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High-Resolution Proton Nuclear Magnetic Resonance Studies of the Glucocerebrosidase Activator Protein from Gaucher Spleen†

Leung Sheh‡ and Robert H. Glew*
Department of Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261
A. A. Bothner-By and P. K. Mishra
Department of Chemistry, Carnegie-Mellon University, Pittsburgh, Pennsylvania 15213
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ABSTRACT: A heat-stable protein factor (HSF) obtained from the spleen of a patient with Gaucher's disease that activates glucocerebrosidase was studied by 600-MHz proton NMR spectroscopy. Assignments for a number of aromatic and aliphatic resonances were made on the basis of spin-decoupling, pH-titration, and resolution-enhancement experiments. The upfield ring current shifted aliphatic region and the downfield aromatic region were examined by nuclear Overhauser effect (NOE) methods using both pulsed Fourier-transform spectroscopy and correlation spectroscopy. It was found that a number of upfield-shifted methyl groups and certain methylene groups of specific aliphatic amino acid residues are in proximity relationships with several aromatic residues, forming a compact hydrophobic clustering site. Of special interest, tyrosine A, phenylalanine A, tryptophan B_1, and tryptophan B_2 were found to be located close to a cluster of aliphatic residues, indicating that the hydrophobic site of the HSF is conformationally rigid and its tertiary structure very compact. A two-dimensional structural model of the hydrophobic site of HSF is proposed.

Gaucher's disease is an inherited lysosomal storage disease in which glucocerebroside accumulates in mononuclear phagocytic cells of the reticuloendothelial system because of a marked deficiency of glucocerebrosidase (glucocerebroside: β-glucosidase; EC 3.2.1.45) activity (Lee, 1968; Fredrickson & Sloan, 1972; Brady & Barranger, 1983). In recent years, much effort has been devoted to improving our understanding of the regulation of the enzyme and to explaining the molecular basis of the distinction between the clinical extremes of Gaucher's disease defined at one end of the spectrum by adults with the type 1 form of the disease who live into the seventh or eighth decade of life without neurologic involvement and at the other end by infants with the type 2 form of the disease who usually die by the age of 2, always with extensive primary central nervous system dysfunction (Fredrickson & Sloan, 1972; Brady & Barranger, 1983).

The search for an explanation of the genetic diversity of Gaucher's disease has focused in part on activators of glucocerebrosidase, namely, acidic phospholipids and a heat-stable factor from spleen first described by Ho & O'Brien (1971) and later purified to homogeneity by Peters et al. (1977). Solubilized preparations of glucocerebrosidase of normal human spleen and liver (Glew et al., 1982; Basu et al., 1984; Prence et al., 1985) and rat liver (Basu & Glew, 1984) are

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‡ Present address: Department of Chemistry, West Virginia University, Morgantown, WV 26505.

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activated markedly by acidic phospholipids (e.g., phosphatidylserine, gangliosides), especially after butanol extraction, and the heat-stable factor greatly decreases the amount of acidic lipid needed to stimulate full glucocerebrosidase activity (Basu et al., 1984; Basu & Glew, 1984). Interest in the heat-stable factor and acidic lipid activators was increased when Wenger & Roth (1982) and Glew et al. (1982) showed that patients with type 1 and type 2 Gaucher's disease could be distinguished on the basis of the responsiveness of the residual glucocerebrosidase to stimulation by phosphatidylserine and the heat-stable factor; the enzyme from type 1 cases was stimulated greatly by the activators, whereas the glucocerebrosidase from type 2 cases was generally unresponsive to these activators.

Structural studies of the heat-stable factor, which is an 11 000-dalton glycoprotein, have been hampered by its resistance to fragmentation into smaller pieces by proteases (e.g., trypsin, chymotrypsin, Pronase), cyanogen bromide (Prence et al., 1985), and mild acid hydrolysis at high temperature (100 °C). Furthermore, efforts by us and others to crystallize the factor have been unsuccessful, thereby precluding X-ray analysis.

Peters et al. (1977) have shown that HSF and human liver glucocerebrosidase both possess significant hydrophobic character. The high content of hydrophobic amino acids together with the hydrophilic carbohydrate portion of HSF could provide the factor with sufficient amphipathic character to interact with glucocerebrosidase and acidic lipids. In an effort to gain insight into the tertiary structure of HSF, particularly specific interactions between aliphatic and aromatic side chains to form a hypothetical hydrophobic pocket, we subjected the HSF to analysis by high-resolution ^{1}H nuclear magnetic resonance. In this work, spin-decoupling and pH-titration experiments were performed to assign the aromatic resonances, which are rather well resolved in the 600-MHz spectra. Nuclear Overhauser effect (NOE) studies were performed to examine the proximity relationships of aromatic and aliphatic residues, and Fourier-transform (FT) and correlation spectra (Dadok & Sprecher, 1974) were recorded.

Experimental Procedures

HSF was purified as described elsewhere (Peters et al., 1977). The protein was dissolved in D_2O and lyophilized 3 times; the final protein concentration was 0.5–1.0 mM in D_2O.

^{1}H NMR spectra were recorded at 600.6 MHz on the spectrometer at the Carnegie-Mellon University NMR Facility for Biomedical Studies. For spectra recorded in the FT mode, the deuterium signal of the solvent was used as the field-frequency lock. Chemical shifts are quoted in parts per million (ppm) relative to internal sodium 4,4-dimethyl-4-silapentanesulfonate (DSS). NOE difference spectra were obtained by subtracting the free induction decay (FID) of reference spectra from the FID of the irradiated spectra with an irradiation period of 0.5 s, at a decoupler attenuation of 5 dB and a 90° pulse length of 12 or 15 μs. The number of scans ranged from 4800 to 18 000. Correlation spectra were recorded in the linear-frequency sweep mode with an internal homonuclear lock on HDO. The sweep was under program control by a Xerox Data Systems (XDS) Sigma 5 computer, and the data were collected by a single Princeton Applied Research Model 121 synchronous detector with an XDS Model MD 51 analog to digital converter. A total of 2K sample points was collected on each sweep. The data were analyzed by a fast Fourier-transform program developed by Singleton (1969). The sweep time was 1.39 s, and the number of scans was typically 13 410, with resolution-enhancement factors of 1.50 and 2.00. pH-titration studies were monitored on an Orion Research Model 601 digital ionalyzer.

Results

Figure 1 shows the upfield and downfield portions of the 600.6-MHz ^{1}H NMR spectra of HSF at pH 6.4 in D_2O. The downfield region is rather well resolved. The region between δ 7.60–9.10 consists mainly of signals from deuterium-exchangeable labile protons (N–H), which disappear on warming the protein solution in D_2O at 100 °C (300-MHz ^{1}H NMR spectrum). The remaining nonexchangeable peaks must be assigned to the one histidine, one phenylalanine, two tyrosines, and two tryptophan residues contained in HSF. Two singlets at δ 8.60 (P_2) and δ 7.28 (P_1) shifted upfield with slight increase in pH and are assigned to be the C2-H and C4-H of the single histidine residue, respectively. Spin-decoupling experiments showed that the doublet at δ 7.23 (M_1) is coupled to the doublet at δ 6.90 (K_1) and the doublet at δ 7.10 (M_2) is coupled to that at δ 6.92 (K_2) (see Figure 2). At pH 10.8, K_2 shifts upfield to δ 6.78, and at pH 11.8, K_1 shifts upfield to δ 6.80 (Figure 3). Thus, K_1 and M_1 are assigned to the ring protons C3-H, C5-H and C2-H, C6-H of Tyr A, respectively. Similarly, K_2 and M_2 are assigned to the ring protons C3-H, C5-H and C2-H, C6-H of Tyr B, respectively. The pH titration curves of Tyr A and Tyr B are shown in Figure 4. It is apparent that both residues have unusually high pK_a values (see Discussion) and that the slope of both curves (K_1 and K_2) do not decrease up to pH 11.8 (pD 12.2). Thus, we estimate the pK_a of Tyr B to be 11.4 or higher and separated by approximately 1 pH unit from that of Tyr A (pK_a $\geq$ 12.0). The top curve in Figure 4 (C2-H, C6-H of Tyr A) begins to deflect at pH 9.5, indicating that Tyr A is probably located close to some residues that titrate in the region of pH 9.0 (e.g., lysine residues). The broad single peak at δ 7.04 (L), which does not shift with increase in pH and is not spin-coupled to any other resonances, was assigned to the ring protons of the single phenylalanine or the ring protons of Trp B_1 and Trp B_2. The broad peaks at δ 7.47 (Q) do not shift despite large increase in pH and were assigned to the C4-H and C7-H of two tryptophan residues (Trp B_1 and Trp B_2) or the ring protons of the single phenylalanine. The other aromatic protons of the three tryptophan residues emerge as multiplets resonating between δ 7.25 and 7.40. Spin-decoupling studies (see Figure 2) showed that the resonance at δ 7.16 (N_1) is coupled to that at δ 7.37 (N_2), neither of which shifted with increase in pH; these resonances were assigned to the ring protons C5-H, C6-H and C4-H, C7-H of a tryptophan residue (Trp A), respectively.

The upfield region is relatively less well resolved. However, six distinct ring current shifted resonances belonging to the methyl groups of the side chains of certain aliphatic residues are apparent. On the basis of the amino acid analysis of HSF (Prence et al., 1985), there are 22 aliphatic residues. It is estimated that A_1, A_2, A_3, A_4, A_4', A_5, and A_6 represent approximately 14 methyl groups (8–12 residues). Resolution enhancement shows that the resonances A_2 and A_3 are triplets, whereas A_4, A_5, and A_7 are doublets (Figure 1A). On this basis, A_2 and A_3 are assigned to isoleucine residues because, of the common amino acids, only isoleucine has a methyl group (δ-methyl) coupled to two vicinal protons. There are four isoleucine residudes in HSF (Prence et al., 1985). The other ring current shifted methyl groups may be attributed to valine, leucine, threonine, or isoleucine. The fact that these six methyl groups are shifted upfield within a range of δ 0.03–0.62 strongly suggests that they are in proximity with certain aromatic amino acid residues. Therefore, these well-isolated methyl groups become good candidates for NOE studies, which

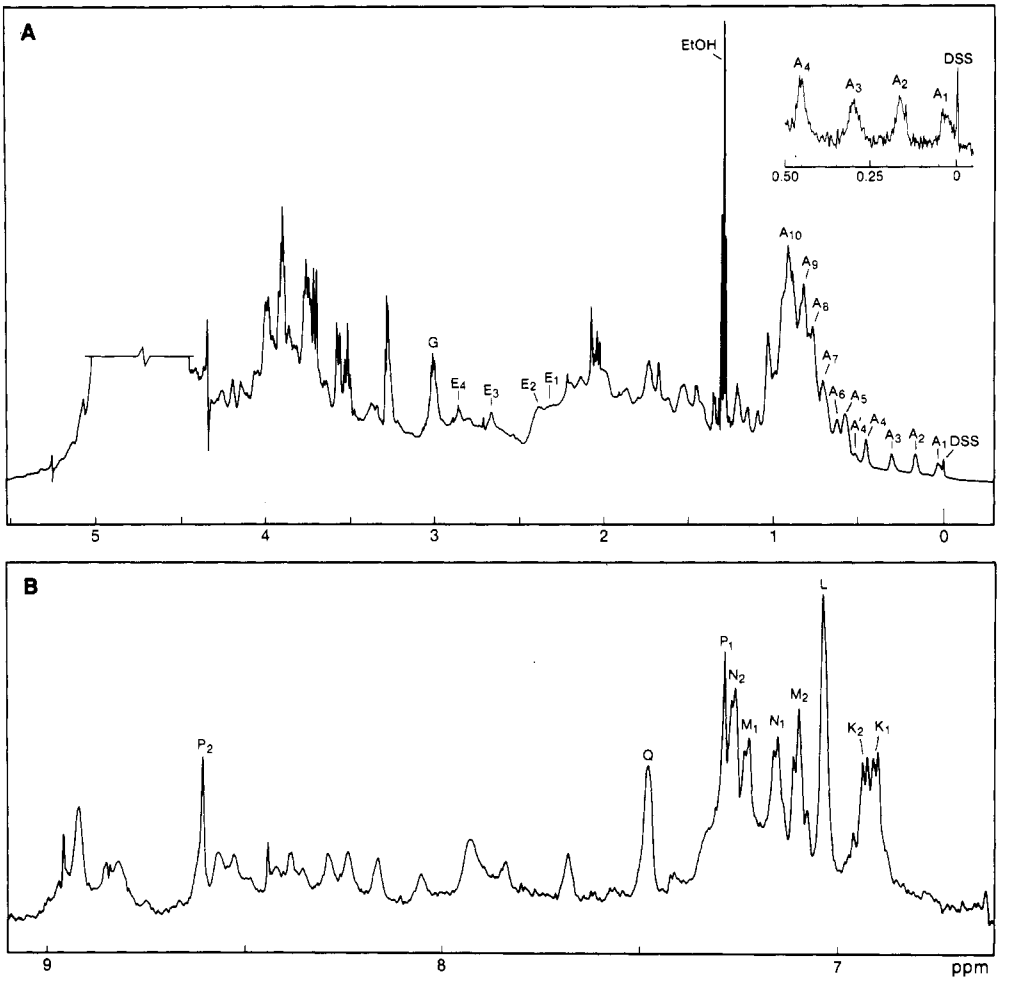

FIGURE 1: (A) ^{1}H NMR spectrum at 600 MHz (FT) of a 0.5 mM solution of HSF in D_2O at pH 6.4. (B) Downfield spectral region of HSF under the same conditions.

may provide useful structural information regarding the hydrophobic pocket of HSF.

The sharp resonances observed between δ 3.30 and 4.00 are considered to be due to the methylene and methine signals of the sugar residues, which are exposed on the protein surface. Upon a decrease in the pH, the multiplets E_1, E_2, E_3, and E_4 shifted significantly downfield (Figure 5). These multiplets were assigned to the γ-methylene resonances of a number of glutamic acid residues known to be present in the HSF. Variations in the structure of the multiplets E_1 and E_2 were observed if the protein was washed with organic solvents [e.g., acetone or 2:1 (v/v) chloroform–methanol] prior to lyophilization. After organic solvent extraction, the multiplets E_1 and E_2 separated into a number of multiplets. This may indicate a degree of distortion of the natural conformation (in D_2O) of some glutamic acid residues by organic solvents with retention of the full glucocerebrosidase-stimulating activity of HSF.

The multiplet G at δ 3.0, which begins to shift upfield at pH 10.0, has been assigned to the ϵ-methylene protons of five lysine residues (Figure 6). We observed that over a wide range of pH below 10.9 the five lysine ϵ-methylene signals emerged as a rather narrow multiplet (base width 0.12 ppm). However, at pH 10.9, a number of resonances from this multiplet shifted upfield such that G becomes a broad multiplet, which overlaps with resonances at E_3 and E_4. Since the five lysine ϵ-methylene resonance are more or less equivalent over a wide range of pH, we suggest that they represent solvent-accessible residues located on the protein surface. At pH 10.9, it is evident that there is significant nonequivalence of these five lysyl residues. This nonequivalence may be due to the interaction of three of these lysyl residues with certain aromatic residues at this alkaline pH. Similar interactions have been reported by Brown et al. (1976), in basic pancreatic trypsin inhibitor.

The NOEs observed in the aromatic resonances by preirradiating the ring current shifted methyl groups are primarily negative NOEs since for a glycoprotein of this size (11 000 daltons) $\omega\tau_c$ is greater than 1 (Balaram et al., 1972; Gordon & Wuthrich, 1978). The validity of the NOEs is confirmed by various difference spectra (i.e., subtraction of the FID of the spectrum while irradiating A_1 from that while irradiating A_2; subtraction of the FID of the spectrum by irradiating A_2 from that while irradiating A_3; subtraction of the FID of the spectrum while irradiating A_4 from that by irradiating A_3, etc.).

Despite repeated attempts, preirradiation of the most upfield shifted resonance A_1 does not give any detectable NOE effects on the aromatic resonances. The reason for this is unclear. Table I shows the NOEs observed when correlation spectroscopy was employed. It is noteworthy that on preirradiation of A_2 (the δ-methyl of isoleucine A) large NOEs were observed on the aromatic resonances C5-H, C6-H and C4-H, C7-H of tryptophan A. On preirradiation of A_3 (the δ-methyl of isoleucine B), an NOE was observed on K_1 (C3-H and C5-H of Tyr A), and M_1 (C2-H and C6-H of Tyr A). On preirradiation of A_4, a strong NOE was observed on K_1 and M_1, and a weak NOE was observed on K_2. It is striking that almost

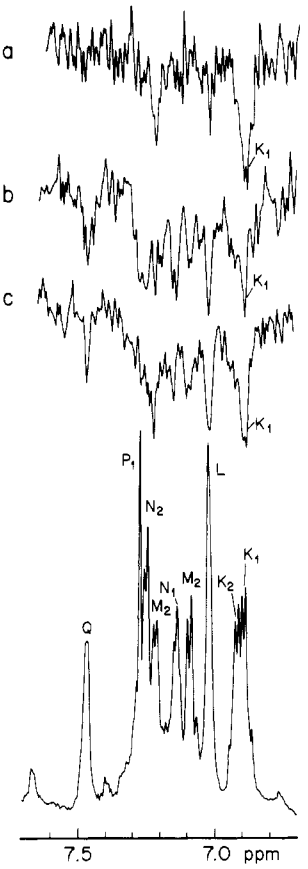

FIGURE 2: Some examples of NOE difference spectra given by HSF in D_2O, from correlation spectroscopy at 600.6 MHz (protein concentration 0.6 mM). Each NOE difference spectrum represents the difference between an accumulation of 400 FIDs of the irradiated spectrum and another accumulation of 400 FIDs of the reference spectrum, with an irradiation period of 0.5 s. The spectra result from irradiation of resonance A_4 (a), resonance A_5 (b), and resonance A_7 (c). Negative NOEs are observed (see text). The selectivity of NOEs for resonance K_1 (C3-H and C5-H of Tyr A) is clearly seen. The bottom spectrum represents the unirradiated downfield aromatic region.

Table I: Nuclear Overhauser Effects (NOE) Observed for HSF in D_2O at 22 °C Using Correlation Spectroscopy[a]

preirradiated signals	effects observed
A_1	N_1
A_2	L (w), N_1, N_2, Q (w)
A_3	K_1, K_2 (w), M_1
A_4	K_1 (s), K_2 (w), M_1
A_4'	K_1, K_2 (w), M_1 (w), N_2 (w)
A_5	K_1, L, M_2 (w), N_1, N_2, Q
A_6	K_1, L, M_2 (w), N_1 (w), M_1 (w), N_2 (w)
A_7	K_1 (s), L (s), M_2 (w), N_1 (w), M_1 (w), N_2 (w), Q
A_8	K_1 (s), L (s), M_2 (w), N_1 (w), M_1, N_2 (w), Q (w)
A_9	K_1, K_2, L (s), M_2 (w), M_1 (w), M_1 (w), N_2 (w), Q
A_{10}	K_1, L (s), N_1, M_1 (w), N_2, Q (s)

[a] The magnitudes of the NOE signals are relative to one another for each difference spectrum. s, strong effect; w, weak effect; unstated, medium effect.

all of the ring current shifted methyl resonances A_1–A_7 show a significant NOE with K_1 (C3-H and C5-H of Tyr A). This indicates that Tyr A, which has a higher pK_a value, is in proximity to the side chains of a number of aliphatic amino acid residues. In other words, the phenolic ring of Tyr A, appears to be located in the core of a group of aliphatic amino acid residues (see Discussion). Preirradiated signals A_8–A_{10} also appeared to have significant NOE relationships with K_1

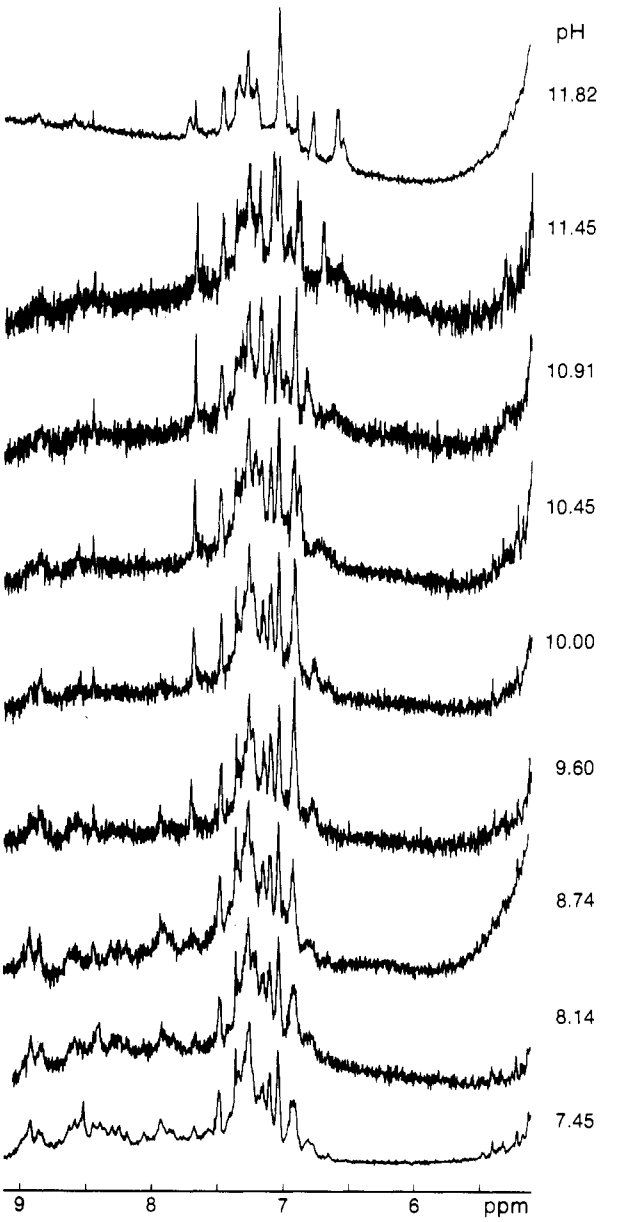

FIGURE 3: ^1H NMR spectra at 600 MHz (FT) of the aromatic region of HSF in D_2O as a function of pH.

and a number of aromatic resonances. These signals overlapped considerably and represent the majority of hydrophobic groups of HSF.

Discussion

Our investigation of human Gaucher's heat-stable protein factor (HSF) by ^1H NMR through tentative assignments, pH titration, and NOE studies has provided some insight into the protein's structure. Nevertheless, at present the scope and depth of structural and biochemical studies of HSF have been restricted by the lack of amino acid sequence data and X-ray structural studies of HSF. These limitations have made it difficult to establish a detailed structural model for HSF, especially one that would indicate the orientation of the side chains in the folded, three-dimensional (tertiary) structure.

The significant pH dependency of the signals of the two tyrosyl residues, one histidyl residue, five lysine residues, and a number of glutamic acid residues indicates that these residues are solvent-accessible and some of them may be located on the protein's surface. The unusually high pK_a's of Tyr A and Tyr B (Figure 4) may indicate that their solvent accessibility

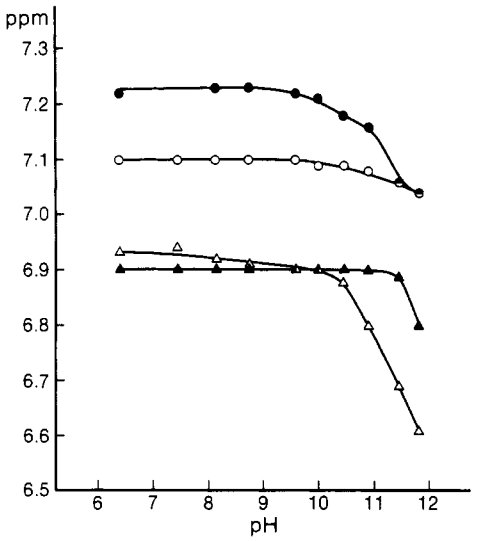

FIGURE 4: Chemical shift as a function of pH meter reading for the aromatic resonances of Tyr A and Tyr B: resonances (●) (○), C2-H and C6-H of Tyr A and Tyr B, respectively; resonances (▲) (△), C3-H and C5-H of Tyr A and Tyr B, respectively.

is limited by the compact hydrophobic clustering of aliphatic residues. Moreover, Tyr A, which has a higher pK_a, is apparently less solvent-accessible than Tyr B. Interesting protein dynamics are observed for three lysyl residues, which may interact with specific aromatic residues at pH values above 10.8.

The most useful structural information about the hydrophobic site of HSF came from the NOE studies. Isoleucine A is close to two tryptophan residues, and isoleucine B lies close to tyrosine A. The NOE data also reveal that Tyr A is in close proximity to the side chains of a number of aliphatic amino acid residues, indicating that this particular aromatic residue (Tyr A) is oriented inside the core of a compact clustering of aliphatic amino acid residues, particularly with A_4, A_7, and A_8. The single phenylalanine residue, or tryptophan B_1 and tryptophan B_2, of HSF appears to be located near a number of aliphatic residues, A_5–A_{10}. These results lead us to propose that the hydrophobic site of HSF is conformationally rigid and its tertiary structure relatively compact. We envision that HSF is composed of three binding sites: a cationic site that is composed of basic amino acid residues such as lysine, histidine, and arginine; an anionic center that is composed of glutamic acid and aspartic acid residues and certain sugar residues; and a hydrophobic pocket.

A rotational correlation time of 27 ns and a spectrometer frequency of 600 MHz combine to produce a value of $\omega\tau_c$ of 101. This is far from the extreme narrowing limit and well into the high-field limit. It is expected therefore that the nuclear Overhauser effect will be close to −1.00 and that the phenomena associated with spin–diffusion in multinuclear systems will be observed. For this reason we do not attempt to draw quantitative conclusions about internuclear distances from the measured NOEs and confine ourselves to qualitative comparisons. Both experimental measurements and theoretical simulations indicate that while one may not draw exact conclusions about distances from such data, it is generally true that direct effects of near neighbors are larger than either direct or indirect effects of more remote protons (Poulsen et al., 1980; Bothner-By & Spevacek, 1982). Thus, a qualitative map of the region may be drawn with good probability.

On the basis of the information yielded by the NOE studies (Table I), we proposed a two-dimensional model of the structure of the hydrophobic site of HSF (Figure 7), in which

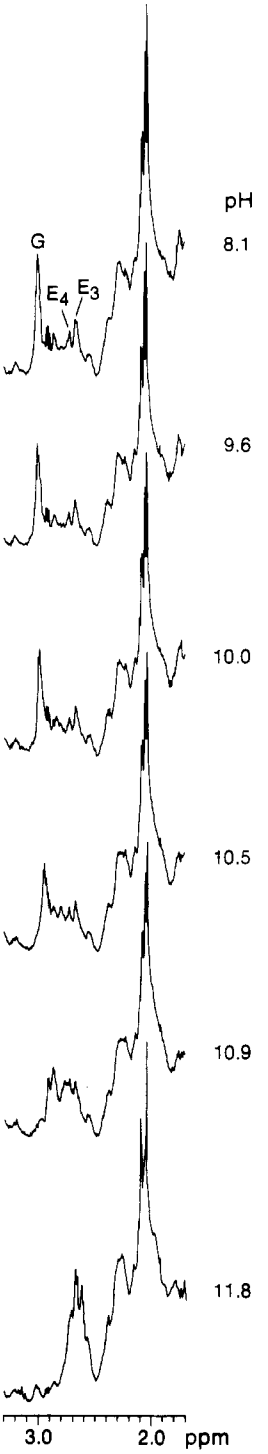

FIGURE 5: Spectral region from 2.0 to 3.3 ppm of ^{1}H NMR spectra at 600 MHz (FT) of HSF in D_2O at the different pH meter readings indicated. G is assigned to ϵ-methylene protons of three lysine residues.

Tyr A is embedded in a helical core of aliphatic residues, represented by the side chains A_3, A_4, A_4', A_6, A_7, A_8, A_9, and A_{10} (where A_3 has been assigned to the δ-methyl group of isoleucine B). The relative locations of the amino acid residues are tentatively determined, on the basis of NOE data.

One interesting question arose concerning whether the appearance of fine aromatic multiplets may reflect certain dynamics of the protein since the rotational movements of tyrosine residues in a compact hydrophobic pocket would be restricted. The line width of signals from individual protons, such as the aromatic protons of the tyrosines, is about 5 Hz. Since resolution is less than 0.5 Hz with the spectrometer, it

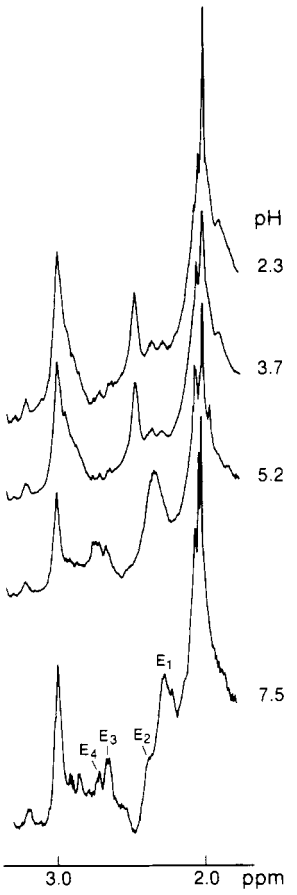

FIGURE 6: Spectral region from 2.0 to 3.3 ppm of ^1H NMR spectra at 600 MHz (FT) of HSF in D_2O at the different pH meter readings indicated. E_1, E_2, E_3, and E_4 are assigned to γ-methylene resonances of a number of glutamic acid residues.

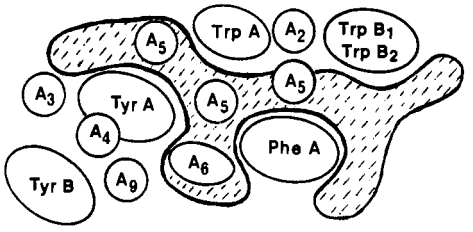

FIGURE 7: Two-dimensional hypothetical structure of the hydrophobic site of HSF. Circled areas represent possible loci of various aromatic and aliphatic residues. Shaded areas represent possible loci of a compact clustering of aliphatic residues A_5, A_6, A_7, A_8, A_9, and A_{10}, all of which contain more than one aliphatic residue.

follows that the natural line width is close to 5 Hz. If the transverse relaxation is entirely due to magnetic dipolar interaction with the ortho proton at a distance of 2.5 Å, the standard formula for T_2 (Abragam, 1961) yields a rotational correlation time of 26 ns for the internuclear axis. Contributions to the relaxation from other nearby nuclei would shorten this calculated time. However, 20–30 ns is the time deduced and expected for other globular proteins of this molecular weight in water solution at room temperature (Oldfield et al., 1975). Thus, it appears likely that the tyrosines are rigidly fixed within the molecular framework and that the rotational motion of the protein as a whole controls the relaxation and line width of the proton signals.

In several cases where tyrosine rings buried deeply in hydrophobic pockets have been studied by high-resolution proton NMR methods, separate signals have been observed for all four protons attached directly to the tyrosine ring (De Marco et al., 1977, 1985). This occurs when the tyrosine ring is sufficiently immobilized, such that the frequency of ring flipping (rotation about the Φ–C_γ bond by 180°) is small compared to the chemical shifts involved. No evidence of such immobilization is observed in the present case, and broadening of the average signals cannot be more than 1–2 Hz. If one assumes a maximum chemical shift of 1 ppm between the signals from protons on opposite sides of the ring, the flipping rate required is 1.4×10^5 s^{-1}. This rate still represents a considerable restriction of motion and is not inconsistent with the proposed model that places a tyrosine ring in a hydrophobic pocket.

The heat-stable glucocerebrosidase activator from Gaucher spleen appears to be similar to pancreatic colipase. Colipase, a heat-stable protein comprised of approximately 100 amino acid residues, activates pancreatic lipase by anchoring the lipase to its hydrophobic substrate, triglyceride (Borgstrom & Erlanson-Albertsson, 1984). There is a highly conserved hydrophobic region in colipase, consisting of the sequence Leu-Tyr-Gly-Val-Tyr-Tyr, which appears to function as the site involved in the binding of bile salt activators of lipase. The hydrophobic pocket in the glucocerebrosidase activator protein we postulate in this paper (Figure 7) resembles that of colipase.

The mechanism by which HSF operates to activate glucocerebrosidase is obscure. In our experience, the principal effect of the factor is to reduce markedly the concentration of phosphatidylserine or ganglioside (e.g., G_{M1}) required for maximum stimulation of glucocerebrosidase (Basu et al., 1984; Basu & Glew, 1985). In a recent study of the activation of rat liver glucocerebrosidase by G_{M1} and HSF, we found that the effects of the HSF are most apparent as ganglioside concentrations where monomers or small micelles predominate; at higher ganglioside concentrations where the lipid is in the form of large micelles, the HSF has little effect on glucocerebrosidase activity (Basu & Glew, 1985). It is tempting to speculate that in the case of gangliosides the HSF acts by facilitating transfer of ganglioside monomers to some specific binding site on the enzyme and that the first step in this mechanism involves the binding of ganglioside to the hydrophobic pocket on the surface of the HSF. Furthermore, the positive charges near the hydrophobic site on the HSF provided by the cluster of lysine residues could stabilize the HSF–lipid interaction by forming ionic bonds with the negatively charged sialic acid residue of the ganglioside (or corresponding negatively charged centers in the acidic phospholipids, which also activate glucocerebrosidase). The negatively charged glutamic acid and aspartic acid residues near the hydrophobic site on HSF may be involved in binding the latter to glucocerebrosidase.

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Methyl-Esterified Proteins in a Mammalian Cell Line†

D. Chelsky,‡ B. Ruskin, and D. E. Koshland, Jr.*
Department of Biochemistry, University of California, Berkeley, California 94720
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ABSTRACT: Methyl esterification of carboxylic acid residues in intact mouse S49 lymphoma cells was examined, and at least 24 proteins were found to be modified. Cell fractionation revealed that a distinct set of these proteins could be found in each of the four fractions. Nuclei contained 11 methyl-esterified proteins at 12, 15.5, 18, 19, 39, 41, 45, 70, 90, 105, and 130 kilodaltons (kDa). Five proteins copurified with the plasma membrane/mitochondrial fraction at 13, 24, 25, 27, and 28 kDa. Two proteins at 32 and 56 kDa were in the microsomal fraction, and six were soluble at 16.5, 21, 24, 26, 34, and 36 kDa. Eleven of these proteins were [^{3}H]methyl esterified when cell homogenates were incubated with *S*-adenosyl-L-[*methyl*-^{3}H]methionine. The steady-state level of methyl group incorporation into protein in intact cells was approximately 118 pmol/mg of protein. Assuming the average protein is 40 kDa, there appears to be 1 methyl group per 210 proteins. This was compared to phosphorylation which gave approximately one phosphoryl group for every four proteins. Exogenously added L-[*methyl*-^{3}H]methionine equilibrated with the cellular *S*-adenosylmethionine pool within 30 min which was sufficiently rapid to allow the rate of methyl group turnover to be determined. Most methyl-esterified proteins demethylated in a pulse–chase experiment with half-lives ranging from 2.6 to 9.3 h. When protein synthesis was blocked with puromycin, amino acid backbone incorporation of methionine was reduced to 2% of control. Methyl group incorporation, however, was 39% of the control. This level of methyl group incorporation could be attained whether label was added 15 or 105 min after the addition of puromycin, indicating that these proteins were being continuously methylated and demethylated. The reversible nature of this modification suggests that it may serve a regulatory function in these cells.

The reversible posttranslational methylation of protein carboxyl groups may be a mechanism for regulation of protein function in mammalian cells (Paik & Kim, 1980a,b; Dilberto, 1982). Methyl esterification activity has been isolated from a wide variety of mammalian tissues. Several proteins have been suggested as possible substrates for this enzyme, but an in vivo role for the modification has not yet been determined (Kim & Li, 1979; Kloog et al., 1980; Freitag & Clarke, 1981; Gagnon et al., 1981; Kloog & Saavedra, 1983; Billingsley et al., 1984). In bacteria, on the other hand, the methyl ester-

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‡ Recipient of a U.S. Public Health Service Individual National Research Service Award. Present address: Central Research and Development Department, E. I. du Pont de Nemours and Co., Experimental Station, Wilmington, DE 19898.

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