Dynamics of a Monomeric Insulin Analogue: Testing the Molten-Globule Hypothesis†

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ABSTRACT: The structure of insulin exhibits local and nonlocal differences among crystal forms and so provides an important model for analysis of protein dynamics. A novel combination of order and disorder has recently been inferred from 2D-NMR studies of the monomeric analogue des-pentapeptide(B26–B30) insulin (DPI) under acidic conditions [the molten-globule hypothesis; Hua, Q. X., Kochoyan, M., & Weiss, M. A. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2379–2383]. Distance-geometry structures are similar in general to crystal structures but differ by rigid-body displacements of α-helices; the hydrophobic core is not well ordered due to insufficient long-range restraints. To test whether such informational uncertainty may represent physical disorder, we have performed complementary studies of the thermal unfolding of DPI and its interaction with 1-anilino-8-naphthalenesulfonate (ANS). Experimental design is based on a predicted analogy between DPI and A-state models of protein-folding intermediates (the "molten globule"). Unfolding is monitored by five distinct biophysical probes: photochemical dynamic nuclear polarization (photo-CIDNP), differential scanning calorimetry (DSC), circular dichroism (CD), ^{1}H-NMR chemical shifts, and slowly exchanging amide ^{1}H-NMR resonances in D_2O solution. The results provide evidence that DPI adopts a compact partially folded state. Because the 2D-NMR spectrum of an engineered insulin monomer under physiological conditions is similar to that of DPI under acidic conditions [Weiss, M. A., Hua, Q. X., Frank, B. H., Lynch, C., & Shoelson, S. E. (1991) *Biochemistry 30*, 7373–7389], we propose that the functional form of insulin is a molten globule.

The relationship between the multiple crystallographic conformations of insulin and its solution structure and function poses a fundamental problem in protein chemistry (Adams et al., 1969; Peking Insulin Structure Group, 1971; Chothia et al., 1983; Baker et al., 1988; Derewenda et al., 1989; Mirmira & Tager, 1989; Badger et al., 1991). Interest in this problem has recently been stimulated by the determination of the X-ray structure (Derewenda et al., 1991) of a completely inactive insulin analogue (mini-proinsulin; Markussen, 1985). Unexpectedly, the structure of mini-proinsulin was observed to be isomorphic to that of native insulin (as a 4-Zn hexamer; Dodson et al., 1979), suggesting that receptor binding requires a change in insulin's conformation. To obtain a complementary description of insulin's structure and dynamics in solution, we and others have undertaken 2D-NMR[1] studies of insulin and insulin analogues (Weiss et al., 1989, 1990, 1991; Kline & Justice, 1990; Roy et al., 1990; Kristensen et al., 1991; Jorgensen et al., 1992). A monomeric insulin analogue, des-pentapeptide(B26–B30) insulin (DPI; Gattner, 1975), has been of particular interest because of its favorable spectroscopic properties. To avoid protein aggregation, 2D-NMR studies of DPI have been conducted under strongly acidic conditions (pH 1.8; Boelens et al., 1990) and in the presence of an organic cosolvent (20% acetic acid; Hua & Weiss, 1990). The present study addresses the question of whether DPI exists as a compact partially folded state under such conditions.

In either solvent the solution structure of DPI, as obtained from distance-geometry and restrained molecular dynamics (DG/RMD) (Hua et al., 1992), retains overall features of the crystal structure (Bi et al., 1984; Dai et al., 1986). Remarkably, however, the solution structure appears to be underdetermined: individual members of the DG/RMD ensemble exhibit rigid-body displacement of α-helices (panels A and B of Figure 1). The range of solution structures is similar to that observed among independent crystallographic protomers of native insulin (Figure 1C; Chothia et al., 1983). To what extent does the DG/RMD ensemble reflect physical motions in the protein? Analysis of simulated NOESY spectra back-calculated from crystal and DG models suggests that the experimental NOESY spectrum is intrinsically underdetermined (Hua et al., 1992). These calculations motivated the hypothesis that DPI provides a novel model of a compact partially folded state (Kawajima et al., 1976; Brazhnikov et al., 1985; Dolgikh et al., 1981, 1985; Pfeil et al., 1986; Ptitsyn, 1987; Baum et al., 1989) with possible implications for the mechanism of insulin action (Baker et al., 1988; Mirmira & Tager, 1989; Derewenda et al., 1991).

In this paper we test predictions of a molten-globule model with respect to binding of 1-anilino-8-naphthalenesulfonate (ANS; Stryer, 1965) and thermal unfolding. Design of these studies is based on previously described properties of equi-

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[1] Abbreviations: ANS, 1-anilino-8-naphthalenesulfonate; BPTI, bovine pancreatic trypsin inhibitor; CD, circular dichroism; DG, distance geometry; DPI, des-pentapeptide(B26–B30) insulin; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser enhancement; photo-CIDNP, photochemical dynamic nuclear polarization; RMD, restrained molecular dynamics; SA, simulated annealing.

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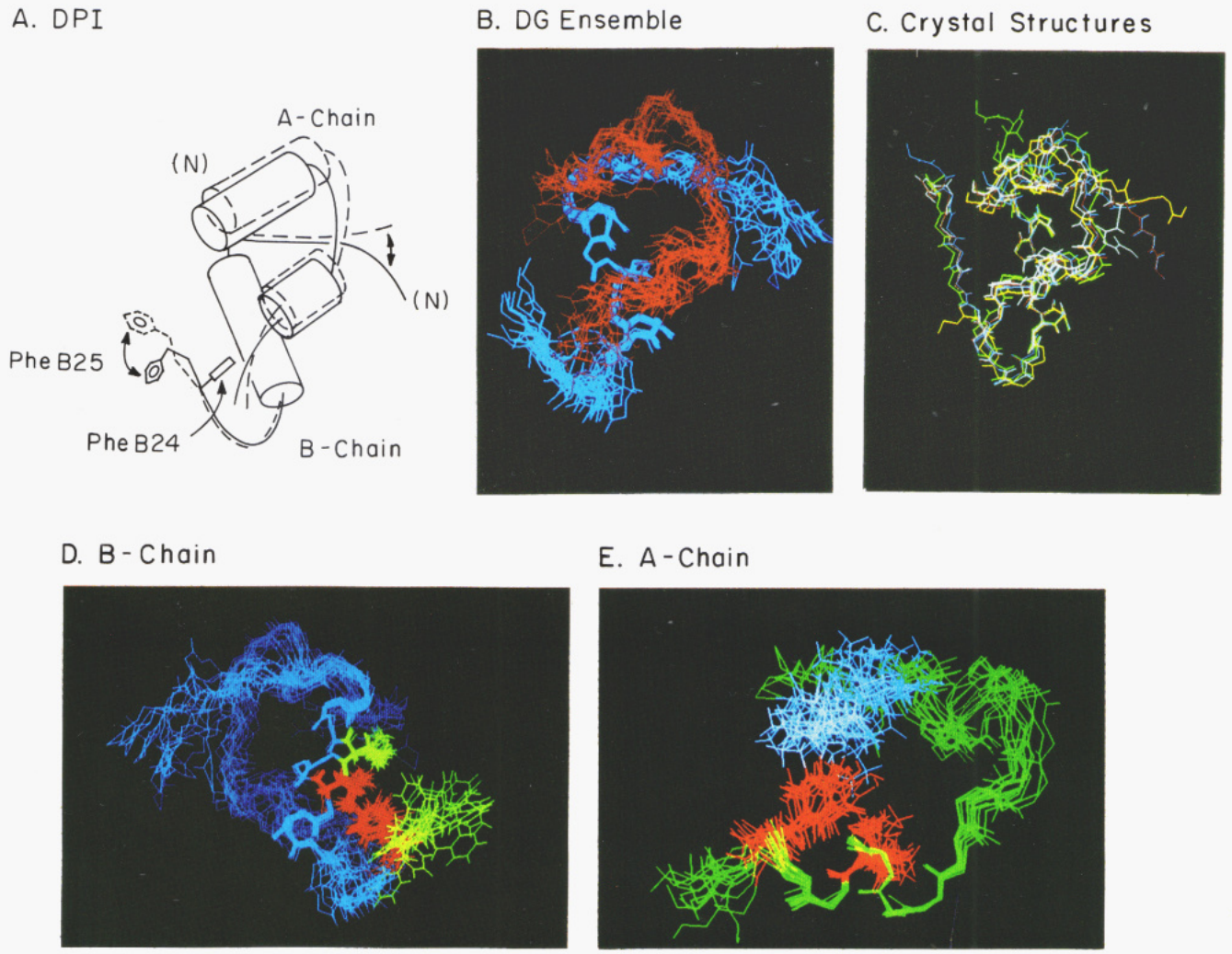

FIGURE 1: (A) Cylinder model of the DPI crystal structure (solid line) and the representative DG structure (dashed line). The A- and B-chains and their N-termini are labeled. PheB24 and PheB25 are also shown. (B) Ensemble of DG structures of DPI in 20% acetic acid (Hua et al., 1992). The A-chain is shown in red and the B-chain in blue. The structures are aligned according to the main-chain atoms of the B-chain α-helix (B9–B19). (C) Set of different crystal forms: 2-Zn molecule 1 (red; Adams et al., 1969; Baker et al., 1988), 2-Zn molecule 2 (blue), cubic (white; Badger et al., 1991), 4-Zn molecule 2 (green; Dodson et al., 1979), and DPI (yellow; Dai et al., 1987). 4-Zn molecule 2 exhibits an α-helical transition for residues B1–B9 as in the phenol-related hexamer (Derewenda et al., 1989; not shown). The crystal structures are aligned as in panel B. (D) B-Chain microdomain with LeuB15 and PheB24 shown in red and ValB12 and PheB25 in green. The B-chain main chain is otherwise shown in blue. The B-chains are aligned as in panel B. (E) A-Chain microdomain with TyrA19 and LeuA16 shown in red and IleA2 and ValA3 in light blue. The A-chain backbone is otherwise shown in green. The A-chains are aligned according to the main-chain atoms of residues A14–A18.

librium models of protein-folding intermediates (Dolgikh et al., 1981, 1985; Pfeil et al., 1986). Evidence that DPI adopts an analogous partially folded state is provided by six complementary probes of protein structure and dynamics: fluorescence studies of ANS binding (Semisotnov et al., 1987; Goto & Fink, 1989), photochemical dynamic nuclear polarization (photo-CIDNP; Kaptein, 1980), differential scanning calorimetry (DSC), circular dichroism (CD), ^{1}H-NMR chemical shifts, and slowly exchanging amide resonances in D_2O solution. Use of multiple biophysical probes strengthens the evidence in favor of the molten-globule hypothesis even though each probe, by itself, does not allow unquestionable interpretation. Bovine pancreatic trypsin inhibitor (BPTI), a globular protein that is similar to insulin in size and in number of disulfide bonds, is used as a control.

MATERIALS AND METHODS

DPI was provided by Eli Lilly & Co. (Indianapolis, IN). Protein concentration was measured by absorbance at 280 nm. 1-Anilino-8-naphthalenesulfonate was purchased from Molecular Probes, Inc. (Eugene, OR). Fluorescence spectra were obtained using a Shimadzu spectrofluorophotometer (Model RF5000U); a 1-cm path-length quartz cuvette was used. CD spectra were obtained using an Aviv spectropolarimeter equipped with a temperature-control unit; a 1-mm path-length quartz cuvette was used. ^{1}H-NMR spectra were obtained at 500 MHz at the Harvard Medical School NMR Facility and MIT Francis Bitter National Magnet Laboratory. Photochemical dynamic nuclear polarization (photo-CIDNP) studies were performed using riboflavin (4 mM) as triplet-state probe; the light source was an Innova-6 continuous argon laser at 488 nm. Light-minus-dark difference spectra were calculated as described (Weiss et al., 1989). ^{1}H-NMR chemical shifts were referenced to the methyl resonance of acetic acid (assumed to be 2.06 ppm). In spectra obtained in H_2O or 20% deuterated acetic acid–80% H_2O the solvent resonance was attenuated by presaturation.

Differential scanning calorimetry (DSC) experiments were performed in a DASM-4 microcalorimeter (Biopribor, Puschino, Russia) under an excess pressure of 2 bar and with a heating rate of 1 K min^{-1}. The proteins were dissolved in H_2O–HCl (pH 1.8) or in 20% CD_3COOD and dialyzed against

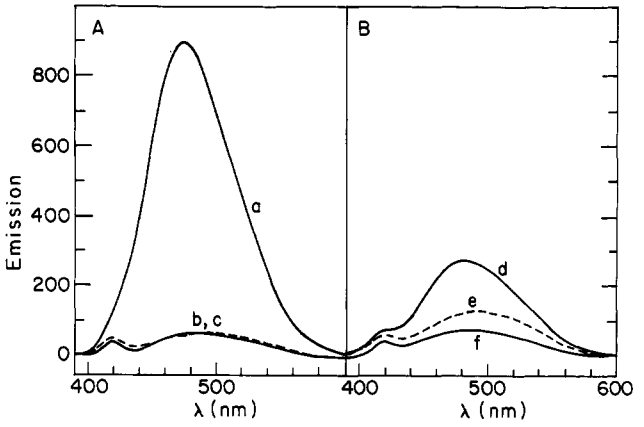

FIGURE 2: Fluorescence emission spectra of ANS in the presence and absence of proteins. (A) H_2O–HCl conditions (pH 1.9). ANS emission spectra in the presence of DPI or BPTI are labeled a and b, respectively; the baseline emission spectrum of ANS in the absence of proteins is labeled c. (B) 20% acetic acid. ANS emission spectra in the presence of DPI or BPTI are labeled d and e, respectively; the baseline emission spectrum of ANS in the absence of proteins is labeled f. In each case the concentration of ANS was 5 μM; the concentrations of DPI and BPTI were 100 μM. Spectra were recorded at room temperature.

the same solutions. The dialysate was used as the reference solution. Instrumental baselines were determined prior to scanning each sample with both cells filled with dialysate. Samples in 20% acetic acid were not degassed due to the high volatility of acetic acid. Reversibility of the observed DSC transition in 20% acetic acid was verified by rapidly cooling the cells to 10 °C and immediately rescanning; denaturation in H_2O–HCl (pH 1.8) was not reversible. Control DSC studies were conducted on bovine pancreatic trypsin inhibitor (BPTI; kindly provided by Prof. G. Wagner), which is similar to insulin in size and number of disulfide bonds. Its DSC trace was obtained under conditions (10 mM sodium acetate, pH 4.5) similar to those in which its solution structure and dynamics have been characterized by 2D-NMR methods.

RESULTS

Overview of Conditions. ANS binding and thermal unfolding experiments were conducted under conditions of two previous 2D-NMR studies; these are H_2O–HCl solution (pH 1.8; Boelens et al., 1990) and 20% acetic acid (pH 1.8; Hua & Weiss, 1990). The NMR data under the two conditions are essentially identical at 25 °C and establish that the solution structure of DPI (Knegtel et al., 1991; Hua et al., 1992) is in overall accord with crystal structures (Bi et al., 1984; Dai et al., 1987). Thermal unfolding is reversible and without aggregation in 20% acetic acid and occurs with irreversible aggregation of the unfolded state in H_2O–HCl solution (pH 1.8).

ANS Binding. Fluorescence emission spectra obtained under the two sets of conditions are shown in Figure 2. In panel A (H_2O–HCl solution) ANS emission spectra in the presence or absence of DPI are labeled a and c, respectively. The substantial increase in fluorescence intensity in spectrum a indicates binding to the protein, presumably to an exposed hydrophobic surface or cavity (Stryer, 1965); such binding is characteristic of ANS binding to kinetic and equilibrium models of protein-folding intermediates (Semisotnov et al., 1987; Goto & Fink, 1989). No increase in fluorescence is observed in the presence of BPTI (spectrum b in panel A), which provides a control as a stably folded globular protein of similar size. Corresponding data obtained in 20% acetic acid are shown in panel B. Although under these conditions ANS fluorescence increases in the presence of DPI (spectrum d; relative to ANS alone, spectrum f), the degree of enhancement is less than in H_2O–HCl solution. Such attenuation is likely to reflect decreased binding of ANS to DPI's hydrophobic core in an organic cosolvent (destabilization of hydrophobic interactions between insulin monomers is also observed in 20% acetic acid; Weiss et al., 1989). Slight enhancement of ANS fluorescence is also observed in the presence of BPTI in 20% acetic acid (spectrum e); the physical state of BPTI under these conditions has not been investigated.

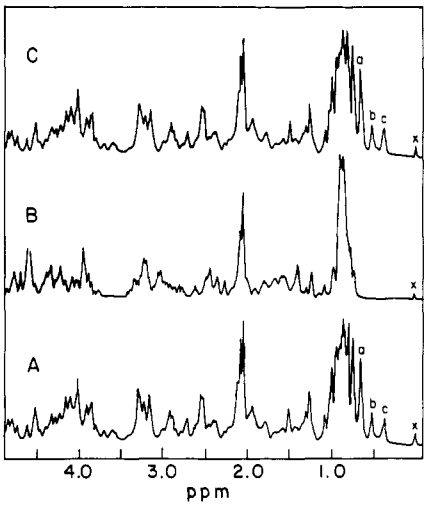

FIGURE 3: Aliphatic region of ^{1}H-NMR spectra of DPI in 20% acetic acid observed at (A) 25 °C (native state), (B) 70 °C (denatured state), and (C) cooled from 70 to 25 °C (refolded state). In panels A and C upfield-shifted resonances are labeled as follows: (a) overlap of A2Ile-γ′CH_3, LeuA13-δ2CH_3, LeuA16–δ1,2CH3, and LeuB6-δ2CH_3; (b) overlap of A2Ile-δCH_3, IleA10-γ′CH_3, LeuB11-δ1CH_3, and LeuB15-δ1CH_3; (c) overlap of LeuB15-δ2CH_3, IleA10-δ2CH_3, and IleA10-H_γ.

Thermal Unfolding Studies. In Figure 3 are shown aliphatic ^{1}H-NMR spectra of DPI at 25 °C (native state; panel A), at 70 °C (denatured state; panel B), and following recooling from 70 to 25 °C (refolded state; panel C). The spectra of the native and refolded species are identical and exhibit significant secondary shifts (e.g., upfield aliphatic resonances a, b, and c; Hua & Weiss, 1990). In the denatured state ^{1}H-NMR resonances assume random-coil values (Wuthrich, 1986) but remain sharp. Thermal unfolding of DPI in 20% acetic acid is thus reversible and without aggregation. In contrast, in H_2O–HCl solution DPI self-associates as it unfolds, leading to broad ^{1}H-NMR resonances and progressive macroscopic precipitation in the NMR tube at temperatures >55 °C (data not shown). Such aggregation is not well characterized but is thought to involve β-sheet formation (B. H. Frank, personal communication).

Circular Dichroism. Far-UV CD measurements could not be performed in 20% acetic acid due to solvent absorbance. In H_2O–HCl solution (pH 1.8) the CD spectrum is in qualitative accord with its secondary structure as determined by 2D-NMR under these conditions (Boelens et al., 1990) (Figure 4A). Far-UV CD spectra of DPI are similar in aqueous solution at pH 1.8 and pH 7 (data not shown) as are corresponding spectra of native zinc-free insulin (Pocker & Biswas, 1980). A thermal unfolding transition is observed in the range 20–80 °C as monitored by mean residue ellipticity at 222 nm (Figure 4B). This transition is broad and not highly cooperative. The expected baseline in the pretransition region ($d[\theta]/dT$ –0.3% deg^{-1}) is shown as a dashed line; the posttransition baseline is unclear due to aggregation in the

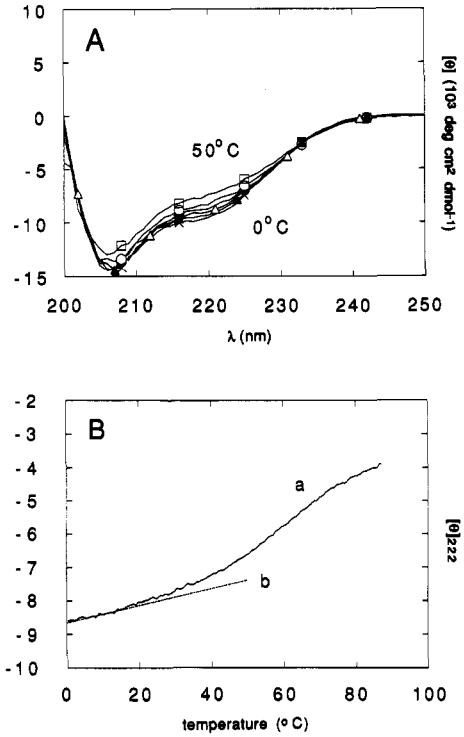

FIGURE 4: (A) Far-UV circular dichroism spectra of DPI in H_2O–HCl (pH 1.8) at different temperatures (0, 10, 20, 30, 40, and 50 °C). The protein concentration was 75 μM. (B) Thermal unfolding transition of DPI in H_2O–HCl (pH 1.8) as monitored by mean residue ellipticity at 222 nm. The pretransition baseline is shown as a dashed line.

denatured state. In accord with NMR studies, thermal unfolding and refolding in aqueous solution is reversible up to 55 °C and irreversible at higher temperatures.

Photo-CIDNP. A complementary probe for protein unfolding is provided by photochemical dynamic nuclear polarization (Weiss et al., 1989; Broadhurst et al., 1991). Photo-CIDNP resonance enhancement of tyrosine, for example, requires collision between its aromatic ring and a photoexcited triplet-state dye to create a transient spin-correlated radical pair (Kaptein, 1980). This technique may thus be used to detect exposure to solvent of nonpolar residues in the hydrophobic core of a protein. Its application to native insulin has previously been described (Muszkat et al., 1984; Weiss et al., 1989). In the native state in 20% acetic acid, the four tyrosines exhibit relative enhancements: A14 (exposed) > B16, B26 ≫ A19 (buried). Upon thermal or urea denaturation their photo-CIDNP enhancements are each similar to that expected of an unstructured polypeptide chain.

DPI contains two tyrosines on the surface of the protein (A14 and B16) and one internal tyrosine (A19). Their relative accessibilities in 20% acetic acid are readily probed by photo-CIDNP enhancement of respective $H_{3,5}$ ring resonances (inset in Figure 5A; Hua & Weiss, 1990). Integration of the photo-CIDNP difference spectrum indicates an enhancement ratio of 2.1 (of 3) in the folded state. The limited exposure of the third tyrosine (A19) to the photo-CIDNP probe (riboflavin) is in accord with surface-accessibility calculations based on the crystal structure (Weiss et al., 1989). An enhancement ratio of 2.8 is observed at 70 °C, presumably reflecting exposure of the hydrophobic core upon thermal denaturation. This increased photo-CIDNP enhancement is reversible on cooling. Unfolding events as monitored by CD and photo-CIDNP are discordant in the pretransition region, as exposure of TyrA19 upon thermal unfolding appears to be highly cooperative.

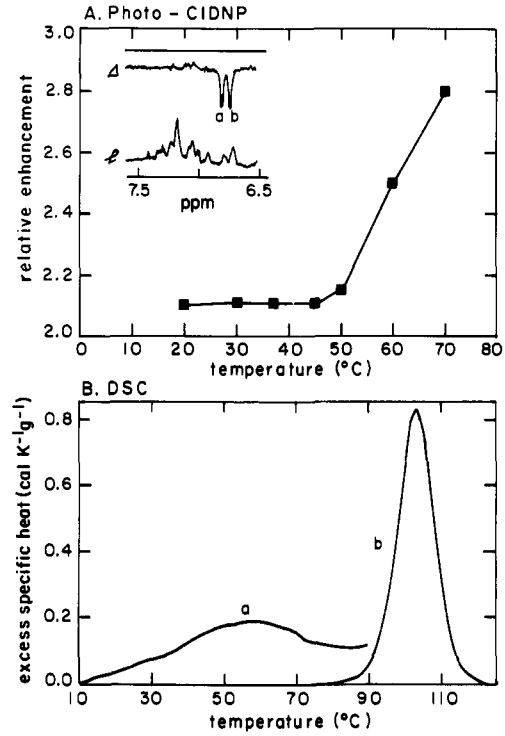

FIGURE 5: (A) Relative photo-CIDNP enhancement of tyrosine $H_{3,5}$ resonances in the ^{1}H-NMR spectrum of DPI as a function of temperature. As previously described (Weiss et al., 1989; Hua & Weiss, 1990), two tyrosine rings are exposed in the pretransition region (A14 and B16); the third, TyrA19, is largely inaccessible in the pretransition region but is exposed in the unfolded state. The enhancement of A14 [which projects from the surface of the protein in the crystal state (Dai et al., 1987) and in solution (Hua et al., 1992)] is assumed to represent the enhancement of a fully exposed tyrosine ring as discussed by Weiss et al. (1989). (Inset) Representative photo-CIDNP difference spectrum at 30 °C: (l) aromatic spectrum prior to laser illumination and (Δ) difference spectrum (unilluminated minus illuminated; difference peak a is assigned to Tyr14 and peak b to TyrB16). The protein concentration was 1 mM. (B) DSC unfolding curves of DPI in 20% acetic acid (curve a) and BPTI in 10 mM sodium acetate (pH 4.5) (curve b). Relative to BPTI (Moses & Hinz, 1983), DPI exhibits a higher apparent heat capacity in the pretransition region, reduced thermal stability, and lower latent enthalpy of unfolding. The concentrations of DPI and BPTI were approximately 0.5 mM.

Differential Scanning Calorimetry. A DSC scan of DPI in 20% acetic acid is shown in Figure 5B (curve A); the transition is reversible. Although similar from 10 to 60 °C, the DSC scan in H_2O–HCl solution (pH 1.8) contains a large negative deflection in the unfolded regime (60–90 °C), presumably due to aggregation of the denatured protein (data not shown). In either solvent a broad transition with T_m at approximately 60 °C is observed, consistent with the photo-CIDNP unfolding curve. In the folded regime (0–40 °C) a linear increase in the molar heat capacity is observed with increasing temperature (dC_p/dT 0.003–0.004 cal K^{-2} g^{-1}), which is consistent in magnitude with that of other globular proteins (Privalov & Khechinashvilli, 1974).

The broad nature of the transition in 20% acetic acid leads to large uncertainty in establishing pre- and posttransition baselines and hence in calculating the latent enthalpy of unfolding (ΔH_{cal}). An upper bound of 2.5 cal g^{-1} may be estimated, which is low for the unfolding of the native state of a globular protein (Table I). A positive but small ΔH_{cal} would be in accord with DSC studies of a compact unfolding intermediate of apomyoglobin at pH 4.2 (Griko et al., 1988). The latter retains more ordered structure than the A-state of α-lactalbumin, whose calorimetric enthalpy of unfolding is

Table I: Calorimetric Enthalpies of Unfolding for Globular Proteins[a]

protein	conditions[b]	T_m (°C)	ΔH_{cal} (cal/g)	reference
BPTI	pH 4.5	100	11.1	this study
λ repressor				
N-terminal domain[c]	pH 5.5	49	7.7	Pabo et al. (1979)
C-terminal domain[c]	pH 5.5	68	11.7	Pabo et al. (1979)
ribonuclease A	pH 2.8	42.6	6.93	Freire and Bilotonen (1978)
T4 lysozyme	pH 2.3	42.9	5.95	Kitamura and Sturtevant (1989)
staphylococcal nuclease[d]	pH 7.0	53.5	4.52	Calderon et al. (1985)
human α-lactalbumin	pH 7	70	4.28	Pfeil et al. (1986)
tryptophan synthase	pH 7.8	57.8	3.26	Matthews et al. (1980)
DPI[e]	20% acetic acid	60	<2.5	this study
human α-lactalbumin[e]	pH 2		0	Pfeil et al. (1986)

[a] The first eight proteins studied represent native states; the last two (DPI and acid-denatured α-lactalbumin) are presumed partially folded states. [b] pH values refer to aqueous solution; 20% acetic acid is pH 1.8 (direct meter reading). [c] Fragments D and C, respectively (Pabo et al., 1979). [d] T_m is concentration dependent. [e] Presumed compact partially folded states.

Table II: Ring-Current Shift Calculations[a] of Upfield Methyl Resonances (ppm)

			individual aromatic contributions							
residue	methyl group	RCS	TyrA14	TyrA19	PheB1	HisB5	HisB10	TyrB16	PheB24	PheB25
IleA2	γCH_3	0.24	0.01	0.22	0.00	0.00	0.00	0.01	0.01	0.00
	δCH_3	**0.90**	0.01	**0.78**	0.00	0.00	0.00	0.03	0.10	0.00
IleA10	γCH_3	0.03	−0.01	−0.01	0.00	0.05	0.00	0.00	0.01	0.00
	δCH_3	0.22	−0.01	0.00	−0.01	0.23	0.00	0.01	0.01	0.00
LeuB15	$\delta 1CH_3$	0.32	0.02	−0.05	0.01	0.00	0.00	0.06	0.29	−0.03
	$\delta 2CH_3$	**1.37**	0.01	0.04	0.00	0.00	0.00	0.11	**1.26**	−0.04

[a] Ring-current shifts (RCS) were calculated as described (Hoch et al., 1982; Weiss & Hoch, 1987) on the basis of the crystal coordinates of DPI at 1.5-Å resolution (Dai et al., 1987) kindly provided by Prof. Liang (Academica Sinica, Beijing, China). Predicted RCS greater than 0.50 ppm are shown in boldface.

negligible (Pfeil, 1981; Dolgikh et al., 1985; Pfeil et al., 1986). As proposed for DPI under strongly acidic conditions (Hua et al., 1992), apomyoglobin at pH 4.2 appears to consist of the nonspecific hydrophobic packing of native elements of secondary structure (Hughson et al., 1990, 1991).

To assess whether the low enthalpy of unfolding of DPI is simply a function of the fact that it is a small protein, comparison was made with bovine pancreatic trypsin inhibitor (BPTI), a protein of similar molecular mass (6.5 kDa) which also has three disulfide bonds. The DSC scan of BPTI is shown in Figure 5B (curve b). In contrast to DPI, a sharp transition is observed (T_m 100 °C). The latent enthalpy of unfolding is 11.1 cal g^{-1}, in accord with a previous study (Moses & Hinz, 1983). Interestingly, the NOESY spectrum of BPTI, unlike that of DPI (Hua et al., 1992), contains the expected number of long-range contacts (Wagner & Wuthrich, 1982ab); its distance-geometry structure is well-defined (Wagner et al., 1987) and is in accord with a high-resolution crystal structure (Deisenhofer & Steigmann, 1975).

Overview of ^{1}H-NMR Chemical Shift Dispersion. Unlike the A-state of α-lactalbumin (Baum et al., 1989), ^{1}H-NMR spectra of DPI in 20% acetic acid or aqueous solution (pH 1.8) exhibit nonrandom chemical shifts (Boelens et al., 1990; Hua & Weiss, 1990). The extent of dispersion is limited in each region of the spectrum, however [i.e., relative to the native states of BPTI (Wagner & Wuthrich, 1982a) and ZFY-6T (Kochoyan et al., 1991)], and in this respect resembles spectra of the partially folded states of apomyoglobin (Hughson et al., 1990) and ubiquitin (Harding et al., 1991). These qualitative observations suggest (but do not prove) the existence of partial averaging of proton microenvironments by protein flexibility. Since native secondary structure is retained (Boelens et al., 1990; Hua & Weiss, 1990), such flexibility would by elimination entail loss of order in the hydrophobic core as previously suggested (Hua et al., 1992).

Secondary shifts in proteins arise from a variety of physical mechanisms (Osapay & Case, 1991); a major contribution to the dispersion of methyl resonances is provided by aromatic ring currents (Hoch et al., 1982). Ring-current shift calculations based on the crystal structure (Dai et al., 1987) predict significant upfield secondary shifts for the methyl resonances of IleA2 and LeuB15 (Table II), which are not observed under these conditions (Table III). Nevertheless, strong NOEs are observed between these residues and that aromatic ring predicted as the predominant source of the upfield shift (IleA2–TyrA19 and LeuB15–PheB24, respectively; boldface in Table II) (Boelens et al., 1990; Hua & Weiss, 1990). The observed secondary shifts of IleA2 and LeuB15 more closely correspond to the ensemble-average of ring-current shifts based on the distance-geometry structures (Table III). This correspondence reflects the extended sampling of relative methyl–ring distances and orientations in the DG ensemble (Figure 1C,D). However, such calculations cannot distinguish between a dynamic mechanism and adoption of a well-defined conformation that differs from the crystal structure.

Table III: Comparison of Observed and Predicted Ring Currents Shifts (ppm)[a]

residue	methyl group	obsd[b]	crystal[c]	⟨ensemble⟩[d]
IleA2	γCH_3	0.22	0.24	0.25
	δCH_3	0.29	**0.90**	0.16
IleA10	γCH_3	0.22	0.24	0.01
	δCH_3	0.29	**0.90**	0.40
LeuB15	$\delta 1CH_3$	0.32	0.29	0.28
	$\delta 2CH_3$	**0.52**	**1.37**	**0.70**

[a] Ring-current shifts were calculated as described (Weiss & Hoch, 1987); those greater than 0.50 ppm are shown in boldface. [b] Observed secondary shifts in 20% acetic acid are obtained by subtracting the random-coil shifts for each residue and atom type as given by Wuthrich (1986) from the observed chemical shifts (Hua & Weiss, 1990). [c] Crystal coordinates were kindly provided by Prof. Liang (Academica Sinica, Beijing, China) as described (Dai et al., 1987). [d] The DG ensemble also predicts significant upfield shifts for the methyl resonances of LeuA16, which are not observed. DG coordinates (Hua et al., 1992) have been deposited in the Brookhaven Data Bank.

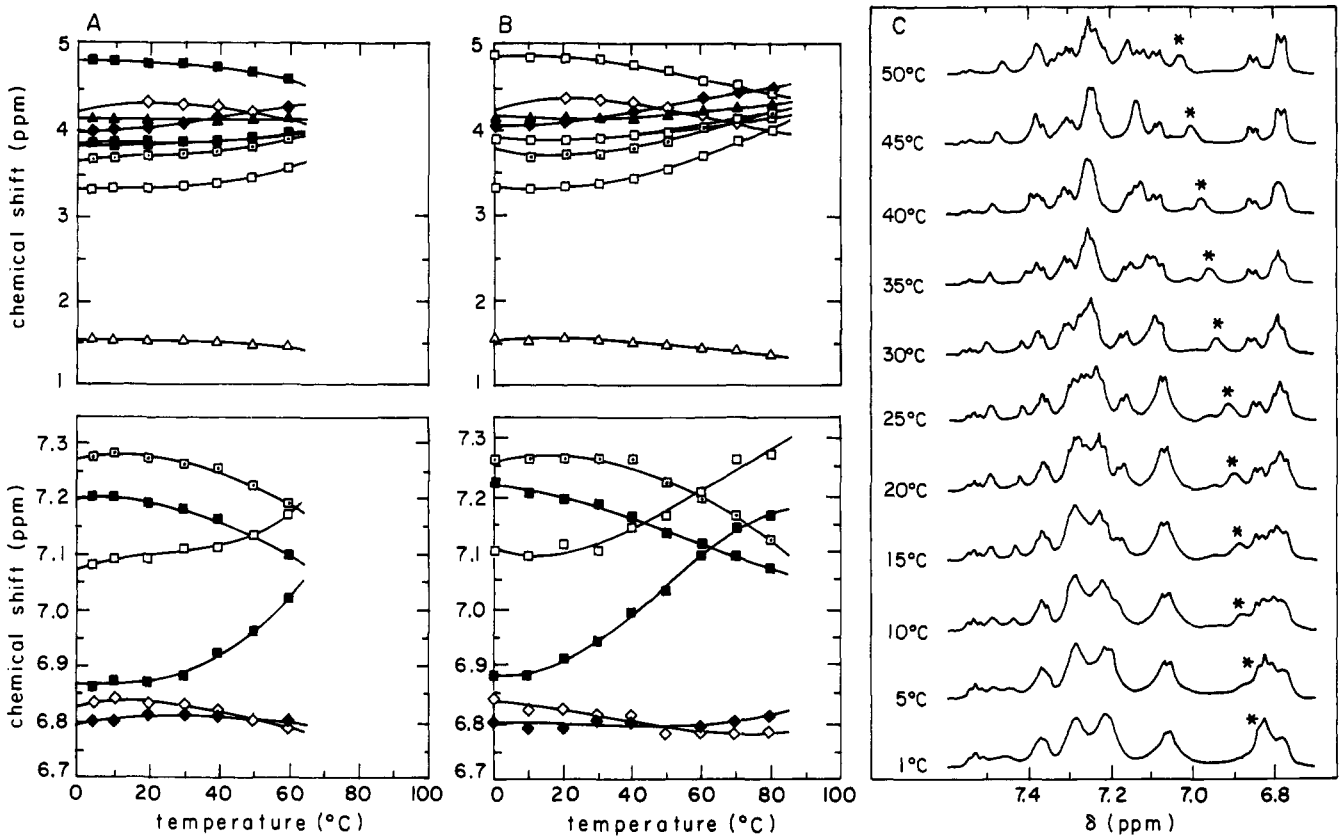

FIGURE 6: ^{1}H-NMR chemical shifts of selected protons resolved in TOCSY spectra of DPI in D_2O–DCl (pD 1.8, direct meter reading) (panel A) and in 20% deuterated acetic acid–D_2O (panel B). In panels A and B assignments (from top to bottom at 10 °C) are as follows: upper boxes, A9-H_α (■ in panel A and □ in panel B), A12-$H_{\beta 2}$ (◇), B14-H_α (▲), A8-H_α (◆), A13-H_α and B18-H_α (unresolved; ■), A3-H_α (⊡), B12-H_α (box), B14-βCH_3 (△); lower boxes, A19-$H_{2,6}$ (⊡), B16-$H_{2,6}$ (■), B24-$H_{3,5}$ (□), B24-$H_{2,6}$ (■), B16-$H_{3,5}$ (◇), and A19-$H_{3,5}$ (◆). (C) Aromatic ^{1}H-NMR resonances of DPI in 20% deuterated acetic acid–D_2O as a function of temperature in the pretransition region; asterisks indicate resonance $H_{3,5}$ of PheB24.

Temperature Dependence of ^{1}H-NMR Chemical Shifts. Dynamic features of protein structure may be probed by the temperature dependence of chemical shifts in the pretransition region. Such changes among nonexchangeable (i.e., carbon-bound) ^{1}H-NMR resonances are ordinarily very small (<0.001 ppm/°C); larger changes are observed among amide resonances (usually 0.001–0.08 ppm/°C). In two small globular proteins (BPTI and eglin C; Hyberts & Wagner, 1990) the latter are observed to correlate with structure (unfolded regions exhibit larger variation with temperature, typically 0.006–0.008 ppm/°C) and formation of amide hydrogen bonds (which are associated with smaller variation with temperature) (Gerhard Wagner, personal communication). Nonexchangeable and amide resonances of DPI exhibit anomalously large variation with temperature as described in turn below.

(*i*) *Nonexchangeable Protons.* The temperature dependence of selected nonexchangeable ^{1}H-NMR resonances of DPI in H_2O–HCl solution (pH 1.8) is shown in Figure 6A. As in the CD transition, gradual changes in chemical shifts are observed in the range 10–55 °C; at higher temperatures irreversible aggregation occurs. Corresponding ^{1}H-NMR studies in 20% acetic acid are shown in Figure 6B; the chemical shifts and NOE patterns of DPI are similar under the two conditions (Boelens et al., 1990; Hua & Weiss, 1990, 1991b). The trend of gradual and progressive changes in chemical shift in the folded state is more apparent in 20% acetic acid. Such changes are particularly clear in the aromatic region (Figure 6C); the $H_{3,5}$ resonance of PheB24 is indicated by an asterisk. In either solvent DPI partially unfolds and refolds reversibly in the range 0–55 °C. In 20% acetic acid complete thermal unfolding is reversible and without significant aggregation, as indicated by narrow resonance line widths (see above).

(*ii*) *Amide Protons.* The amide resonances of DPI in 20% deuterated acetic acid–80% H_2O are shown as a function of temperature in the pretransition region in Figure 7A. Overall loss of chemical shift dispersion is observed with increasing temperature. The chemical shifts of four amide resonances resolved in the downfield region of the spectrum (assigned to residues A11, B9, B8, and B6; respectively labeled a–d in Figure 7A) are shown as a function of temperature in panels A (20% deuterated acetic acid) and B (aqueous solution at pH 1.8) of Figure 8. Their variation with temperature is unusually large (0.013–0.020 ppm/°C in the range 10–40 °C). These amide resonances also exhibit anomalous broadening, which has been attributed to exchange among conformational substates (Weiss et al., 1989; Kline & Justice, 1990).

The side chains of A11, B6, and B8 participate in or are adjacent to contacts between the A- and B-chains; B9 is the N-terminal residue of the B-chain α-helix (Baker et al., 1988; Hua et al., 1992). In the crystal structure the A11 amide forms an interchain hydrogen bond to the B4 carbonyl; among crystal forms of insulin interchain hydrogen bonds are variably observed from the B6 amide to A6 carbonyl or via a bound water to the A3 carbonyl; the B8 and B9 amides are exposed to solvent. Consistent with this hydrogen-bonding pattern, the A11 and B6 amide resonances are slowly exchanging in D_2O whereas those of B8 and B9 are not. In either solvent slowly exchanging amide resonances are observed in α-helical segments at 0–50 °C (Figure 7B). Preservation of slowly exchanging amide resonances in D_2O throughout this tem-

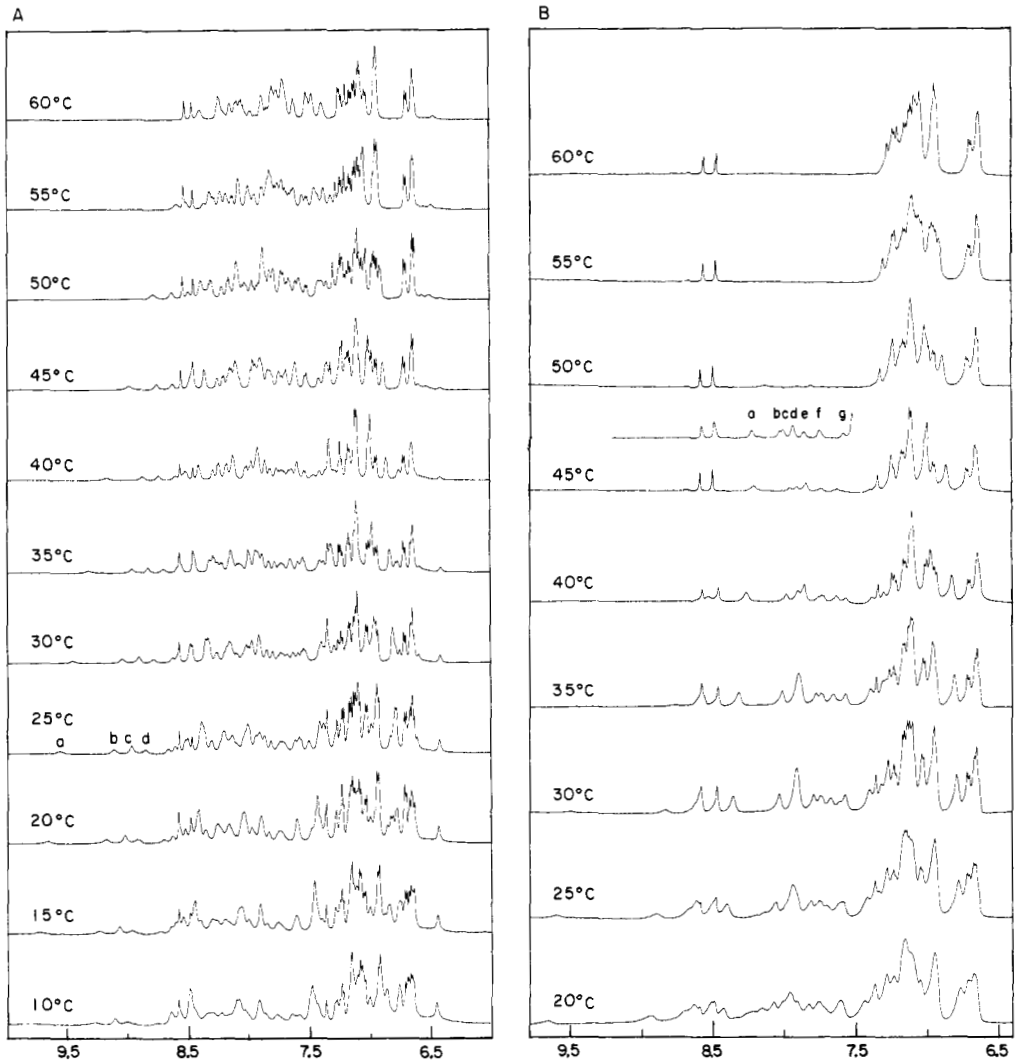

FIGURE 7: (A) 1D ^{1}H-NMR spectra of DPI in 20% deuterated acetic acid–80% H_2O as a function of temperature. Four amide resonances resolved in the downfield region are labeled (a) A11, (b) B9, (c) B8, and (d) B6. (B) Slowly exchanging amide resonances in 20% deuterated acetic acid–80% D_2O as a function of temperature. Assignment of such resonances at 25 °C is as described (Hua & Weiss, 1990). The inset at 45 °C is a freshly dissolved sample with slowly exchanging amide resonances: (a) A6 or A7, (b) A8 or B16, (c) A16 or A17, (d) B10 or B13 (e) A19, (f) B14 or B17, and (g) A14 or A15.

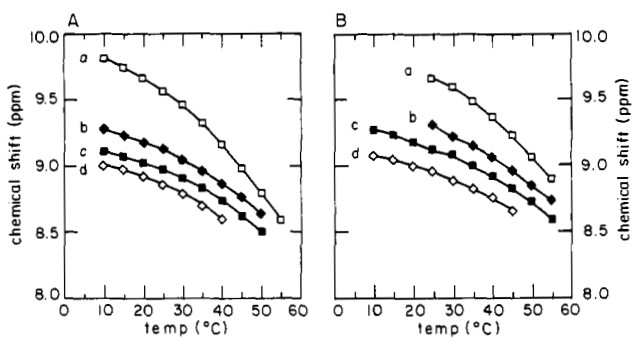

FIGURE 8: Temperature dependence of the four resolved downfield-shifted amide resonances in 20% deuterated acetic acid (panel A) and in H_2O–HCl (pH 1.8) (panel B): A11 (a, □), B9 (b, ◆), B8 (c, ■), and B6 (d, ◇). The environments of these protons are influenced by contacts between the A- and B-chains. In panel B chemical shifts are not shown for A11 (a) and B9 (b) for temperatures <20 °C due to extreme resonance line broadening; such line broadening reflects intermediate exchange mechanisms in the monomer (Weiss et al., 1989) and partial self-association in the absence of acetic acid (Boelens et al., 1990).

perature range suggests that the progressive convergence of chemical shifts toward random-coil values (Wuthrich, 1986) in the pretransition region is not due to averaging between α-helical and nonhelical structures, since the latter fluctuations would be associated with loss of amide hydrogen bonds and rapid solvent exchange.

DISCUSSION

States of protein organization intermediate between the native state and an unstructured polypeptide chain (the random coil) are of fundamental interest in relation to mechanisms of protein folding and stability (Kim & Baldwin, 1982). Insight into such intermediate states has been obtained by studies of partially folded forms of α-lactalbumin (the A-state; Kuwajima et al., 1976; Pfeil, 1981; Dolgikh et al., 1985; Pfeil et al., 1986; Baum et al., 1989), apomyoglobin (Griko et al., 1988; Hughson et al., 1990, 1991), cytochrome c (Jeng et al., 1990), and ubiquitin (Harding et al., 1991). Although these equilibrium models differ in the extent to which native structure is retained, each exhibits greater loss of tertiary than secondary structure. Nonlocal stabilization of secondary structure in a compact form is proposed to be mediated by nonspecific hydrophobic interactions without the precise side-chain interactions characteristic of the native state (the molten globule). In thermal unfolding studies partially folded proteins exhibit reduced (Griko et al., 1988) or absent (Pfeil et al., 1981, 1986; Dolgikh et al., 1985) cooperativity and calorimetric enthalpy.

In the present study we have focused on a monomeric insulin analogue (DPI), which lacks residues B26–B30. Crystal structures of DPI demonstrate that the basic insulin fold is retained, although in the absence of residues B26–B30 the underlying hydrophobic surface of the A-chain is partially exposed (Bi et al., 1984; Dai et al., 1987). The 2D-NMR structure of DPI has been determined in 20% acetic acid and in aqueous solution at pH 1.8 (Boelens et al., 1990, 1991; Hua & Weiss, 1990). In either solvent the NOESY data appear to be underdetermined (Hua et al., 1992), and resulting distance-geometry ensembles contain stably folded elements of secondary structure with a range of tertiary orientations (Figure 1B). The geometry of its hydrophobic core is not well-defined by the NMR data but appears as a coalescence of distinct α-helix-associated microdomains (Karplus & Weaver, 1976) (Figure 1D,E; Hua et al., 1992). The NMR studies may thus depict an insulin A-state; alternatively, it is possible that DPI consists of a set of conventional native states whose relative populations and interconversion rates could account for the observed NOESY spectrum.

To distinguish between these models, we have undertaken the present study of ANS binding and thermal unfolding. Like kinetic protein-folding intermediates (Semisotnov et al., 1987) and their equilibrium models (Goto & Fink, 1989), DPI binds ANS. Such binding indicates the exposure of a hydrophobic surface or pocket (Stryer, 1965) and is uncommonly observed in a conventional native state. Complementary insight is provided by studies of protein unfolding, which have used five biophysical probes (circular dichroism, photo-CIDNP, DSC, ^{1}H-NMR chemical shifts, and slowly exchanging amide resonances). Taken together, results of these probes support the molten-globule hypothesis even though each by itself would permit wider interpretation. Their overall nonconcordance, for example, provides evidence of a partially folded state whose features are similar to those of a partially folded intermediate of apomyoglobin (helices A, G, and H; Griko et al., 1988; Hughson et al., 1990, 1991). The partially folded states of DPI and apomyoglobin differ from the A-state of α-lactalbumin by retention of nonrandom ^{1}H-NMR chemical shifts and of small but distinguishable calorimetric transitions. Further unfolding of apomyoglobin (as the pH is lowered) or DPI (as the temperature is raised in the pretransition region) is likely to proceed with progressive loss of tertiary structure. DPI differs in turn from the apomyoglobin intermediate in the extent to which native elements of secondary structure are retained (completely in DPI and incompletely in apomyoglobin).

NMR studies of the structure and dynamics of partially folded proteins are likely to provide a detailed view of states of intermediate organization. Such studies pose a general question in NMR spectroscopy: what is the physical interpretation of imprecision in a distance-geometry ensemble? Such calculations cannot distinguish physical uncertainty (absence of restraint information due to internal motions) from informational uncertainty (absence of restraint information due to incomplete data analysis). The latter may be due, for example, to resonance overlap, absence of stereospecific resonance assignment, and coarse interpretation of NOEs as strong, medium, or weak. In a previous study (Hua et al., 1992) the distinction between physical and informational uncertainties was addressed by matrix-relaxation calculations (Keepers & James, 1984). Comparison of the observed NOESY spectrum of DPI with those back-calculated from DG models demonstrates consistency of short- and medium-range NOEs (i.e., contacts that define the α-helices and β-turn) but significant underrepresentation of predicted long-range contacts (the NOESY "paradox"; Hua et al., 1992). The observed subset of long-range NOEs corresponds to spatial features *shared in common* by the various DG models, whereas the "missing" NOEs provide restraints that would specify one particular model or another. The present study demonstrates the utility of complementary studies of protein unfolding by independent biophysical methods. Use of ANS is of particular interest as a probe for exposure of hydrophobic surfaces. Future studies of partially folded states may be facilitated by development of additional probes specific for classes of nascent structure (Lynn & Fasman, 1968).

Polypeptide hormones can adopt a variety of conformations depending on environmental conditions and state of self-association. Glucagon, for example, has been studied as an α-helical trimer in the crystal state (Sasaki et al., 1975) and in aqueous solution at high peptide concentration (Wagman et al., 1980), as a random coil in aqueous solution (Boesch et al., 1978), as a complex with dodecylphosphocholine micelles (Wider et al., 1982; Braun et al., 1983), and in nonaqueous solvents (Gratzer et al., 1968). Analysis of such structural transitions provides insight into what conformational changes are possible on receptor binding and has application to the design of agonists and antagonists (Frandsen et al., 1985; Gysin et al., 1986). Like glucagon, insulin exhibits conformational variability (Chothia et al., 1983) but with an essential difference: unlike smaller peptide hormones or their fragments, insulin contains stably folded elements of secondary structure that pack to define an interior and exterior. In this respect it must be regarded as a protein rather than as a peptide. We propose that its conformational repertoire among crystal forms represents alternative late steps in the folding of a molten monomer.

Is the molten globule the functional form of insulin in the bloodstream? The solution structure of insulin or an insulin analogue has not yet been determined under native conditions, and so it is not known whether A-state features of DPI in 20% acetic acid or in aqueous solution (pH 1.8) differ from those of insulin at pH 7. Nevertheless, four types of studies, taken together, raise the provocative possibility that the insulin monomer in fact functions as a molten globule. (i) 2D-NMR studies of a fully active engineered insulin monomer (DKP-insulin), conducted under physiological conditions (Roy et al., 1990), demonstrate attenuation of tertiary NOEs (relative to matrix relaxation calculations) similar to that of DPI (Weiss et al., 1991). (ii) Flexibility in the tertiary organization of the insulin monomer is also suggested by molecular dynamics simulations (Kruger et al., 1987; Caves et al., 1990; Mark et al., 1991). (iii) The relative bioactivities of insulin analogues are difficult to rationalize from static crystal models and suggest that protein flexibility is required for receptor binding (Baker et al., 1988; Mirmira & Tager, 1989). (iv) The complementary results of recent crystallographic and NMR studies of inactive and active insulin analogues (mini-proinsulin and GlyB24-insulin, respectively) imply that a change in the conformation in the C-terminal region of the B-chain is required for receptor binding; specifically, detachment of the B-chain β-strand (B23-B28) is proposed to expose an alternative protein surface (Derewenda et al., 1991; Hua et al., 1991).

In summary, we have investigated the dynamics of a monomeric insulin analogue (DPI) by analysis of thermal unfolding and ANS binding under acidic conditions. The

results support a model of DPI as a compact partially folded state (Hua et al., 1992). A functional requirement for reorganization of discrete protein surfaces on receptor binding would provide a biological rationale for such a molten structure. Future studies of the dynamics of insulin under physiological conditions would provide further insights into the mechanism of receptor binding and have application to design of insulin agonists.

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