H}/^{14}\text{C}$ -peptides were transferred into vials containing Aquasol-2-scintillation cocktail (5 ml). The  $^3\text{H}/^{14}\text{C}$  ratios were quantified on an LKB RackBeta liquid scintillation counter.

### 2.12. Nonaqueous Fluorescent Labeling of Carboxyl Groups in Acetylated Bovine Serum Albumin

Acetylated bovine serum albumin (5 mg, *LpH* 7.0) was sealed in an evacuated reaction vessel with ethoxyformic anhydride (2  $\mu\text{l}$ ) and incubated in an oven at 65°C for 22 hr. The reagent chamber was placed in liquid nitrogen to remove any unreacted reagent and the vacuum seal was broken. The protein was suspended in an Eppendorf tube containing the fluorescent amine (100  $\mu\text{g}$ ) dissolved in *N,N*-dimethylformamide (100  $\mu\text{l}$ ). The coupling reaction was terminated after 1 hr by repeated extraction of the reagent from the insoluble protein using *N,N*-dimethylformamide.

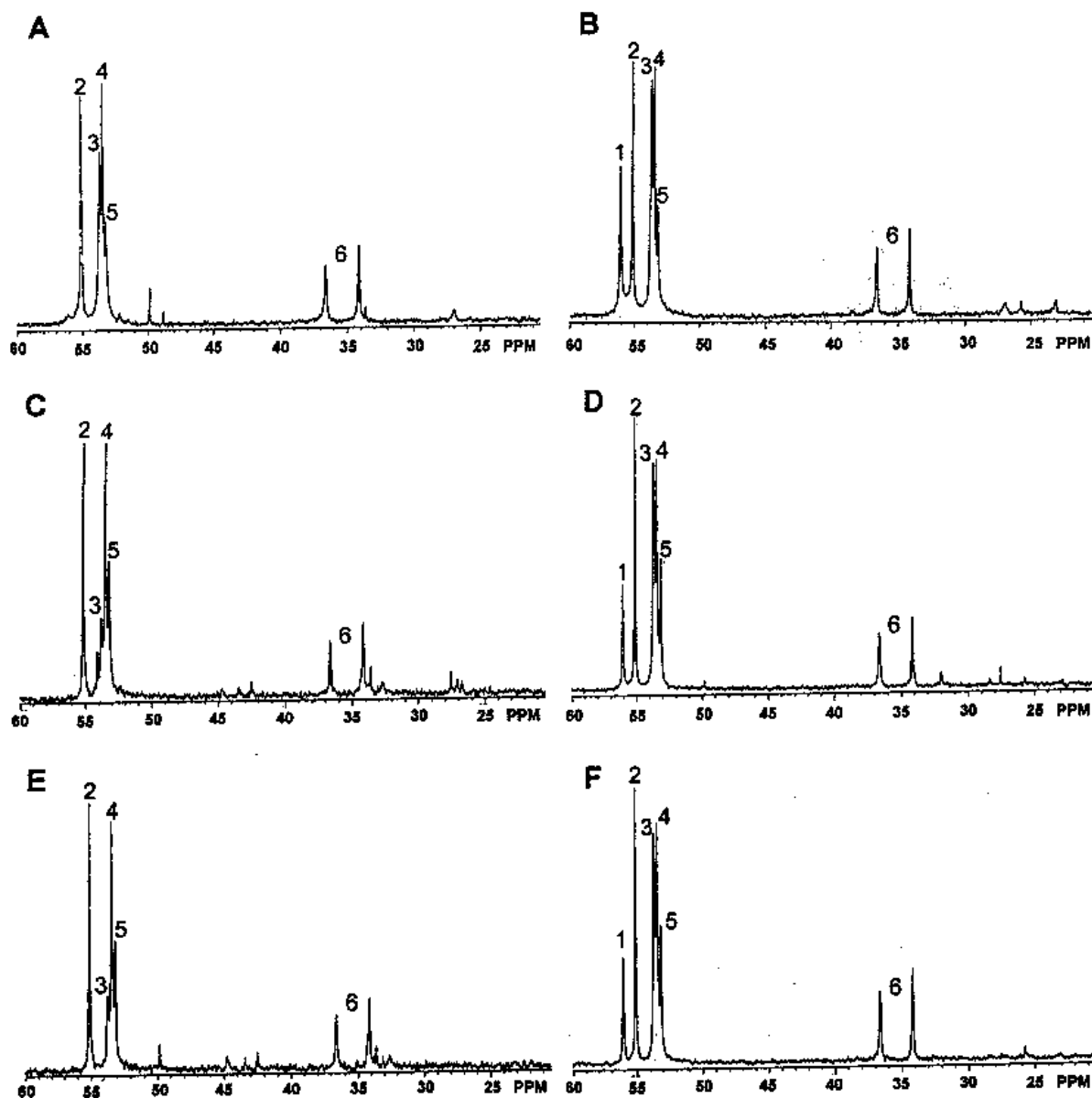
### 2.13. NMR Spectra

[ $^{13}\text{C}$ ]NMR spectra were obtained using a Gemini 200-MHz spectrometer. For the aqueous reactions, derivatized proteins were dialyzed against 10 mM HCl and lyophilized. Methylated protein samples and standards were analyzed in 8 M urea (90%  $\text{D}_2\text{O}$ , 100 mM sodium phosphate) which gave a *pH*-meter reading of 8. Acetonitrile (30  $\mu\text{l}$ ), with a  $^{13}\text{C}$ -chemical shift of its methyl group of 1.70 ppm (Breitmaier and Voelter, 1987, p. 109), was added to reference peak resonances.

## 3. RESULTS

Two experimental approaches for chemical modification of proteins in nonaqueous media were investigated. The first parallels the approach developed by Klivanov (Klivanov, 1984; Zaks and Klivanov, 1988; Broos *et al.*, 1995) for enzymatic reactions in organic solvents. A protein solution is adjusted to the desired *pH* value, lyophilized, and dispersed in octane. The modifying reagent is added to the protein dispersion and the reaction mixture is stirred in a temperature-controlled bath. The modified protein can be isolated simply by filtration or centrifugation, washed with octane, and residual organic solvent removed under vacuum. In a second approach, the reaction is carried out directly on the lyophilized protein. A reaction vessel with two compartments is employed. Protein solution is lyophilized in one compartment and then modifying reagent is added to the other, which is immersed in liquid nitrogen. The reaction vessel is sealed under vacuum and placed in a temperature-controlled oven. To terminate the reaction, the unreacted reagent is trapped out by placing the reagent compartment in liquid nitrogen and releasing the vacuum. The modified protein from either procedure is dissolved in an aqueous medium for analysis by NMR or other analytical procedure. The corresponding aqueous reactions were carried out in the usual manner (Imoto and Yamada, 1989) by adding reagents to stirred protein solutions.

The reactions of insulin with iodomethane in water at *pH* 7.5 and *pH* 10 were compared with the nonaqueous reactions of insulin at *LpH* 7.5 (i.e., lyophilized at *pH* 7.5) and at *LpH* 10. The potential reactive functional groups in insulin are the  $\alpha$ -amino termini of glycine and phenylalanine, the  $\epsilon$ -amino group of lysine, four tyrosine phenolic hydroxyl groups, and two histidine imidazole



**Fig. 1.** Expanded 200-MHz  $^{13}\text{C}$ -NMR spectra (1024 transients) of 20 mg of bovine insulin reacted with  $^{13}\text{C}$ iodomethane. Reaction conditions were: (A) water pH 7.5, 24 hr, 37°C; (B) water pH 10, 24 hr, 37°C; (C) *in vacuo* LpH 7.5, 24 hr, 75°C; (D) *in vacuo* LpH 10, 12 hr, 75°C; (E) octane LpH 7.5, 24 hr, 75°C; (F) Octane LpH 10, 12 hr, 75°C. Peak resonances correspond to the methyl groups in (1) Tyr(OMe); (2)  $\text{Me}_3\text{N}^+\text{-Gly}$ ; (3) Lys( $\epsilon\text{-NMe}_3$ ); (4)  $\text{Me}_3\text{N}^+\text{-Phe}$ ; (5)  $\alpha\text{-NMe}_3$  unidentified; (6) His(Im $^+\text{Me}_2$ ).

groups. Resonances corresponding to the methylated derivatives of all these groups were observed in NMR spectra of the  $^{13}\text{C}$ -labeled proteins (Fig. 1). Peak resonances were assigned to their methylated derivatives by comparing their chemical shifts with those of  $^{13}\text{C}$ methylated standard compounds containing the expected functional groups (Table I). The following similarities in the water, octane, and *in vacuo* reactions were observed with insulin: (a) the same functional groups are modified in the water, octane, and *in vacuo* reactions, (b) the same

derivatives of the various functional groups are obtained, namely amino groups are trimethylated to the quaternary state, histidine forms the dimethylimidazolium cation derivative, and tyrosine forms the phenolic O-methyl derivative, and (c) the phenolic hydroxyl of tyrosine does not react at pH 7.5 or LpH 7.5, but reacts at pH 10 and LpH 10. However, the octane and *in vacuo* reactions differed from the aqueous reaction in that the degree of methylation of the phenylalanine  $\alpha$ -amino, glycine  $\alpha$ -amino, and, most significantly,

**Table I.** Chemical Shifts in ppm for [<sup>13</sup>C]Methyl Groups of Amino Acid Standards, Methylated Insulin, and Methylated  $\alpha$ -Chymotrypsin

[ <sup>13</sup> C]Methyl standards	Chemical shift (ppm)		
	Standard	Insulin	$\alpha$ -Chymotrypsin
Ac-NH-Tyr(OMe)-NH <sub>2</sub>	56.12	56.00	56.18
Me <sub>3</sub> Ne <sup>+</sup> -Gly-Leu	55.06	55.07	—
H <sub>2</sub> N-Lys( $\epsilon$ - <sup>+</sup> NMe <sub>3</sub> )	53.66	53.62	53.69
[Me <sub>3</sub> N <sup>+</sup> -Cys(NH <sub>2</sub> )-S-] <sub>2</sub>	53.42	—	53.50
Me <sub>3</sub> N <sup>+</sup> -Phe-Gly-Gly	53.41	53.48	—
Me <sub>3</sub> N <sup>+</sup> -Ile-NH <sub>2</sub>	53.03	—	53.21
Me <sub>3</sub> N <sup>+</sup> -Ala-Ala	52.60	—	52.71
H <sub>2</sub> N-Lys( $\epsilon$ - <sup>+</sup> NHMe <sub>2</sub> )	43.42	—	43.37
Ac-NH-His(Im <sup>+</sup> Me <sub>2</sub> )-NH <sub>2</sub>	34.04, 36.46	34.10, 36.58	34.08, 36.59
H <sub>2</sub> N-Lys( $\epsilon$ - <sup>+</sup> NH <sub>2</sub> Me)	33.59	—	33.55
<sup>+</sup> SMe <sub>3</sub>	27.5 <sup>a</sup>	—	27.54
H <sub>2</sub> N-Met( <sup>+</sup> SMeMe)	25.41	—	25.65

<sup>a</sup> Breitmaier and Voelter (1987), p. 234.

the lysine  $\epsilon$ -amino group was considerably less at  $LpH$  7.5 than at  $LpH$  10. These differences are greater than is apparent in Fig. 1 because the vertical scales used in Figs. 1D and 1F are attenuated with respect to Figs. 1C and 1E. Insulin reacted under aqueous conditions (24 hr,  $T = 37^\circ C$ , and  $pH = 10$ ) or nonaqueous conditions *in vacuo* (12 hr,  $T = 75^\circ C$ , and  $LpH = 10$ ) tested negative with Pauly's diazo reagent (Darbre, 1986, p. 260) in both cases, weakly ninhydrin-positive for the nonaqueous sample, and ninhydrin-negative for the aqueous sample. An extra resonance at 53.12 ppm in the chemical shift region for the  $\alpha$ -amino termini is present in the spectrum of methylated insulin (Fig. 1). This resonance was not always observed with other preparations of bovine insulin. It is therefore believed to be due to the presence of some insulin with an internal proteolytic cleavage in this particular commercial preparation.

The aqueous reaction of diisopropylphosphoryl (DIP)- $\alpha$ -chymotrypsin at  $pH$  7.5 and  $pH$  10 with [<sup>13</sup>C]iodomethane (Figs. 2A and 2B) gave all the methylated derivatives observed with insulin. In addition, the dimethylsulfonium derivative of the methionine side chain, which is not present in insulin, was observed. The nonaqueous reaction of  $\alpha$ -chymotrypsin with [<sup>13</sup>C]iodomethane differs from the aqueous reaction (Fig. 2B) in that no O-methyltyrosine is observed at  $LpH$  10 (Fig. 2D and 2F). Methylation of ribonuclease paralleled that of chymotrypsin in that under aqueous conditions tyrosine was methylated at  $pH$  10 but not at  $pH$  7.5, and not methylated under nona-

queous conditions at  $LpH$  10.  $\alpha$ -Chymotrypsin has three amino termini and three resonances are therefore expected in the chemical shift region of trimethylated  $\alpha$ -amino groups as observed in the nonaqueous reaction (Fig. 2C, peaks 3–5). Peak 3 for the trimethylated cystine  $\alpha$ -amino terminus is not visible in Figs. 2A, 2B, 2D, and 2F because of the very intense neighboring trimethylated lysine  $\epsilon$ -amino resonance, but is resolved in higher field spectrometers. It was necessary to attenuate the vertical scale in Figs. 2B, 2D, and 2F in order to accommodate the intense resonance at 53.69 ppm, due to the superimposition of the 14 lysine residues in  $\alpha$ -chymotrypsin after reaction at  $pH$  10 and  $LpH$  10. For this reason the peak intensities for the other resonances at  $pH$  10 and  $LpH$  10 appear weaker than they do in the spectra for reactions at  $pH$  7.5 and  $LpH$  7.5 (Figs. 2A and 2C), but the degree of methylation of these groups is at least as great or greater. When chymotrypsin was not inactivated with diisopropylfluorophosphate (DFP), more than three multiple-peak resonances were observed in the aqueous reaction (Fig. 2E), indicating that, unlike the nonaqueous reaction, autolysis had occurred generating additional  $\alpha$ -amino groups. Also observed are resonances at 49.79 ppm (<sup>13</sup>CH<sub>3</sub>OH), probably due to traces of water reacting with iodomethane, 43.37 ppm (<sup>13</sup>C- $\epsilon$ -dimethylamino lysine), and 33.55 ppm (<sup>13</sup>C- $\epsilon$ -monomethylamino lysine) due to incomplete methylation at  $LpH$  7.5, and 27.54 ppm (trimethylsulfonium cation), possibly arising from breakdown of disulfide bridges. These are variable resonances

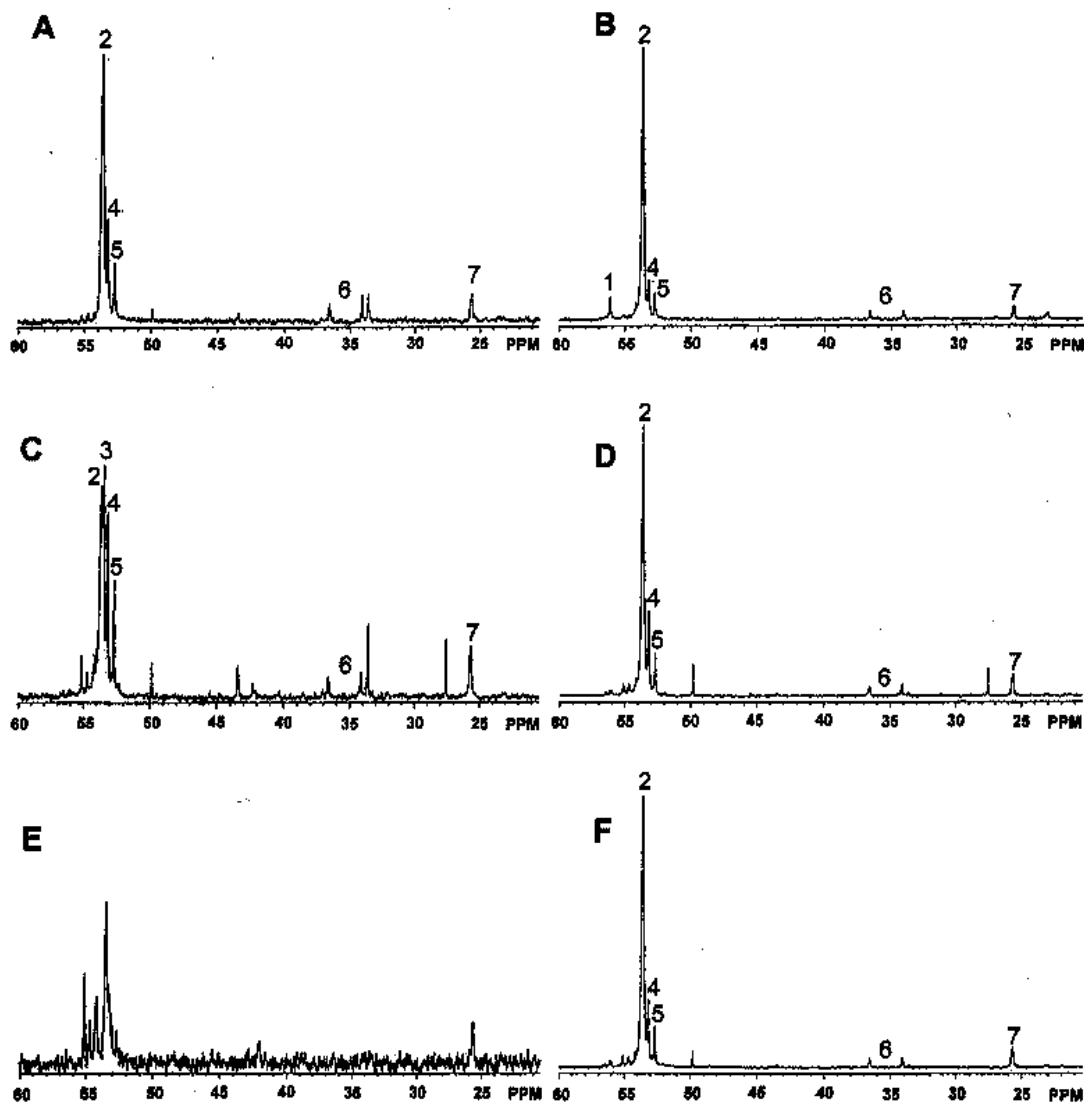


Fig. 2. Expanded 200-MHz  $^{13}\text{C}$ -NMR spectra (1024 transients) of 20 mg of bovine  $\alpha$ -chymotrypsin and DIP- $\alpha$ -chymotrypsin reacted with  $^{13}\text{C}$ iodomethane. Reaction conditions were: (A) DIP- $\alpha$ -chymotrypsin, water pH 7.5, 24 hr, 37°C; (B) DIP- $\alpha$ -chymotrypsin, water pH 10, 24 hr, 37°C; (C)  $\alpha$ -chymotrypsin, *in vacuo* LpH 7.5, 24 hr, 75°C; (D)  $\alpha$ -chymotrypsin, *in vacuo* LpH 10, 12 hr, 75°C; (E)  $\alpha$ -chymotrypsin, water pH 7.5, 24 hr, 37°C; (F)  $\alpha$ -chymotrypsin, octane LpH 10, 12 hr, 75°C. Peak resonances correspond to the methyl groups in (1) Tyr(OMe); (2) Lys( $\epsilon$ - $^+\text{NMe}_3$ ); (3)  $\text{Me}_3\text{N}^+$ -Cys; (4)  $\text{Me}_3\text{N}^+$ -Ile; (5)  $\text{Me}_3\text{N}^+$ -Ala; (6) His( $\text{Im}^+\text{Me}_2$ ); (7) Met( $^+\text{SMeMe}$ ).

arising from incomplete derivatization or minor side-reactions. With aqueous modifications in general, it is common practice to dialyze proteins following reaction. For this reason, small breakdown products are usually not observed. This is best illustrated by the weak  $^{13}\text{C}$ -NMR signal obtained in Fig. 2E, where a large portion of the autolysis products have been removed by dialysis, requiring expansion of the vertical scale. For the nonaqueous reactions reported here the proteins need not be dialyzed, so all minor methylated derivatives are also observed.

Modification with acetic anhydride was carried out on  $\alpha$ -chymotrypsin *in vacuo* with protein at LpH 9 using excess  $^3\text{H}$ acetic anhydride (Table II). The reaction was monitored by quantifying the extent of tritium incorporation of acetylated peptides (Kaplan, 1972). Complete acetylation of amino groups of proteins is readily achieved in water at pH 9, but in the nonaqueous reaction only 25% of the  $\epsilon$ -amino groups and 90% of the  $\alpha$ -amino groups were modified after prolonged reaction times. It also appears that the acetylation of the available  $\epsilon$ -amino groups reached completion

**Table II.** Relative Incorporation of Acetic Anhydride into Amino Groups of  $\alpha$ -Chymotrypsin by Nonaqueous Derivatization

Amino group	Relative incorporation at given reaction time <i>in vacuo</i> (LpH 9.0)			
	1 hr	8 hr	24 hr	60 hr
$\alpha$ -Amino	0.34	0.42	0.58	0.87
$\epsilon$ -Amino	0.21	0.23	0.25	0.26

<sup>a</sup> Fraction relative to complete derivatization in water,  $T = 75^\circ\text{C}$ .

much more rapidly than the acetylation of the  $\alpha$ -amino groups.

$\alpha$ -Chymotrypsin, which was lyophilized at pH 8 in presence of the competitive inhibitors indole and N-acetyl-L-tryptophan, was reacted *in vacuo* with iodomethane. In the absence of any inhibitor or with indole the enzyme was completely inactivated by reaction with iodomethane. In sharp contrast, even after 24 hr reaction, substantial activity was retained when N-acetyl-L-tryptophan was present (Table III).

Acetic anhydride forms a mixed anhydride with the side-chain carboxyl groups of proteins in water, but these are rapidly hydrolyzed either by the direct action of water or via participation of an oxazolinone intermediate (Darbre, 1986, p. 551). In a nonaqueous medium hydrolysis cannot occur. Indirect evidence for the formation of anhydride was obtained from the observation that *in vacuo*  $^3\text{H}$ -acetylated protein lost over 50% of its radioactivity after dialysis in 8 M urea. Direct evidence for the formation of anhydrides under nonaqueous conditions was obtained by acetylating all the amino groups of bovine serum albumin under aqueous conditions, reacting the lyophilized acetylated protein *in vacuo* at LpH 7 with

ethoxyformic anhydride, and then incubating with a small amount of fluorescent amine (N-1-[5-dimethylamino-1-naphthalenesulfonyl]-2-hydroxy-1,3-diaminopropane) in N,N-dimethylformamide. An intense fluorescence was observed under UV with the *in vacuo* modified protein, but not in the control protein, which was incubated *in vacuo* without ethoxyformic anhydride. Although amino groups do not necessarily have to be blocked, acetylated bovine serum albumin was used as a test protein to ensure that carboxyl groups were the predominant nucleophile available in the protein for reaction with ethoxyformic anhydride.

#### 4. DISCUSSION

Klibanov and his co-workers (Klibanov, 1984, 1989; Zaks and Klibanov, 1988; Wescott and Klibanov, 1994; Broos *et al.*, 1995) observed that enzymes, and by inference, proteins, demonstrated certain properties in organic solvents which potentially can be very advantageous for elucidating structure-function relationships of proteins. The most important property is that they appear to retain their native conformation. Proteins in organic solvents have a greatly enhanced thermostability, which would permit the use of elevated temperatures to accelerate the modification reaction without unfolding the native structure. For the *in vacuo* approach, it is expected that the thermal stability of the protein will be as great, if not greater, than in organic solvents. The thermal stability most likely arises from the rigidity of the protein structure in nonaqueous media (Zaks and Klibanov, 1988; Broos *et al.*, 1995). Reactivity data from nonaqueous reactions may therefore be more readily interpreted than in solution, where interpretation is complicated by the need to take into consideration the effect of all the dynamic conformational equilibria on the observed chemical properties (Young and Kaplan, 1989; Kaplan *et al.*, 1971).

Reductive methylation of amino groups with formaldehyde and borohydride seldom results in disruption of the protein structure or loss of biological activity (Means and Feeney, 1971; Glazer *et al.*, 1976; Means, 1977; Lundblad and Noyes, 1984; Imoto and Yamada, 1989; Lundblad, 1995) and for this reason it is widely used in protein structure-function studies (Zhang and Vogel, 1933; Zhang *et al.*, 1994). Surprisingly, iodomethane has been used so infrequently for protein methylation

**Table III.** Activity of Lyophilized  $\alpha$ -Chymotrypsin (LpH 8.0) in Presence and Absence of Competitive Inhibitors after Reaction with Iodomethane at  $75^\circ\text{C}$  for 24 hr

Inhibitor	% activity <sup>a</sup>
No inhibitor	0
Indole	0
N-Acetyl-L-tryptophan	68 $\pm$ 2

<sup>a</sup> Average of two trials relative to untreated  $\alpha$ -chymotrypsin.

that it is not usually included in a catalogue of chemical reagents for protein modification (Lundblad and Noyes, 1984). While reductive methylation proceeds to give at most the dimethylamino derivative, iodomethane gives the quaternary trimethyl derivative. Although quaternization of amino groups is known to occur *in vivo* as a posttranslational modification of protein (Paik and Kim, 1975), this modification, to our knowledge, has not been reported as an *in vitro* chemical modification for amino groups in native proteins and provides a means for placing a permanent positive charge on the  $\alpha$ - and  $\epsilon$ -amino groups at all pH values. The formation of a dimethylimidazolium cation derivative with the side chain of histidine was unexpected since this derivative has not been reported as either an *in vitro* chemical modification of a native protein or as an *in vivo* posttranslational modification. Like trimethylation of amino groups, dimethylation of the imidazole function also provides a means for placing a positive charge on the side chain of histidine residues at all pH values.

It appears from the results obtained in this initial investigation that most of the factors which affect the relative reactivity of the various functional groups of proteins in solution are retained by the lyophilized protein in the nonaqueous environment. These are the intrinsic nucleophilicity of the group, the microenvironment, and the ionization state of the group (Kaplan *et al.* 1971; Young and Kaplan, 1989). The reaction of  $\epsilon$ -amino groups with acetic anhydride reached completion faster than that of the  $\alpha$ -amino groups (Table II), which is expected on the basis of their relative nucleophilicities. However, the degree of derivatization of the  $\epsilon$ -amino groups is much less than that of the  $\alpha$ -amino groups. This would be expected on the basis of the pH memory effect (Zaks and Klibanov, 1988), where ionizable groups in nonaqueous media retain the ionization state they had in the solution from which they were lyophilized. With  $pK_a$  values approximately 7–8, more than 50% of the  $\alpha$ -amino groups would be expected to be derivatized at  $LpH$  9, whereas with  $pK_a$  values approximately 10–11, less than 50% of the  $\epsilon$ -amino groups would react (Creighton, 1993, p. 6). This pH memory effect is also evident in the reactions of the  $\epsilon$ - and  $\alpha$ -amino groups and imidazole groups with iodomethane. At  $LpH$  7.5, the degree of reaction of the  $\epsilon$ -amino groups relative to that of the  $\alpha$ -amino groups is clearly much less than at  $LpH$  10, while the imidazole

group with a  $pK_a$  value approximately 6–7 (Creighton, 1993, p. 6) shows a much smaller difference (Fig. 2). Since functional groups in proteins have  $pK_a$  values (Creighton, 1993, p. 6) varying from 3.5 to 12, this phenomenon of pH memory has the potential to be utilized as a means of achieving selective chemical modifications of ionizable functional groups by controlling the pH of lyophilization.

The tyrosine phenolic hydroxyl function is readily methylated under aqueous conditions at  $pH$  10 with model compounds and proteins used in this study (Figs. 1B and 2B). There was, however, a notable difference in the reactivity of this group between insulin and both  $\alpha$ -chymotrypsin and ribonuclease in a nonaqueous environment, in that only in insulin was this function methylated at  $LpH$  10. This suggests that the phenolic side chains are buried in the major conformational states of  $\alpha$ -chymotrypsin and ribonuclease in solution at  $pH$  10. The reason these side chains react in an aqueous, but not in a nonaqueous environment is presumed to be due to the dynamic equilibrium that exists between the various conformational states in solution and, although the tyrosine side chains are exposed only in minor conformations, this can lead to substantial reaction over a period of time due to Le Châtelier's principle. In the nonaqueous state no such dynamic equilibria exist, so that no substantial modification can occur. Insulin, being a very small protein, is unfolded to a large extent in solution at  $pH$  10, and at  $LpH$  10, exposing some or all of its tyrosine side chains.

Proteolysis is a common problem in the study of proteins (Scopes, 1987; Volkin and Klibanov, 1989). Chemical modification of proteins often disrupts the native structure, making the modified protein extremely sensitive to degradation by trace amounts of proteolytic enzymes. It is particularly difficult to chemically modify proteases such as  $\alpha$ -chymotrypsin under conditions where the enzyme is catalytically active without some autolysis. In the present study evidence for substantial hydrolytic breakdown was obtained in the aqueous reaction of  $\alpha$ -chymotrypsin with iodomethane at  $pH$  7.5 (Fig. 2E). In contrast, the *in vacuo* reaction gave peak resonances only for the expected three methylated amino termini (Fig. 2C), demonstrating that nonaqueous conditions can be used to perform the derivatization while eliminating hydrolytic breakdown of the protein. In the case of insulin (Fig. 1), the presence of an extra  $\alpha$ -amino

resonance indicated a heterogeneity in the commercial insulin preparation. This ability to detect free  $\alpha$ -amino termini demonstrates that the nonaqueous methylation procedure with [ $^{13}\text{C}$ ]iodomethane can be used to assess the homogeneity of some protein preparations or provide evidence for the presence of blocked amino termini.

Ligands which bind to proteins have been shown to protect functional groups in the binding regions from chemical modification (Means and Feeney, 1971, pp. 24–34; Bosshard, 1979). However, this approach for identifying functional groups in the active sites of enzymes has found only limited application. Because most competitive inhibitors have relatively weak binding affinities, active-site functional groups are still readily modified even in the presence of these inhibitors, due to the dynamic binding equilibria that exist in aqueous media. In contrast, no such dynamic equilibria can exist under *in vacuo* conditions, so if a nonvolatile ligand is bound, it cannot dissociate and the residues in that binding site will not be expected to react.

The results reported in Table III show that lyophilized  $\alpha$ -chymotrypsin is inactivated by methylation. As histidine-57 is essential for catalytic activity (Creighton, 1993, pp. 419–421), methylation of this residue is expected to result in loss of activity. Indole, which is a good competitive inhibitor ( $K_1 = 0.72$  mM at 25°C) (Barman, 1969), does not offer any protection, whereas N-acetyl-L-tryptophan, which is a much weaker inhibitor ( $K_1 = 17.5$  mM at 25°C) (Barman, 1969), gives substantial protection.  $\alpha$ -Chymotrypsin was lyophilized from a solution containing these inhibitors at a concentration of 10 mM. It is therefore expected that virtually all the indole-binding sites in the lyophilized enzyme are occupied, whereas a substantial protection of the binding sites are free in the case of N-acetyl-L-tryptophan. The reason there is such a striking difference in protection is that the N-acetylpropionyl moiety of the latter inhibitor covers the catalytic site. If we make the assumption that 68% of the enzyme has the inhibitor bound and 32% is free, we calculate a  $K_f = 15$  mM at 0°C, which is in good agreement with the experimental value reported at 25°C. Another interesting inference that can be made from the results is that dimethylation of the other histidine residue and trimethylation of the amino groups does not inactivate the enzyme. It is significant to note that such information would be

very difficult to obtain in an aqueous medium and provides yet another example of the potential utility of the chemical modification of proteins in nonaqueous environments.

Activated carboxyl groups are short-lived in water, which requires the use of high concentrations of activating reagents and nucleophiles to achieve extensive modification of carboxyl groups (Means and Feeney, 1971; Glazer *et al.*, 1976; Lundblad and Noyes, 1984; Imoto and Yamada, 1989; Lundblad, 1995). As demonstrated with acetylated bovine serum albumin, another advantage of nonaqueous conditions is that stable activated derivatives of carboxyl groups in proteins can be prepared with relatively small amounts of reagents. Couplings with nucleophiles to form water-stable derivatives then can be carried out. In the present study coupling was carried out with a fluorescent amine in N,N-dimethylformamide. However, there are a large number of procedures that could be used for such couplings and further investigation is required to determine the best strategies and their limitations.

The results of the present study show that nonaqueous modification of lyophilized proteins in octane or *in vacuo* is feasible and practical. Modification in organic solvent has the advantage that nonvolatile modifying reagents can be used. With the *in vacuo* procedure, stirring is not required to maintain the protein in a dispersed state, the reaction temperature is not limited by the boiling point of the solvent, recovery of unreacted reagent is much simpler, and no further manipulation of the modified protein is required. Regardless of which nonaqueous procedure is used, there are significant advantages over aqueous modification: (1) Water is not present as a competing reactant, so much smaller amounts of reagents are required, which can be useful when expensive reagents are used. In the present study, for example, 10  $\mu\text{l}$  of [ $^{13}\text{C}$ ]iodomethane was used in the *in vacuo* reaction at an approximate cost of \$1, whereas the equivalent aqueous reaction required 125  $\mu\text{l}$  at a cost of \$12.50. This is a substantial saving and would be even more substantial with costlier reagents such as radiolabeled compounds. (2) Unreacted reagents can be recovered, which again can add to the cost-efficiency of the chemical modification. (3) Water-insoluble reagents can be employed at high concentrations or pressures, thereby increasing their effectiveness as modifying reagents. (4) The derivatized protein is easily

isolated. (5) Derivatives which are unstable in water, such as activated carboxylic acids, can be prepared as reactive intermediates. (6) Hydrolytic degradation of proteins can be prevented.

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