due to an increased physical expansion of the molecule at low ionic strength over the values observed at high ionic strength.

In summary, at ionic strengths in excess of 0.07 with a 1% BSA solution, BSA expands as the pH is lowered below the isoelectric point. No further physical expansion occurs when the ionic strength is lowered.

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Fluorine Nuclear Magnetic Resonance Studies of Trifluoroacetyl-insulin Derivatives. Effects of pH on Conformation and Aggregation†

Richard A. Paselk and Daniel Levy*

ABSTRACT: ^{19}F nuclear magnetic resonance techniques have been used to study the effects of solvent and pH on the conformation and aggregation properties of several trifluoroacetyl derivatives of insulin. Sedimentation velocity studies were also performed in an effort to obtain independent data on the aggregation state of these derivatives at pH 2.0 and 6.8. At pH 2.0, insulin and the trifluoroacetyl-insulin derivatives exist largely as dimers, as suggested by the $s_{20,w}$ values and the three narrow ^{19}F resonance peaks derived from the trifluoroacetyl moiety bound to the α-amino groups of the glycine^{A-1} and phenylalanine^{B-1} and the ϵ-amino group of lysine. At pH 6.8, high molecular weight aggregates were indicated by the $s_{20,w}$ values and the considerable broadening of the ^{19}F resonance spectra. Spectral analysis at this pH suggested that the trifluoroacetyl group on glycine^{A-1} possessed significantly more motional freedom than on phenylalanine^{B-1} or lysine^{B-29}. Further elevation of the pH to 8.7 resulted in disaggregation of the trifluoroacetyl-insulin derivatives, as indicated by the line widths of the ^{19}F nuclear magnetic resonance spectra. Spectral analysis of the chemical shifts at pH 8.7 also suggested conformational alterations in the regions of phenylalanine^{B-1} and lysine^{B-29}

The use of nuclear magnetic resonance (nmr) techniques to study structural and conformational properties of protein molecules has afforded a wealth of information. Proton magnetic resonance spectroscopy affords extremely complex spectra due to the large number of equivalent protons and the broadening of the various peaks resulting from the incorporation of the absorbing species into a macromolecule. Nevertheless, considerable information has been obtained from the examination of those resonance peaks which are resolved from the main absorption envelope (Jardetsky and Wade-Jardetsky, 1971).

In an effort to simplify these complex spectra, several studies have been concerned with the chemical introduction of fluorine as the trifluoroacetyl group to serve as an environmental probe at defined sites in a macromolecule. This procedure allows for the identification of individual resonance peaks and the analysis of observed spectral alterations resulting from differences in

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local environment, and modifications caused by conformational changes and aggregation phenomena. ^{19}F-Nmr techniques have several important advantages: the ^{19}F chemical shifts are very sensitive to alterations in the environment; the probe is small, and can be easily introduced into protein molecules, thus allowing for a minimum alteration in native structure and biological activity. The trifluoroacetyl moiety has been chemically bound to ribonuclease and hemoglobin and studied using ^{19}F-nmr techniques (Huestis and Raftery, 1971, 1972a-c). ^{19}F-Nmr has also been used to study the conformation of angiotensin II analogs (Vine *et al.*, 1973).

A variety of studies have been concerned with the relationship of structure to function for the hormone, insulin. The complex aggregation properties and conformation of this molecule have been shown to be dependent on pH, salt concentration, metal ions, protein concentration, and temperature as well as chemical and enzymatic modifications of the native structure. Various studies have been undertaken to explore this complex behavior, utilizing sedimentation velocity and equilibrium measurements (Jeffrey and Coates, 1966a,b; Pekar and Frank, 1972; Slobin and Carpenter, 1966; Fredericq, 1956), ultraviolet spectroscopy (Rupley *et al.*, 1967; Frank *et al.*, 1972; Lord *et al.*, 1973), and optical rotatory dispersion and circular dichroism (Carpenter, 1966; Levy and Carpenter, 1970; Morris *et al.*, 1968; Mercola *et al.*, 1967, 1972; Ettinger and Timasheff, 1971). The availability of the high-resolution X-ray crystallographic data for zinc insulin (Blundell *et al.*, 1972; Adams *et al.*, 1969) further enables the analysis of the results obtained from these solution studies.

In an effort to further understand the effects of pH and solvent on the complex aggregation behavior of insulin as well as effects on conformation, the trifluoroacetyl moiety has been introduced at various points in the molecule (Figure 1; R = H or CF_3CO[1]) to serve as an environmental probe utilizing ^{19}F-nmr techniques. The derivatives obtained have been shown to possess high biological activity and immunoreactivity (Levy and Paselk, 1973; Geiger and Obermeier, 1973; Paselk and Levy, 1974). This report concerns the ^{19}F-nmr spectra of several trifluoroacetyl-insulin derivatives and appropriate model compounds. The nature of the aggregation phenomena and conformational changes induced by alterations in pH are discussed.

Experimental Section

Materials

Ethyl thioltrifluoroacetate was the product of Pierce Chemical Co. Glycylglycylglycine and phenylalanylglycylglycine were obtained from Sigma Chemical Co. L-Glycine and L-phenylalanine were obtained from Calbiochem. DL-Lysine monohydrochloride was the product of Nutritional Biochemical Co. L-1-Tosylamido-2-phenylethyl chloromethyl ketone (Tos-PheCH_2Cl) treated trypsin was purchased from Worthington Biochemical Corp. The chromatography resins (Sephadex G-50, fine; Sephadex G-10, fine) were obtained from Pharmacia Fine Chemicals, Inc. Dowex 50W-X2 was obtained from Bio-Rad laboratories. MN polygram SIL N-HR precoated plastic sheets were obtained from Brinkmann Instruments, Inc. Bovine zinc insulin (lot No. 493-88GPD-017) was the product of Eli Lilly and Co. All other chemicals were of analytical grade and were obtained from Mallinckrodt Chemical Co.

N-Trifluoroacetylglycine, *N*-trifluoroacetylphenylalanine, N^{ϵ}-trifluoroacetyllysine, and *N*-trifluoroacetylglycylglycylglycylglycine were prepared according to the procedures of Schallenberg and Calvin (1955): *N*-trifluoroacetylglycine, reported mp 114–116°, found 115–117°; *N*-trifluoroacetylphenylalanine, reported mp 119.4–120.6°, found 120–121°; N^{ϵ}-trifluoroacetyllysine, reported mp 226–231°, found, 224–230° (Schallenberg and Calvin, 1955); *N*-trifluoroacetylglycylglycylglycylglycine, reported mp 225–228°, found 227–229° (Weygand and Röpsch, 1959).

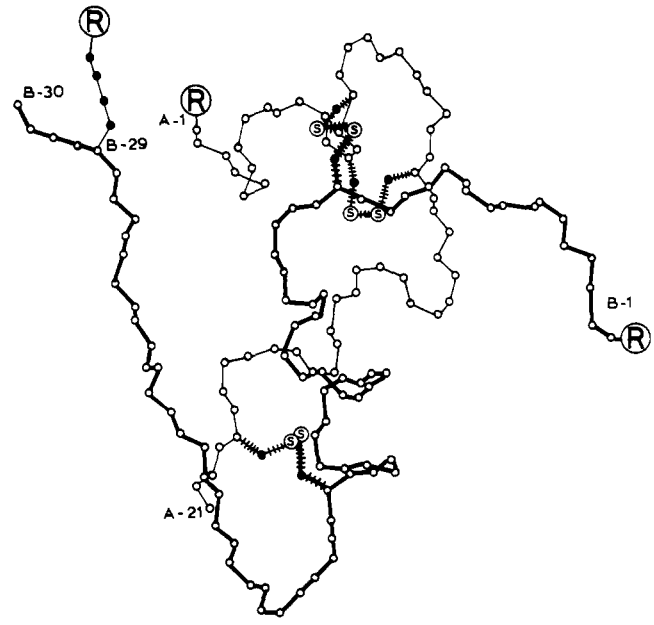

FIGURE 1: Structure of the insulin monomer (redrawn from Blundell *et al.*, 1972) indicating the location of the fluorine probes (R = H or CF_3CO): A chain (—O—); B chain (—O—); disulfide bridges (⫴⫴●⫴⫴).

N-Trifluoroacetylphenylalanylglycylglycine. Phenylalanylglycylglycine was treated with ethyl thioltrifluoroacetate by the method of Schallenberg and Calvin (1955), and recrystallized from ethyl acetate–petroleum ether (bp 30–60°): mp 174–176°; yield 50%. This product was shown to be ninhydrin negative and homogeneous on thin-layer chromatography using two solvent systems (ammonium hydroxide–1-propanol, 30:70; and chloroform–methanol–acetic acid, 95:5:1).

Anal. Calcd for $C_{15}H_{16}F_3O_5$: C, 48.00; H, 4.30; F, 15.19. Found: C, 48.15; H, 4.33; F, 14.92.

N^{ϵ}-Lysine$^{B\text{-}29}$-trifluoroacetyl-insulin-octapeptide. Tritrifluoroacetyl-insulin (Levy and Paselk, 1973) (170 mg, 28 μmol) was dissolved in bicarbonate buffer, pH 8.0. To this solution was added 14.2 ml of a Tos-PheCH_2Cl–trypsin solution (pH 3.0, 1 mM $CaCl_2$, 1.2 mg/ml) and the reaction was stirred for 4 hr at 38°. The reaction mixture was chromatographed on Sephadex G-50, followed by chromatography on Sephadex G-10, as previously described (Levy and Paselk, 1973), to afford the N^{ϵ}-trifluoroacetyl$^{B\text{-}29}$-octapeptide derivative.

Glycine$^{A\text{-}1$-trifluoroacetyl-S-sulfo-insulin A Chain. Insulin hydrochloride (164 mg, 28 μmol) was subjected to sulfotolysis according to the procedures of Dixon and Wardlaw (1960) to afford the *S*-sulfo derivatives of the A and B chains. The purified *S*-sulfo A chain (75 mg, 28 μmol) was trifluoroacetylated in dimethyl sulfoxide instead of dimethylformamide to increase the solubility of the peptide, using procedures previously described (Levy and Paselk, 1973). The reaction mixture was lyophilized and the product purified on Sephadex G-25, eluting with 2.5 N acetic acid, affording the ninhydrin negative peptide.

Glycine$^{A\text{-}1$-phenylalanine$^{B\text{-}1$-trifluoroacetyl-desoctapeptide-

[1] Abbreviations used are: CF_3CO, trifluoroacetyl; Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

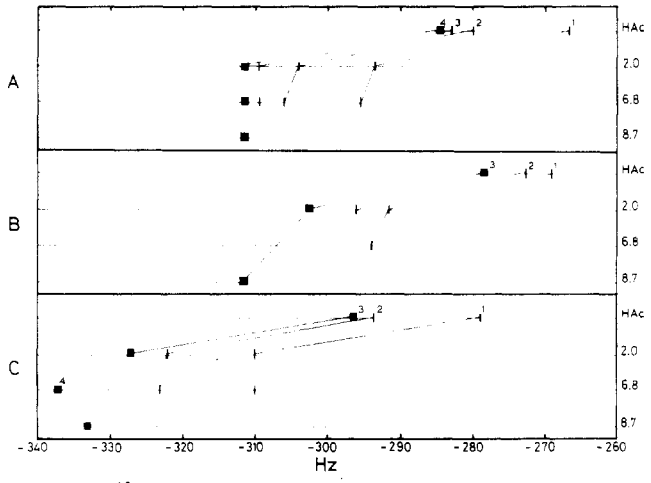

FIGURE 2: ^{19}F chemical shifts of trifluoroacetylamino acids, peptides, and insulin derivatives as a function of solvent (5 N acetic acid [HAc] or water) and pH. (A) Trifluoroacetyl group bound to the α-amino group of glycine: (1) *N*-trifluoroacetylglycine; (2) *N*-trifluoroacetylglycylglycylglycine; (3) glycine^{A-1}-trifluoroacetyl-*S*-sulfo-insulin A chain; (4) trifluoroacetylglycine-insulin derivatives. (B) Trifluoroacetyl group bound to the ϵ-amino group of lysine: (1) N^{ϵ}-trifluoroacetyllysine; (2) lysine^{B-29}-trifluoroacetyl-insulin-octapeptide; (3) trifluoroacetyllysine-insulin derivatives. (C) Trifluoroacetyl bound to the α-amino group of phenylalanine: (1) *N*-trifluoroacetylphenylalanine; (2) *N*-trifluoroacetylphenylalanylglycylglycine; (3) trifluoroacetylphenylalanine-insulin derivatives; (4) glycine^{A-1}-phenylalanine^{B-1}-trifluoroacetyl-desoctapeptide-insulin.

insulin. Insulin hydrochloride (100 mg, 17.2 μmol) was treated with Tos-PheCH$_2$Cl-trypsin as previously described (Levy and Paselk, 1973) to afford desoctapeptide-insulin, which was purified on Sephadex G-25, eluting with 0.05 M ammonium acetate (pH 7.5). Trifluoroacetylation of the insulin derivative was effected in dimethylformamide with ethyl thioltrifluoroacetate (Levy and Paselk, 1973). The product was purified on Sephadex G-50, eluting with 0.05 M ammonium acetate (pH 7.5). The product was characterized by ninhydrin analysis and by deamination followed by amino acid analysis (Levy and Paselk, 1973).

Trifluoroacetylation of Insulin. The additional synthesis of the mono- and bis(trifluoroacetyl)insulin derivatives were performed. The details of these procedures along with the biological properties of these derivatives are described elsewhere (Paselk and Levy, 1974).

Methods

Amino acid analyses were performed on a Technicon amino acid analyzer (Spackman *et al.*, 1958). Samples were hydrolyzed in 6 N HCl at 120° for 6 hr. Ultraviolet absorption measurements were taken on a Zeiss spectrophotometer (PMQ II). pH measurements were made on a Radiometer Copenhagen Model 26 or 4 pH meter. Elemental analysis was performed by Elek Laboratories, Los Angeles, Calif. Melting points were taken on a Fischer-Johns melting point apparatus. Thin-layer chromatography was performed on polygram SIL N-HR precoated plastic sheets. ^{19}F nuclear magnetic resonance spectra were recorded on a Varian HA-100-15 spectrometer modified to operate at 94.1 MHz. Spectra were accumulated on a Fabritek Model 1074 computer of average transients. Sedimentation velocity measurements were made on a Beckman-Spinco Model E analytical ultracentrifuge equipped with a Schlieren optical system and rotor temperature controls.

Nuclear Magnetic Resonance Measurements. The trifluoroacetylamino acids, peptides, and insulin derivatives were dissolved in water and the pH adjusted with HCl or NaOH. Amino acid and peptide derivatives were run at a concentration of 0.05 M. *N*-Trifluoroacetylglycine was also run at a concentration of 1.5 mM. Insulin derivatives were run at a concentration of 10 mg/ml. All spectra were measured in 12-mm tubes (Wilmad Glass Co., No. 514A-7PP) at a probe temperature of 35 ± 1°, using a concentrically held 4 mM capillary tube of trifluoroacetic acid as an external reference standard. All samples were allowed to equilibrate to the temperature of the probe before spectra were accumulated. Peak widths were measured at half-maximum intensity.

Sedimentation Velocity Studies. Samples of the trifluoroacetyl-insulin derivatives (10 mg/ml) were prepared in an identical fashion to the solutions used in ^{19}F magnetic resonance studies. Samples were sedimented at 59,790 rpm using conventional sedimentational velocity techniques. Sedimentation coefficients were corrected to $s_{20,w}$ using a partial specific volume of 0.73 ml/g (Pekar and Frank, 1972) and a solvent correction for 0.01 N NaCl.

Results

Several trifluoroacetyl derivatives of insulin have been prepared along with the appropriate amino acid and peptide model

Gly - Phe - Phe - Tyr - Thr - Pro - Lys - Ala
└CF$_3$CO

compounds. Lysine^{B-29}-trifluoroacetyl-insulin-octapeptide was obtained utilizing trypsin cleavage of tritrifluoroacetyl-insulin. The identity of this product was established by amino acid analysis. No alanine was released on enzymatic hydrolysis, indicating that the ϵ-amino group of lysine was blocked with the trifluoroacetyl moiety. It was also observed that the conditions used to effect the cleavage of the octapeptide resulted in a slight hydrolysis of the trifluoroacetyl group on glycine^{A-1}, as assessed by ninhydrin analysis and amino acid analysis of the deaminated product. The preparation of glycine^{A-1}-phenylalanine^{B-1}-trifluoroacetyl-desoctapeptide-insulin was thus prepared directly from desoctapeptide-insulin using the same procedures as described for native insulin, to afford a homogeneous product which was ninhydrin negative. *S*-Sulfoinsulin A chain was also trifluoroacetylated on glycine^{A-1} as previously described, yielding a ninhydrin negative product which was further characterized by amino acid analysis of the deaminated derivative.

The ^{19}F-nmr spectra of the amino acids, peptides, and insulin derivatives were taken in 5 N acetic acid and at pH 2.0 ± 0.1, 6.8 ± 0.1, and 8.7 ± 0.2. The chemical shifts of *N*-trifluoroacetylglycine, *N*-trifluoroacetylphenylalanine, and N^{ϵ}-trifluoroacetyllysine in 5 N acetic acid, pH 2.0, 6.8 and 8.7, are shown in Figure 2A–C. A pronounced downfield shift is observed when these compounds are transferred from 5 N acetic acid to water (pH 2.0); however, the introduction of a negative charge on the carboxyl group when the pH was raised to 6.8 had only a minimal effect on the glycine and lysine derivatives, and no effect on the phenylalanine derivative. There was also no effect of concentration of the ^{19}F chemical shift of *N*-trifluoroacetylglycine in the range 50–1.5 mM at pH 2.0.

The effect of modifying the α-carboxyl group in these trifluoroacetyl-model compounds by the addition of amino acids to form *N*-trifluoroacetylglycylglycylglycylglycine, *N*-trifluoroacetylphenylalanylglycylglycine, and lysine^{B-29}-trifluoroacetyl-insulin-octapeptide resulted in downfield shifts in all solvents and pH values (Figure 2A–C). Again, the downfield shifts observed when the compounds were transferred from 5 N acetic acid to water were quite marked, but were relatively insensitive to alterations in pH.

TABLE I: ^{19}F Nuclear Magnetic Resonance Peak Widths (Hz) for Monotrifluoroacetyl-insulin Derivatives as a Function of pH.

	Gly^{A-1},[a] G[b]	Phe^{B-1},[a] P
5 N acetic acid	3.1	2.7
pH 2.0 ± 0.1	3.2	3.7
pH 3.0 ± 0.1	5.6	Insoluble
pH 6.8 ± 0.1	12 ± 1	*c*

[a] -trifluoroacetyl-insulin. [b] Trifluoroacetyl peak position, Figure 3. [c] No ^{19}F-nmr peak observed.

Lysine^{B-29}-trifluoroacetyl-insulin-octapeptide could not be run at pH 6.8 because of its low solubility. Glycine^{A-1}-trifluoroacetyl-*S*-sulfo-insulin A chain was shifted further downfield than the smaller model compounds, and again the chemical shift was markedly altered on transferring the compound from 5 N acetic acid to water (pH 2.0), but was also insensitive to a pH alteration to 6.8 (Figure 2A). The line widths of all of these trifluoroacetyl derivatives were narrow (2–3 Hz) in both 5 N acetic acid and in water, and were essentially invariant over the pH range investigated.

The ^{19}F nmr spectra of the various trifluoroacetyl-insulin derivatives were studied under the identical conditions used for the model compounds. The spectrum of glycine^{A-1}-phenylalanine^{B-1}-lysine^{B-29}-trifluoroacetyl-insulin in 5 N acetic acid is shown in Figure 3a. The assignment of each of the three peaks was established by obtaining a spectrum of the two monotrifluoroacetyl-insulin derivatives and the two bis(trifluoroacetyl)-insulin derivatives. The ^{19}F resonance positions for the three trifluoroacetyl groups went from low to high field in the order: phenylalanine, glycine, lysine. The line widths in the two mono-, two bis-, and one tris(trifluoroacetyl)-insulin derivatives were all quite narrow (Tables I and II). The chemical shifts were located at lower fields than the various model compounds (Figure 2).

As in the case of the various model compounds, the transfer of the trifluoroacetyl-insulin derivatives from 5 N acetic acid to water (pH 2.0) resulted in downfield shifts of 24 to 31 Hz (Figure 2). A composite spectrum derived from the two bis(trifluoroacetyl)insulin derivatives is shown in Figure 3b. The spectra of glycine^{A-1}-trifluoroacetyl-insulin and phenylalanine^{B-1}-trifluoroacetyl-insulin further corroborated the peak assignments. As shown in Tables I and II the peak widths again remained quite narrow. The tris(trifluoroacetyl)-insulin derivative was insoluble at pH 2.0, and thus could not be studied at this pH. When the pH of glycine^{A-1}-trifluoroacetyl-insulin was adjusted to pH 3.0, the ^{19}F-nmr spectrum indicated a significant broadening of the resonance peak (Table I), suggesting the formation of higher aggregates in solution. All other derivatives were insoluble at this pH and thus could not be studied.

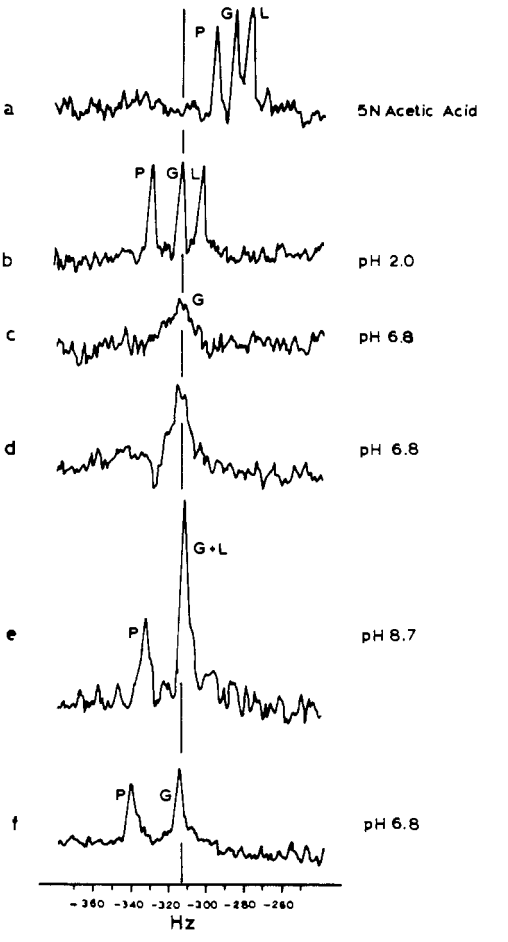

FIGURE 3: ^{19}F-nmr spectra of trifluoroacetyl-insulin derivatives: (a) glycine^{A-1}-phenylalanine^{B-1}-lysine^{B-29}-trifluoroacetyl-insulin; (b) composite from glycine^{A-1}-phenylalanine^{B-1}-trifluoroacetyl-insulin and glycine^{A-1}-lysine^{B-29}-trifluoroacetyl-insulin; (c) glycine^{A-1}-trifluoroacetyl-insulin; (d) glycine^{A-1}-phenylalanine^{B-1}-lysine^{B-29}-trifluoroacetyl-insulin; (e) glycine^{A-1}-phenylalanine^{B-1}-lysine^{B-29}-trifluoroacetyl-insulin; (f) glycine^{A-1}-phenylalanine^{B-1}-trifluoroacetyl-desoctapeptide-insulin. Abbreviations used in the figure are: P, phenylalanine; G, glycine; L, lysine; DOP, desoctapeptide.

The ^{19}F-nmr spectrum of tris(trifluoroacetyl)-insulin at pH 6.8 afforded a broadened peak of 9.0 ± 1 Hz (Figure 3d, Table II), whose center corresponded to the ^{19}F resonance peak of glycine^{A-1}-trifluoroacetyl-insulin at pH 2.0. When the mono derivative (glycine^{A-1}-trifluoroacetyl-insulin) was studied at pH 6.8, it afforded a broad peak of 12 ± 1 Hz (Figure 3c, Table I), as did the two bis(trifluoroacetyl)-insulin derivatives (Table II); however, when phenylalanine^{B-1}-trifluoroacetyl-insulin was studied at pH 6.8, no ^{19}F magnetic resonance peaks

TABLE II: ^{19}F Nuclear Magnetic Resonance Peak Widths (Hz) for Bis- and Tris(trifluoroacetyl)-insulin Derivatives.

	Gly^{A-1}-Phe^{B-1} [a]		Gly^{A-1}-Lys^{B-29} [a]		Gly^{A-1}-Phe^{B-1}-Lys^{B-29} [a]			Gly^{A-1}-Phe^{B-1} [c]	
	G[b]	P	G	L	G	P	L	G	P
5 N acetic acid	2.9	2.8	2.7	3.3	3.0	3.0	3.1	3.5	3.3
pH 2.0 ± 0.1	3.3	3.2	3.2	3.2	Insoluble				
pH 6.8 ± 0.1	12 ± 1		12 ± 1		9 ± 1			3.7	4.6
pH 8.7 ± 0.2	4.1	4.8			4.4[d]	4.3			

[a] -trifluoroacetyl-insulin. [b] Trifluoroacetyl peak position, Figure 3. [c] -desoctapeptide-trifluoroacetyl-insulin. [d] Width of trifluoroacetylglycine + trifluoroacetyllysine peak.

TABLE III: Svedberg Constants ($s_{20,w}$) of Insulin and Trifluoroacetyl-insulin Derivatives as a Function of pH.[a]

Compound	pH 2.0 ± 0.1	pH 6.8 ± 0.1
Insulin	1.4	3.5
Glycine^{A-1}[b]	1.5	3.5
Phenylalanine^{B-1}[b]	1.8	3.6
Glycine^{A-1}-phenylalanine^{B-1}[b]	1.6	3.2
Glycine^{A-1}-phenylalanine^{B-1}-lysine^{B-29}[b]		2.8
Glycine^{A-1}-phenylalanine^{B-1}[c]		1.4

[a] All samples were run at a concentration of 10 mg/ml. [b] -trifluoroacetyl-insulin. [c] -trifluoroacetyl-desoctapeptide-insulin.

were observed. Glycine^{A-1}-phenylalanine^{B-1}-trifluoroacetyl-desoctapeptide-insulin, which would not be expected to undergo extensive aggregation based on the behavior of desoctapeptide-insulin (Arquilla *et al.*, 1969), afforded a two-peak spectrum (Figure 3f) with narrow peak widths at pH 6.8 (Table II). In addition to the observed peak widths, a downfield spectral shift of 11 Hz was also observed for the trifluoroacetyl group bound to phenylalanine^{B-1}, while the trifluoroacetyl group on glycine^{A-1} indicated a negligible shift (Figure 2).

Glycine^{A-1}-phenylalanine^{B-1}-trifluoroacetyl-insulin and glycine^{A-1}-phenylalanine^{B-1}-lysine^{B-29}-trifluoroacetyl-insulin were also studied at pH 8.7. The spectrum of the tris(trifluoroacetyl)-insulin derivatives is shown in Figure 3e. This spectrum indicates two narrow resonance peaks (Table II), whose areas are in the ratio 1:2. Spectral assignments were based on the positions observed for the glycine^{A-1}-phenylalanine^{B-1}-trifluoroacetyl-insulin derivative at this pH. In addition to the narrow resonance peaks, downfield spectral shifts of 7 and 10 Hz were observed for the trifluoroacetyl moiety on phenylalanine^{B-1} and lysine^{B-29}, respectively, between pH 2.0 and 8.7 (Figure 2).

Sedimentation Velocity Studies. In an effort to correlate the observed ^{19}F-nmr spectral peak width changes with alterations in the aggregation state of the trifluoroacetyl-insulin derivatives, $s_{20,w}$ values for several derivatives at pH 2.0 ± 0.1 and 6.8 ± 0.1 are listed (Table III). At pH 2.0, sedimentation values indicated the presence of insulin dimers, while at 6.8, the $s_{20,w}$ values indicated considerable aggregation, while the desoctapeptide-insulin derivative still had a sedimentation value similar to the insulin derivatives at pH 2.0. The significantly lower $s_{20,w}$ value for the tris(trifluoroacetyl)-insulin derivative is also reflected in a narrower ^{19}F resonance peak width (Table II).

Discussion

Using the techniques of ^{19}F-nmr spectroscopy, these studies have been concerned with the effects of pH on the conformation and complex aggregation properties of trifluoroacetyl-insulin derivatives with high biological and immunological activity. Several model compounds have also been studied in reference to effects of solvent and pH on ^{19}F resonance chemical shifts as well as on peak widths. The synthesis of several trifluoroacetyl-insulin derivatives has afforded the possibility of studying the environment at three distinct locations in the molecule.

Model Trifluoroacetyl Derivatives. All of the trifluoroacetylamino acids and peptide derivatives experienced a downfield shift of the ^{19}F resonance peaks when transferred from 5 N acetic acid to water (pH 2.0). Such deshielding effects have been observed when trifluoroacetylphenylalanine was transferred from nonpolar solvents to water (Zeffren and Reavill, 1968; Zeffren, 1970). The ^{19}F resonance peaks were essentially insensitive to alterations in pH, suggesting that the presence or absence of a negative charge on the carboxyl group had little influence on the environment of the *N*-trifluoroacetyl moiety, these results again consistent with the work of Zeffren and Reavill (1968). Alterations in the environment of the *N*-trifluoroacetyl group on the amino acids and peptides were observed, however, when the α-carboxyl group was modified by the addition of various numbers of amino acids as shown in Figure 2. The ^{19}F resonance peaks from these peptides, as in the case of the amino acid derivatives, were also insensitive to alterations in pH. For all the model compounds, the line widths remained quite narrow (2–3 Hz), suggesting little restriction of motion.

Trifluoroacetyl-insulin Derivatives. The ^{19}F nuclear magnetic resonance spectra of the trifluoroacetyl-insulin derivatives suggested several important facts concerning the effects of pH on aggregation and the environments of the introduced ^{19}F probes. The chemical shifts of the ^{19}F resonance peaks from the trifluoroacetyl moiety on glycine in the various insulin derivatives at pH 2.0, 6.8, and 8.7 were essentially identical, suggesting similar environments at this position in the molecule under these conditions. The chemical shift is very close to that of glycine^{A-1}-trifluoroacetyl-*S*-sulfo-insulin A chain, indicating a high degree of exposure to the aqueous environment. The ^{19}F chemical shifts of the trifluoroacetyl moiety bound to phenylalanine and lysine in the insulin derivatives were, however, sensitive to changes in pH from 2.0 to 8.7, resulting in downfield shifts. This deshielding effect could be caused by alterations in the magnetic environment around the ^{19}F probe resulting from a pH-induced conformational change (Morris *et al.*, 1968), or titration of a specific group, independent or in conjunction with the state of insulin aggregation at pH 8.7.

Based on the results of Zeffren and Reavill (1968), these downfield shifts could be caused by increased exposure to the aqueous environment or, as mentioned above, exposure to a charged species in a relatively nonpolar environment. The chemical shifts at pH 6.8 for the trifluoroacetyl group bound to lysine and phenylalanine could not be determined because of the extreme width of the peaks. The trifluoroacetyl moiety on phenylalanine^{B-1} in the trifluoroacetyl-desoctapeptide-insulin derivative, however, was observable, indicating a shift to lower field, suggesting an alteration in its environment. Conformational alterations in desoctapeptide-insulin have been suggested by Mercola *et al.* (1967) using circular dichroism and by Carpenter (1966) using optical rotatory dispersion.

The ^{19}F-nmr spectra of the trifluoroacetyl-insulin derivatives also exhibited considerable variations in peak widths as a function of pH. An increased width can be interpreted in terms of a restriction of molecular motion of a specific group. All of the trifluoroacetyl-insulin derivatives had narrow resonance peaks at pH 2.0. Glycine^{A-1}-trifluoroacetyl-insulin exhibited a significant increase in peak width at pH 3.0 (Table I), suggesting that this group was now held more rigidly as a result of a conformational change, or that aggregation was beginning to restrict the motion of this group. As the pH was raised to pH 6.8, further broadening was observed to 12 ± 1 Hz. When phenylalanine^{B-1}-trifluoroacetyl-insulin was studied at pH 6.8, no observable resonance peak was obtained, suggesting that this group was held even more rigidly than the trifluoroacetyl moiety on glycine. As shown in Table II, the two bis(trifluoroacetyl) derivatives also afforded a broad peak of 12 ± 1 Hz, suggesting that the signal originated from the trifluoroacetyl

moiety on glycine, and that no signal was obtained from phenylalanine^{B-1}. The shape and intensity of the peak from glycine^{A-1}-lysine^{B-29}-trifluoroacetyl-insulin also suggested that the trifluoroacetyl group on lysine^{B-29} is also highly restricted and thus affords an extremely broad peak that cannot be observed above background. Because the ^{19}F-resonance peaks from the trifluoroacetyl groups on glycine and lysine occur closer together than the peak from trifluoroacetylphenylalanine, we cannot exclude the possibility that there may be some slight contribution to this broad peak at pH 6.8 from the trifluoroacetyllysine group. Sufficient lysine^{B-29}-trifluoroacetyl-insulin was not available to run a spectrum to absolutely establish the contribution by the trifluoroacetyllysine group. The tris(trifluoroacetyl)-insulin derivative also afforded a broad peak, although somewhat reduced in width (Table II, Figure 3d), suggesting that the aggregation of the tris derivative was somewhat reduced under these conditions. Recent studies have suggested that modifications of the three amino groups of insulin could alter the aggregation behavior of the molecule (Massey and Smyth, 1972; Arquilla *et al.*, 1969).

Several studies were performed to establish that the ^{19}F peak broadening reflected the aggregation state of insulin. As mentioned above, desoctapeptide-insulin has been shown not to undergo extensive aggregation at pH 7.4–7.5 (Arquilla *et al.*, 1969). The ^{19}F spectrum of the glycine^{A-1}-phenylalanine^{B-1}-trifluoroacetyl derivative of this compound at pH 6.8 supported this proposal, affording a well-resolved two-peak spectrum (Table II, Figure 3f). Several studies have suggested that insulin disaggregates at pH values above 8 (Fredericq, 1956; Slobin and Carpenter, 1966). Accordingly, glycine^{A-1}-phenylalanine^{B-1}-trifluoroacetyl-insulin and glycine^{A-1}-phenylalanine^{B-1}-lysine^{B-29}-trifluoroacetyl-insulin were studied at pH 8.7. As seen in Table II and Figure 3e, the peak widths have narrowed considerably, suggesting a disaggregation of the trifluoroacetyl-insulin derivatives.

In an effort to further establish the relationship between aggregation states and ^{19}F-nmr peak widths, sedimentation velocity studies were carried out on insulin and several of the trifluoroacetyl-insulin derivatives. At pH 2.0, glycine^{A-1}-trifluoroacetyl-insulin, phenylalanine^{B-1}-trifluoroacetyl-insulin, and glycine^{A-1}-phenylalanine^{B-1}-trifluoroacetyl-insulin had $s_{20,w}$ values quite close to zinc-free insulin. Under similar conditions insulin has been shown to exhibit a weight-average molecular weight of 12,000 daltons (Jeffrey and Coates, 1966a,b). The $s_{20,w}$ values obtained are slightly lower than values reported in the literature (Fredericq and Neurath, 1950; Cunningham *et al.*, 1955; Gutfreund, 1952), probably resulting from the low salt concentration (0.01 M NaCl) which was used in order to duplicate the condition of the ^{19}F-nmr experiments. At pH 6.8, the broad ^{19}F-nmr peaks for the trifluoroacetyl derivatives are paralleled by a substantial increase in the $s_{20,w}$ values, as shown in Table III. The slightly narrower peak width for the tris(trifluoroacetyl)-insulin derivative is again reflected in the $s_{20,w}$ value which suggests a lower molecular weight than that of native insulin or the other mono- and bis(trifluoroacetyl) derivatives. Finally, the suggestion that the trifluoroacetyl derivative of desoctapeptide-insulin does not undergo extensive aggregation at pH 6.8 is corroborated by the $s_{20,w}$ value of 1.4.

In summary, ^{19}F-nmr studies have shown that the trifluoroacetyl moiety bound to the three amino groups in insulin can serve as reporter groups at these locations. At pH 2.0 the trifluoroacetyl-insulin derivatives afford three narrow peaks with different chemical shifts reflecting differences in the three environments. From the analysis of the ^{19}F-nmr spectra of these derivatives at pH 6.8, it appears that significant aggregation occurs where the trifluoroacetyl moiety on glycine^{A-1} appears to be on the surface of the complex, resulting in a relatively high degree of motional freedom as suggested by the broadened but observable ^{19}F-resonance peak. The trifluoroacetyl moiety on phenylalanine^{B-1}, however, appears to be highly restricted in its motion at this pH, as suggested by the absence of any observable ^{19}F resonance peak, this restriction probably resulting from the ^{19}F probe being located within the insulin aggregate. Furthermore, when the aggregate is dissociated at pH 8.7 there appears to be a significant alteration in the environment around the trifluoroacetyl probes in lysine^{B-29} and phenylalanine^{B-1}, as suggested by a deshielding of the ^{19}F resonance peaks resulting from an alteration in conformation and/or charge. Although the quarternary structure of zinc-free insulin may be different from that of zinc insulin, the results obtained in these solution studies concerning the differential alterations in the environment about the trifluoroacetyl groups on glycine^{A-1} and phenylalanine^{B-1} upon insulin aggregation are consistent with the X-ray crystallographic data of Blundell *et al.* (1972) which indicate that phenylalanine^{B-1} is found in a hydrophobic pocket within the insulin hexamer, while glycine^{A-1} is found on the surface of the aggregate.

The comparison of structure of highly complex aggregating systems such as insulin in solution and in the crystalline state is of obvious importance. Further studies utilizing various spectroscopic methods should thus serve to resolve many important questions concerning conformation and aggregation in this system under a variety of conditions. Further studies are under way using ^{19}F-nmr techniques concerning the effects of metal ions, temperature, chaotropic agents, and detergents on protein folding and quaternary structure.

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Linear Electric Field Effect Measurements of Variant Low-Spin Forms of Ferric Cytochrome *c*†

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ABSTRACT: The linear electric field induced *g* shifts in electron paramagnetic resonance have been measured at 4.2°K for two low-spin forms of cytochrome *c*. At pH 6.6, with the magnetic and electric fields aligned approximately normal to the heme plane (C. Mailer and C. P. S. Taylor (1972), *Can. J. Biochem. 50,* 1040), the shift parameter $S = (1/E(\Delta g/g) = 0.95 \times 10^{-9}$ cm V^{-1}. At pH 10.0 the shift parameter for the same field orientations increases to 3.3×10^{-9} cm V^{-1}, verifying a ligand exchange. This result also indicates that the difference in crystal field strength of the axial ligands is considerably greater in the pH 10.0 form than in the pH 6.6 form of the protein.

The electron paramagnetic resonance (epr) spectrum of mammalian cytochrome *c*, which is a low spin ($s = \frac{1}{2}$) ferric heme protein, has been studied both in frozen solution (Salmeen and Palmer, 1968) and in single crystals (Mailer and Taylor, 1972). As in other low-spin ferric heme proteins the results can be described by a *g* tensor with three principal *g* values. At near neutral pH these values are 3.07, 2.23, and 1.21. At elevated pH the spectrum is, however, dominated by another low-spin heme species with *g* values 3.4, 2.07, and 1.5 (Blumberg *et al.*, 1973; Lambeth *et al.*, 1973).

It has been unequivocably demonstrated by X-ray analysis of single crystals (Dickerson *et al.*, 1971; Takano *et al.*, 1973) and in part verified by nuclear magnetic resonance (nmr) spectroscopy of protein solutions (Wüthrich, 1970; Redfield and Gupta, 1971; McDonald and Phillips, 1973) that the axial ligands in the neutral pH form of cytochrome *c* are methionine sulfur and histidine imidazole nitrogen, confirming the earlier suggestion of Harbury *et al.* (1965). No X-ray studies have been made on the high pH form, but since the *g* values lie close to the values obtained for carboxymethyl cytochrome *c* (Schejter and Aviram, 1970) where nmr evidence (Gupta and Koenig, 1971) indicates that an amine from an endogenous lysine nitrogen atom has been substituted for methionine sulfur, it is provisionally concluded that the ligands in the high pH form of cytochrome *c* are amine nitrogen and imidazole nitrogen. Comparison of the *g* values to those of similar heme compounds would tend to reinforce this conclusion (Seamonds *et al.*, 1972; Peisach *et al.*, 1973a). It cannot be definitely inferred, however, whether the imidazole ligands in each case are in the neutral form, or in an ionized form with a proton removed at the N-3 position (Peisach *et al.*, 1973a).

For both forms of ferric cytochrome *c* the iron site is noncentrosymmetric and we may, therefore, expect to observe a linear shift in *g* values when an electric field is applied to the sample.

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