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Clearly, the phenomenon of pH memory has the potential to be utilized as a means of achieving selective chemical modification of ionizable functional groups, even for those within the same class, by controlling the pH of lyophilization.

**Table 2:** Relative Incorporation\* of Acetic Anhydride into Amino Groups of  $\alpha$ -Chymotrypsin by Nonaqueous Derivatization

Amino Group	Reaction Time In <i>Vacuo</i> (LpH 9.0)		
	1 h	8 h	24 h
$\alpha$ -amino	0.34	0.42	0.58
$\epsilon$ -amino	0.21	0.23	0.25

\*fraction relative to complete derivatization in water. T = 75°C

The results of the present study show that nonaqueous modification of lyophilized proteins in octane or *in vacuo* is feasible and practical. The increased temperature stability (27,34) of proteins in the lyophilized state permits the use of elevated temperatures to accelerate the reactions. Modification in organic solvent has the advantage that volatile and nonvolatile 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 procedures. A case in point is the reaction with iodomethane, which because of its low solubility and slow reaction in water, has been so infrequently used for protein modification that it is not usually included in catalogues of reagents for protein modification (1,3). While the results show that iodomethane does react with proteins in water to form the same derivatives as under nonaqueous conditions, the long reaction time and rapid agitation required to disperse the iodomethane makes it very difficult to prevent denaturation or hydrolytic breakdown. It is expected that the same will be true for all insoluble modifying reagents that require long reaction times. In contrast, the nonaqueous reaction, whether in organic solvent or *in vacuo*, is facile with such reagents, and the possibility of irreversible structural damage is greatly reduced. Another significant advantage is that the pH memory effect observed with lyophilized proteins can be exploited to improve the selectivity of modification, even

for groups within the same functional class by controlling the pH of lyophilization. The ability to use nonaqueous conditions opens the door to the use of novel protein modifying reagents and should provide the opportunity, as in the case of iodomethane, for the preparation of novel derivatives to explore structure-function relationships in proteins.

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the side-chain of histidine was unexpected since this derivative has not been reported as either an *in vitro* chemical modification of a native

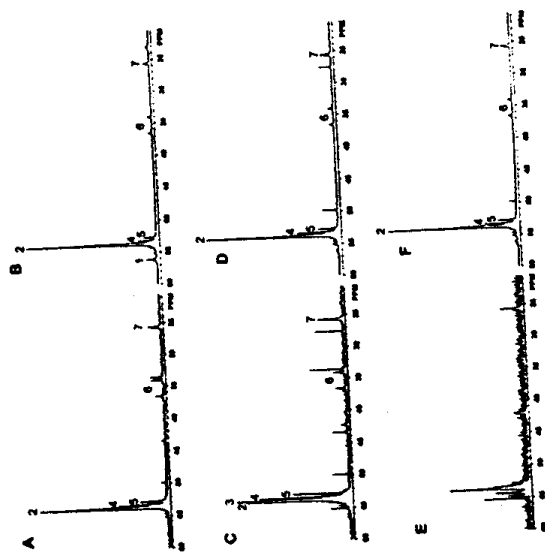


Figure 2. NMR Spectra of  $\alpha$ -Chymotrypsin and DIP-Chymotrypsin. A) DIP-Chymotrypsin, water, pH 7.5; B) DIP-Chymotrypsin, water, pH 10; C)  $\alpha$ -Chymotrypsin, *in vacuo*, LpH 7.5; D)  $\alpha$ -Chymotrypsin, *in vacuo*, LpH 10; E)  $\alpha$ -Chymotrypsin, water, pH 7.5; F)  $\alpha$ -Chymotrypsin, water, LpH 10. Peak resonances correspond to: 1- Tyr(Ome); 2- Lys( $\epsilon$ -NMe<sub>2</sub>); 3- Me<sub>2</sub>N<sup>+</sup>-Cys; 4- Me<sub>2</sub>N<sup>+</sup>-Ile; 5- Me<sub>2</sub>N<sup>+</sup>-Ala; 6- His(Im<sup>+</sup>Me<sub>2</sub>); 7- Met(<sup>+</sup>SMe<sub>2</sub>).

protein or as an *in vivo* post-translational modification. Like the trimethylation of amino groups, dimethylation of the imidazole function also provides a means for placing a positive charge on the side-chain at all pH values.

The tyrosine phenolic hydroxyl function was readily methylated to form a methyl ether under aqueous conditions at pH 10 with the proteins

used in this investigation and with model compounds. To our knowledge this modification reaction with iodomethane has also not been previously reported as an *in vitro* chemical modification of native proteins. There was, however, a notable difference in the reactivity of this group between insulin (Figure 1d and 1f) and both  $\alpha$ -chymotrypsin (Figure 2d and 2f) and ribonuclease (spectrum not shown) 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 comparable dynamic equilibria exist, so that the tyrosine side-chains never become exposed. 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.

The most significant difference between the aqueous and nonaqueous reactions was that for the nonaqueous reaction the relative degree of methylation of  $\epsilon$  and  $\alpha$ -amino groups depended on the pH of lyophilization. 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<sub>a</sub> value approximately 6 to 7 (33) shows a much smaller difference (Figures 1c & 1d, 1e & 1f and 2c & 2d). This can be explained on the basis of the pH memory effect (27), where ionizable groups "remember" the ionization state they had in the solution from which they were lyophilized. In nonaqueous media there is no water present for a dynamic equilibrium to be established between the two ionization states and therefore only the deprotonated species will be derivatized while the protonated form remains unmodified. This interpretation is consistent with the results obtained for the *in vacuo* reaction of the  $\epsilon$  and  $\alpha$ -amino groups of lyophilized  $\alpha$ -chymotrypsin at LpH 9 with acetic anhydride at 75°C (Table 2). It was found that after 60 h of reaction, 87% of the  $\alpha$ -amino groups were acetylated, but in contrast, only 26% of the lysine  $\epsilon$ -amino groups were acetylated. With pK<sub>a</sub> values approximately 7 to 8, more than 50% of the  $\alpha$ -amino groups would be expected to be derivatized at LpH 9, whereas with pK<sub>a</sub> values approximately 10 to 11, less than 50% of the  $\epsilon$ -amino groups would react (33).

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 and glycine  $\alpha$ -amino groups, and most significantly the lysine  $\epsilon$ -amino group was considerably less at LpH 7.5 than at LpH 10. These differences are greater than is apparent in figure 1 because the vertical scales used in figures 1d and 1f are attenuated with respect to figures 1c and 1e, in order that all the resonances be on scale. Insulin reacted under aqueous conditions (24 h, T = 37°C and pH = 10) or nonaqueous conditions *in vacuo* (24h, T=75°C and LpH =10) tested negative with Pauly's diazo reagent (31) in both cases, weakly ninhydrin positive for the nonaqueous sample and ninhydrin negative for the aqueous sample, showing that the reactions proceeded to completion.

The aqueous reaction of DIP- $\alpha$ -chymotrypsin at pH 7.5 and pH 10 with [ $^{13}\text{C}$ ]iodomethane (Figures 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 reactions of  $\alpha$ -chymotrypsin with [ $^{13}\text{C}$ ]iodomethane differs from the aqueous reaction (Figure 2b) in that no O-methyltyrosine is observed at LpH 10 (Figures 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 nonaqueous 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 (Figure 2c, peaks 3, 4 and 5). Peak 3 for the trimethylated cystine  $\alpha$ -amino terminus is not visible in figures 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 figures 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 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 (Figures 2a and 2c) but the degree of methylation of these groups is at least as great or greater. When chymotrypsin was not inactivated with DFP, more than three multiple peak resonances were observed in the

aqueous reaction (Figure 2e) indicating that, unlike the nonaqueous reaction, autolysis had occurred generating additional  $\alpha$ -amino groups. With aqueous modifications, it is usually necessary to dialyze proteins following reaction with a derivatizing reagent and as a result small breakdown products are not observed. This is illustrated in figure 2e where a large portion of the protein has autolyzed and small methylated peptides have been removed by dialysis, requiring expansion of the vertical scale in order to observe the weak resonances of the remaining fragments. In contrast, for the nonaqueous reactions reported here the proteins need not be dialyzed so resonances corresponding to low molecular weight products and minor side reactions can also be observed.

Table 1: Chemical shifts in ppm for [ $^{13}\text{C}$ ]Methyl Groups of Amino Acid Standards, Methylated Insulin and Methylated  $\alpha$ -Chymotrypsin

[ $^{13}\text{C}$ ]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> N <sup>+</sup> -Gly-Leu	55.06	55.07	
H <sub>2</sub> N-Lys( $\epsilon$ - <sup>+</sup> N Me <sub>3</sub> )	53.66	53.62	53.69
[Me <sub>3</sub> N <sup>+</sup> -Cys(NH <sub>2</sub> )-S]-I <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(lm <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
+SMe <sub>3</sub>	27.5 <sup>(39)</sup>		27.54
H <sub>2</sub> N-Met( <sup>+</sup> SMeMe)	25.41		25.65

The reaction of iodomethane with amino groups proceeds to give the quaternary trimethylamino derivative. In contrast reductive methylation yields at most the dimethylamino derivative (1-5). Although quaternization of amino groups is known to occur *in vivo* as a post-translational modification of proteins (32), 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. Similarly, the formation of a dimethylimidazolium cation derivative with

### III. RESULTS AND DISCUSSION

The chemical modification of insulin with [ $^{13}\text{C}$ ]iodomethane was employed to compare the reaction of the lyophilized protein under nonaqueous conditions, *in vacuo* and in octane, with that of the solubilized protein under aqueous conditions. Reactions were carried out at pH 7.5 and 10 for the aqueous reaction and LpH 7.5 and 10 for the nonaqueous conditions. Peak resonances (Figure 1) corresponding to methylated derivatives of functional groups were assigned from the chemical shifts of methylated standard compounds (Table I). The following similarities in the water, octane and *in vacuo* reactions were observed: 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, viz. amino groups are trimethylated to the quaternary state, histidine forms the

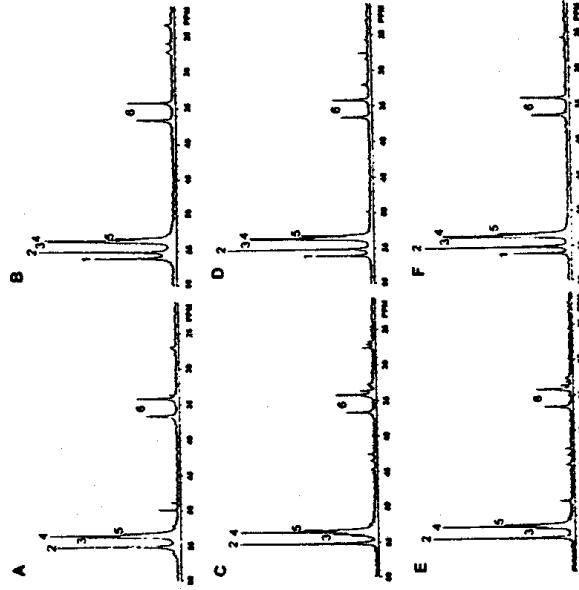


Figure 1. NMR Spectra of  $^{13}\text{C}$ -Methylated Insulin. A) Water, pH 7.5; B) Water, pH 10; C) *In vacuo*, LpH 7.5; D) *In vacuo*, LpH 10; E) Octane, LpH 7.5; F) Octane, LpH 10. Peak resonances correspond to: 1- Tyr(OMe); 2-  $\text{Me}_3\text{N}^+\text{-Gly}$ ; 3- Lys( $\epsilon$ - $\text{NMe}_2$ ); 4-  $\text{Me}_3\text{N}^+\text{-Phe}$ ; 5-  $\alpha$ - $\text{NMe}_2$ (unidentified); 6- His( $\text{Im}^+\text{Me}_2$ ).

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

***In vacuo* methylation of proteins at LpH 7.5 and LpH 10.** The proteins were lyophilized as described above for the octane reaction in the two-compartment reaction vessel. [ $^{13}\text{C}$ ]iodomethane (10  $\mu\text{l}$ ) was transferred into the reagent chamber which was submerged in liquid nitrogen. The open end of the reaction vessel was fitted with a vacuum hose, the reaction vessel was sealed under vacuum and incubated at  $75^\circ\text{C}$ . To terminate the reaction, the reagent chamber was placed in liquid nitrogen, the vacuum seal was broken and the modified protein was removed.

***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 1N 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 chambers 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  $75^\circ\text{C}$ . Reaction vessels were then removed at various time points and the reactions terminated and the protein isolated as described above.

**Quantification of  $^3\text{H}$  incorporation into amino groups.** The quantification procedure employed was that used in the competitive labeling technique (30) with [ $^{14}\text{C}$ ]acetic anhydride (25 mCi/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 (26). Aliquots of the  $^3\text{H}/^{14}\text{C}$ -peptides were transferred into vials containing Aquasol-2 scintillation cocktail (5 ml) and the  $^3\text{H}/^{14}\text{C}$ -ratios were quantified on a LKB RackBeta liquid scintillation counter.

**NMR Spectra.** [ $^{13}\text{C}$ ]NMR analyses (1024 transients) were obtained using a Gemini 200 MHz spectrometer. Methylated protein samples were analyzed in an 8M urea, 90%  $\text{D}_2\text{O}$  solution of 100 mM sodium phosphate which gave a pH meter reading of 8. For the aqueous reactions derivatized proteins were dialyzed against 10 mM HCl and lyophilized prior to the addition of 8M urea. Acetonitrile (30  $\mu\text{l}$ ) was added to reference peak resonances.

differences among proteins in this regard, but the results of extensive studies with lyophilized enzymes in organic solvents provide strong evidence that most proteins in the lyophilized state retain the essential elements of their native structure (11-13). It is therefore expected that reactivity of functional groups in lyophilized proteins will reflect their properties in solution and provide information on the solution structure. The present study reports that modification of lyophilized proteins in a nonaqueous environment has significant advantages over aqueous procedures.

## II. MATERIALS AND METHODS

### A. Proteins, reagents and amino acid derivatives

**Proteins.** Bovine insulin,  $\alpha$ -chymotrypsin and ribonuclease were purchased from Sigma Chemical Company. Inactivated diisopropylphosphoryl(DIP)- $\alpha$ -chymotrypsin was prepared by incubation with diisopropylfluorophosphate(DFP) (25).

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

**$^{13}C$ -Methylated amino acids were prepared as follows:** N-Acetyl-L-tyrosine-amide, polylysine-HBr, Gly-Leu, Ile-NH $_2$ , Ala-Ala, Phe-Gly-Gly, histidine amide, and cystine dimethylester dihydrochloride were methylated with iodomethane to serve as standards for the assignment of peak resonances in methylated proteins.

N- $\alpha$ -Amino acids (10 mg) were typically dissolved in 200 mM, pH 10 sodium metaborate buffer (1 ml), and methylated directly by the addition of a 1:1 v/v solution (20  $\mu$ l) of [ $^{13}C$ ]iodomethane in acetonitrile, with rapid mixing of the sealed biphasic mixture at 37°C for 24 h. Cystine dimethyl ester, in particular, was first converted to the diamide at pH 9.5 using ammonia. Histidine amide was first acetylated prior to reaction with iodomethane.

Side-chain methylated amino acids were prepared from N- $\alpha$ -blocked starting materials in the same manner, and in the case of methylated polylysine, was followed by acid hydrolysis of the peptide bonds.

**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  $\mu$ l) of [ $^{13}C$ ]iodomethane in acetonitrile was added, the vial was sealed tightly and the biphasic mixture shaken at 37°C for 24 h.

### B. Modification of lyophilized proteins under nonaqueous conditions

Two experimental approaches for nonaqueous chemical modification of proteins lyophilized at specific pH values (L-pH) were investigated. The first parallels the approach developed by Klibanov (27-29) 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 oven. 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 glass reaction vessel with two compartments is employed. Protein solution is lyophilized in one compartment and then modifying reagent is added to the other compartment, which is immersed in liquid nitrogen. The reaction vessel is sealed under vacuum and placed in an 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 procedures.

**Methylation of proteins at L-pH 7.5 and L-pH 10 in octane.** Proteins were lyophilized directly in the reaction vessels. Only in the case of insulin at L-pH 7.5 was it necessary to lyophilize the protein from a large 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, at which time [ $^{13}C$ ]iodomethane (100  $\mu$ l) was added. To prevent the loss of

# Nonaqueous Chemical Modification of Lyophilized Proteins

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## I. INTRODUCTION

Chemical modification has been widely used to investigate structure-function relationships in native proteins. Comprehensive descriptions of techniques, reagents and strategies for the modification of proteins in aqueous environments are available in general reviews of the field (1-6). The 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 protein functional groups. Indeed, nonaqueous chemistry has been employed in amino and carboxyl terminal sequencing methodologies (7,8) and in the derivatization of peptides for applications in mass spectrometry (9,10). However, these applications did not focus on the native structure of the protein and the procedures were devised for denatured or fragmented proteins. Nevertheless, they show the potential advantages of a nonaqueous environment for the modification of proteins.

It is now well established that the catalytic properties of a wide variety of enzymes remain intact in organic solvents (11-13). These findings imply that proteins may also retain their native structures when lyophilized and dispersed in organic solvents. Evidence has been obtained that crystallized proteins have essentially the same structure in water and organic solvent (14,15). In the lyophilized state, proteins are also in a nonaqueous environment and it is expected their physico-chemical properties will differ from that in solution, as the dynamic conformational equilibria that exists in solution will be absent. Some physico-chemical studies indicate that the structure of the lyophilized state is very similar to that in solution (16-18), while others indicate that there is some limited but reversible conformational change (19-24). There are likely to be