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Experimental approaches to protein folding based on the concept of a slow hydrogen exchange core

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In a review of protein hydrogen exchange, we concluded that the slow exchange core is the folding core. By this we mean that the elements of secondary structure carrying the slowest exchanging backbone amides will tend to be the elements of secondary structure to fold first, that partially folded proteins will tend to be most organized in the core, and that peptides made to mimic the slow exchange core will tend to show nativelike structure. These generalizations have led us to ask several experimental questions that will be examined here: (1) In partially folded and unfolded proteins, how do the dynamics and structure of core regions differ from noncore regions? (2) Can we make protein 'core modules' as peptides corresponding to the slow exchange core? Can core modules be covalently linked to make a native state in which one conformation is significantly more stable than all other accessible conformations? (3) In a mutant perturbed outside the core, what are the effects on hydrogen exchange and folding? © *2001 by Elsevier Science Inc.*

INTRODUCTION

The slow exchange core of a protein refers to the mutually packed elements of secondary structure that are most refractory to hydrogen exchange. The rates of hydrogen isotope exchange of backbone amide N^1H with aqueous solvent ^2H_2O monitors protein flexibility.[1] NH groups on the surface of a native protein exchange rapidly, with rates approaching those of amides in small peptides in water. NH groups that are buried and intramolecularly H-bonded in the crystal structure exchange more slowly, with rates that vary widely throughout the folded protein. Buried amides in more mobile regions exchange from several-fold to several orders of magnitude more slowly than model peptides, while buried amides in less flexible regions exchange many orders of magnitude more slowly. Under some conditions, the slowest amides do not exchange until the protein unfolds. The group of amides that are the very last to exchange in a folded protein define the slow exchange core.

Dynamics and Structure of Core and Noncore Regions

(1) In partially folded and unfolded proteins, how do the dynamics and structure of core regions differ from noncore regions? Partially folded and unfolded proteins sample numerous arrangements of the backbone, like a spaghetti noodle in a pot of boiling water, and are best envisioned as an equilibrium ensemble of more and less extended conformations. Spectroscopic measurement of a partially folded or unfolded protein gives a snapshot of signals emanating simultaneously from all conformations in the ensemble. In an NMR spectrum, each NH is observed as one average peak if the conformations sampled by that amide group interconvert rapidly on the NMR time scale (<ms). However, if two or more conformations sampled by an amide interconvert slowly (msec or less), then two or more peaks may be observed for each NH. In NMR analyses this is explained by slow chemical exchange (not to be confused with hydrogen isotope exchange). When spectra show slow exchange, each peak represents a family of conformations. Within a family, conformational interchange is rapid, while between families interchange is slow. Slow chemical exchange is inferred from several types of NMR spectra.[2]

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[2] In rotating frame NOE spectroscopy (ROESY), the cross peaks due to chemical exchange have the same sign as the diagonal peaks, while cross peaks due to the NOE are of opposite sign. In NOESY spectra, cross peaks due to chemical exchange are observed in addition to cross peaks due to NOE, if the exchange rate is of the same order of magnitude as the NOESY

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When analyzing NMR parameters of reporter groups in rapid chemical exchange, it is useful to bear in mind that chemical shift and coupling constants report the ensemble average of rapidly interconverting conformations, while NOEs may report minor populations. NOEs in native proteins measure interatomic distance, but in partially folded and unfolded ensembles NOE intensity reflects the combined effect of proximity and population. Weak NOE intensity may arise from a long-range distance between reporter nuclei in a large fraction of conformations, or from short- to medium-range distance between nuclei in a small fraction of conformations. In addition to chemical exchange and hydrogen isotope exchange, we use various NMR relaxation methods to characterize the local dynamics of specific NH groups. These provide microscopic probes of local flexibility monitored simultaneously at numerous points throughout the molecule. Dynamic parameters measured include heteronuclear NOEs, order parameters, and spectral density functions. A high, positive heteronuclear NOE implies a rigid amide N—H bond vector, while one that is negative implies a flexible vector. Order parameters range from 0 to 1, from least to most ordered. Spectral density functions[3] and order parameters are a measure of rotational fluctuations of the atomic bond vector with respect to the magnetic field.[5,6] Our experiments are carried out on partially folded and fully unfolded derivatives of bovine pancreatic trypsin inhibitor (BPTI). These are prepared by solid-phase synthesis[7] to give species not amenable to production by molecular biological techniques due to intracellular proteolysis. Our approach is to remove intramolecular disulfide bonds by synthesizing BPTI variants with cysteine residues replaced by α-amino-*n*-butyric acid (Abu). Abu was chosen over Ala or Ser as it is isosteric with Cys (-SH replaced by -CH_3). By replacing Cys with Abu, partially and fully unfolded proteins are obtained without addition of high concentrations of denaturants or extremes of pH. Natural BPTI has three disulfide bonds. Partially folded species, called $[14\text{-}38]_{Abu}$, retain one natural disulfide bond, between residues 14 and 38, which is outside the slow exchange core. Fully unfolded species, called $[R]_{Abu}$, are missing all three disulfides (R stands for "fully reduced"). An additional feature of the approach is to incorporate ^{15}N-labeled amino acids at selected points in the sequence to provide specific ^{15}N probes for NMR experiments. For fully unfolded BPTI, amide groups undergo extensive conformational averaging, but NOEs indicate that detectable populations of nativelike conformations are present in the unfolded ensemble,[8] relaxation measurements indicate that groups in the core are less mobile than in noncore residues [unpublished results] and hydrogen exchange of core amides is slowed to a small reproducible degree [results to be published]; hydrodynamic measurements indicate that $[R]_{Abu}$ is more collapsed on average than expected for a random coil of the same amino-acid sequence.[9] The implication is that among the large number of conformations visited by an unfolded protein, nativelike conformations are present, core residues tend to be less flexible (more organized) than noncore residues, and the average molecular radius is less than a 'random coil.' The unfolded ensemble contains some collapsed conformations, some residues in nativelike structure, and more order in core residues than in the rest of the molecule. This suggests that the unfolded ensemble contains conformations approaching the native protein. For the partially unfolded ensemble the picture is more complex, due to slow conformational exchange.[10–12] The $[14\text{-}38]_{Abu}$ ensemble contains two (in one case, three) families of conformations detectable as slow exchange peaks for individual ^{15}N-labeled amides. This does not mean that the molecule as a whole has two families of conformations. Rather, each NH samples two conformations, and core residues sample nativelike conformations more than noncore residues. In NMR spectra in which slow exchange is observed, for example Figure 1, the slow exchange cross peaks for each NH are referred to as (f) and (u) peaks for that amide (more folded and more unfolded). The conformational families giving rise to (f) and (u) cross peaks are called, respectively, P_f and P_d (P for partially folded, f for more folded, and d for more denatured). P_f and P_d families are each composed of numerous conformations in rapid exchange, while P_f–P_d interconversion is slow (rate constants in the range 0.045 to 0.88 sec^{-1}).[11] NOE and chemical shift analysis under a variety of conditions tells us that P_f families are not the same for all amides. P_f families for core residues on average are very nativelike, while P_f families of noncore residues on average are not nativelike.

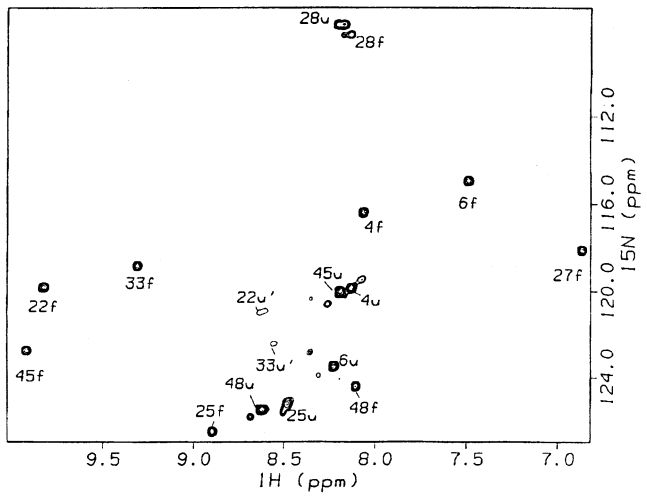

Figure 1. ^{1}H-^{15}N HSQC spectrum of $[14–38]_{Abu}$ with ^{15}N labels inserted at nine sites. For each labeled NH there is an (f) peak and one or more (u) peaks. For the same residue number, peaks (f) and (u) represent, respectively, P_f conformations of that amide group, which are partially folded and more ordered, and P_d conformations that are partially folded and less ordered. P_f and P_d are in slow exchange; they interconvert on a time scale <ms. A relaxation delay of 3 s was used, which is sufficiently longer than T_1 values to permit quantitative volume integration. The relative population of P_f and P_d are determined from peak intensities. Ala 27 (u) is too broad and hence not observed at the noise level plotted here. The spectrum was acquired at pH 5.0 and 5°C. At higher temperature, the intensities of the (f) peaks decrease while those of (u) peaks increase. Reprinted with permission from Barbar.[13]

the mixing time. In total correlated spectroscopy (TOCSY), cross peaks in the amide–amide region are easily distinguished because no other correlations are generally observed in this region.

[3] Spectral densities, J(0), J(wN) and J(wH) are evaluated at different frequencies and sample a range of time scales for motions of the N—H bond vector.[5,6,12] Greater mobility is indicated by lower J(0), lower J(wN), and higher J(wH).

Unfolding/folding analysis of microscopic probes indicates that the P_f to P_d fluctuations are local in nature and do not involve global cooperative folding/unfolding.[13,14] The ensemble structure derived from our NMR studies is drawn schematically in Figure 2. In addition to chemical exchange, relaxation parameters were measured to assess the internal motions of partially folded and unfolded BPTI variants. Relaxation analysis tells us that core amides are less flexible, and more organized, than noncore residues.[11,12] Further, P_d conformations of core residues are less mobile than P_d conformations of noncore residues. Finally, our findings with $[14\text{-}38]_{Abu}$, taken together with results of other single disulfide mutants of BPTI, indicate that any single native disulfide bond favors formation of a partially folded ensemble with nativelike structure in the core and more disorder in the rest of the molecule. This leads us to conclude that the folding 'pathway' of fully reduced BPTI is not critically dependent on the order of disulfide bond formation.[11,12] The slow conformational interchange phenomena in partially folded BPTI variants may be general for partially folded proteins. There are examples in the literature of intermediate exchange behavior of other partially folded ensembles. In α-lactalbumin, the absence of observable cross peaks under molten globule conditions, along with the presence of sharp peaks under unfolding conditions, is explained by broadening in the molten globule due to intermediate exchange.[15] In denatured staphylococcal nuclease there is a similar situation. Some residues are apparently obscured by broadening; their intermediate chemical exchange is inferred from appearance of their cross peaks in spectra of the fully unfolded protein.[16] In thioredoxin, reconstituted fragments give heteronuclear NMR spectra in which peaks missing under milder conditions are present under denaturing conditions, implying that under milder conditions the protein is partially folded and the peaks in question are in intermediate exchange.[17] If it turns out that in partially folded proteins, core backbone atoms are, in general, in slow–intermediate exchange between nativelike and disordered conformational families that interconvert by local motions, this means that families of locally fluctuating conforma-

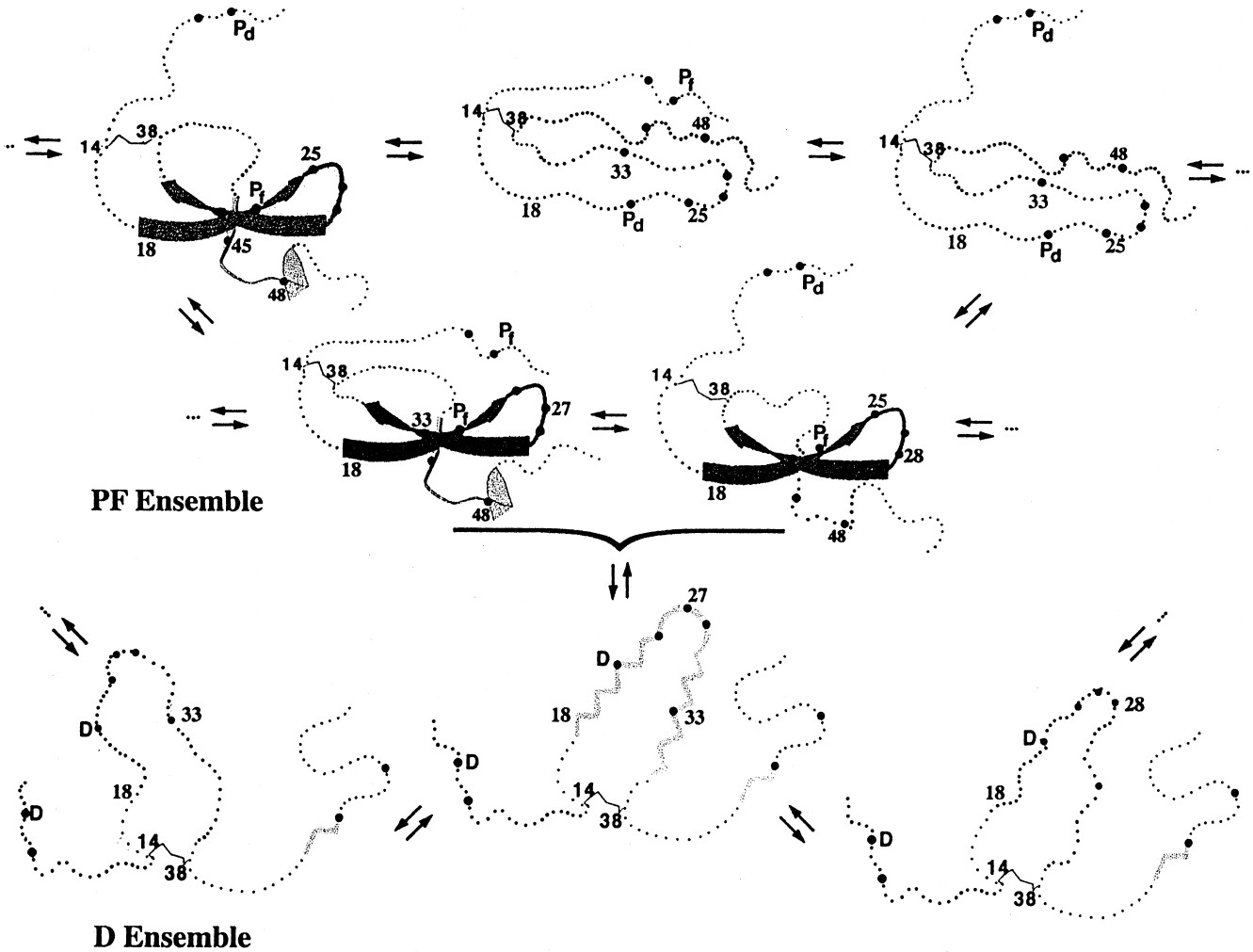

Figure 2. Schematic representation of the conformational ensembles of $[14\text{-}38]_{Abu}$ in the partially folded state, PF, and the unfolded state, D. larger circles show the positions of residues labeled with ^{15}N. For each ^{15}N-bound ^{1}H in partially folded $[14\text{-}38]_{Abu}$, there are both slow (≥ms) local fluctuations (top) between P_f, a more folded conformation, and P_d, a more disordered conformation. At higher temperature the NH may also be in D, a globally denatured conformation (bottom). The labels P_f, P_d, and D are for residues 4 and 22 in each specific conformation. Reprinted with permission from Barbar et al.[12]

tions are separated by an energy barrier, the nature of which may be important in understanding early events in protein folding.

Protein Core Modules

(2) Can we make protein 'core modules' as peptides corresponding to the slow exchange core? Can core modules be covalently linked to make a native state in which one conformation is significantly more stable than all other accessible conformations? We are exploring a strategy for design and construction of peptides that acquire a 'native' state in which one conformation (or ensemble of closely related conformations) is significantly more stable than all other accessible conformations of that polypeptide. The strategy is based upon two hypotheses: that peptides corresponding to core elements (the slow exchange core) of a protein will favor nativelike structure; and that incorporation of a well-placed cross link will eliminate high-entropy extended conformations and stabilize the more favored collapsed forms. An oxidized core module (OxCM) of BPTI has been designed (Figure 3), synthesized, and characterized by NMR and circular dichroism.[18] Core elements of BPTI are two long strands of antiparallel β-sheet (18–24, 29–35) and a small β-bridge (43–44). OxCM spans residues 14–38, has neutral end groups, replaces A27 by D27 to stabilize a type I β-turn, and retains the natural 14–38 disulfide. To address our hypothesis about the role of a cross link, a reduced core module (RedCM) with the same sequence as OxCM but lacking a disulfide was also synthesized. ^{1}H NMR and simulated annealing studies indicate that the OxCM is an equilibrium ensemble of conformations among which a major population is similar to the nativelike 4:4 β-hairpin and a minor population approximates 3:5 β-hairpins (Figure 4). RedCM NOEs are similarly consistent with rapid equilibria between 4:4 and 3:5 β-hairpins. However, NOEs for RedCM are clearly fewer and weaker than for OxCM. These results[18]

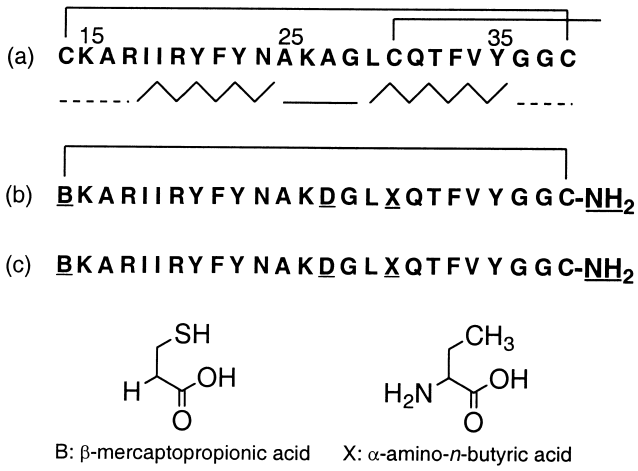

Figure 3. Design of BPTI core modules. Sequences for (a) core template, (b) oxidized core module, and (c) reduced core module. Differences from the native sequence are underlined. The structures of the nonnatural amino acids B, β-mercaptopropionic acid, and X, α-amino-n-butyric acid are drawn at the bottom. Reprinted with permission from Carulla et al.[18]

support our expectation that core elements favor nativelike structure, and form the basis of the next step in the project, now ongoing. In the current phase of this research, two core modules are linked by methods designed to stabilize nativelike structure in both, and thereby mimic a true native state having one unique conformational family. Our first attempt is to link two BPTI oxidized core modules. The rationale is that dimeric BPTI exists in solution and undergoes rapid association-dissociation,[19,20] and molecular modeling studies indicate that residues at the monomer–monomer interface are primarily in the core. In the dimer model, R17 in monomer 1 is near l29 in monomer 2. Our general approach to production of a core module dimer is illustrated in Figure 5. The R17 side chain in one monomer is replaced by one group (triangle) and the L29 side chain in a different monomer is replaced by a second group (grooved rectangle). The groups are designed to react with each other to form a stable cross link (Figure 5). In current experiments, one replaced side chain carries a aminooxyacetyl moiety while the other carries a glyoxylyl moiety. Under mild conditions, these react with each other to produce an oxime linkage between monomer modules. The length of the replacement side chains can be varied by adding methylene (—CH_2—) groups. The molecules have been synthesized in NMR quantities, and their biophysical characterization is in progress.

Core vs. Non-core Mutants

(3) In a mutant perturbed outside the core, what are the effects on hydrogen exchange and folding? BPTI contains a central β-sheet in its slow exchange core, packed by N- and C-terminal helices that are stabilized by buried disulfide bonds (5–55 and 30–51). Two overlapping loops that comprise the binding site for proteases are connected by a disulfide bond (14–38) that is somewhat solvent exposed. High resolution crystal structures of core mutants (F22A, Y23A, N43G, and F45A) show that the replacements create 'crevices' with very little rearrangement of surrounding amino acids.[21] Their loss of stability ($\Delta\Delta G \sim$ 2–7 kcal/mol) can be accounted for by the additional surface area exposed to solvent.[22] In contrast, a substitution outside the core, Y35G, has large rearrangements of loops 11–17 and 35–43 near the mutation, and altered internal H-bonds.[23]

In the active site 35–43 loop, there is an unusual polar interaction between G37 backbone NH and Y35 aromatic ring.[24,25] The NH–aromatic interaction (Figure 6A) is thought to be electrostatic in nature, with the partial positive dipole of the NN group interacting with the partial negative charge in the center of the ring. A similar interaction is proposed in packing of arginine and lysine side chains against aromatic rings.[26] Calculated estimates of the NH–aromatic interaction energy *in vacuo* suggest that it can be worth 1–3 kcal/mol.[27,28] G37A was designed to perturb the NH–aromatic interaction, as G37 in WT BPTI has backbone angles that lie in the region of the ϕ/ψ plot disallowed for a β-carbon amino acid. The G37A mutation produces a large destabilization of the protein (~5 kcal/mol), which is unusual for the addition of a single methyl group. We have fully assigned and calculated the three-dimensional NMR structure and dynamics of G37A.[29] In contrast to Y35G, another other loop mutation, G37A, does not result in large structural rearrangements of the protein backbone, even though it is highly destabilized. G37A retains the structure of wild-

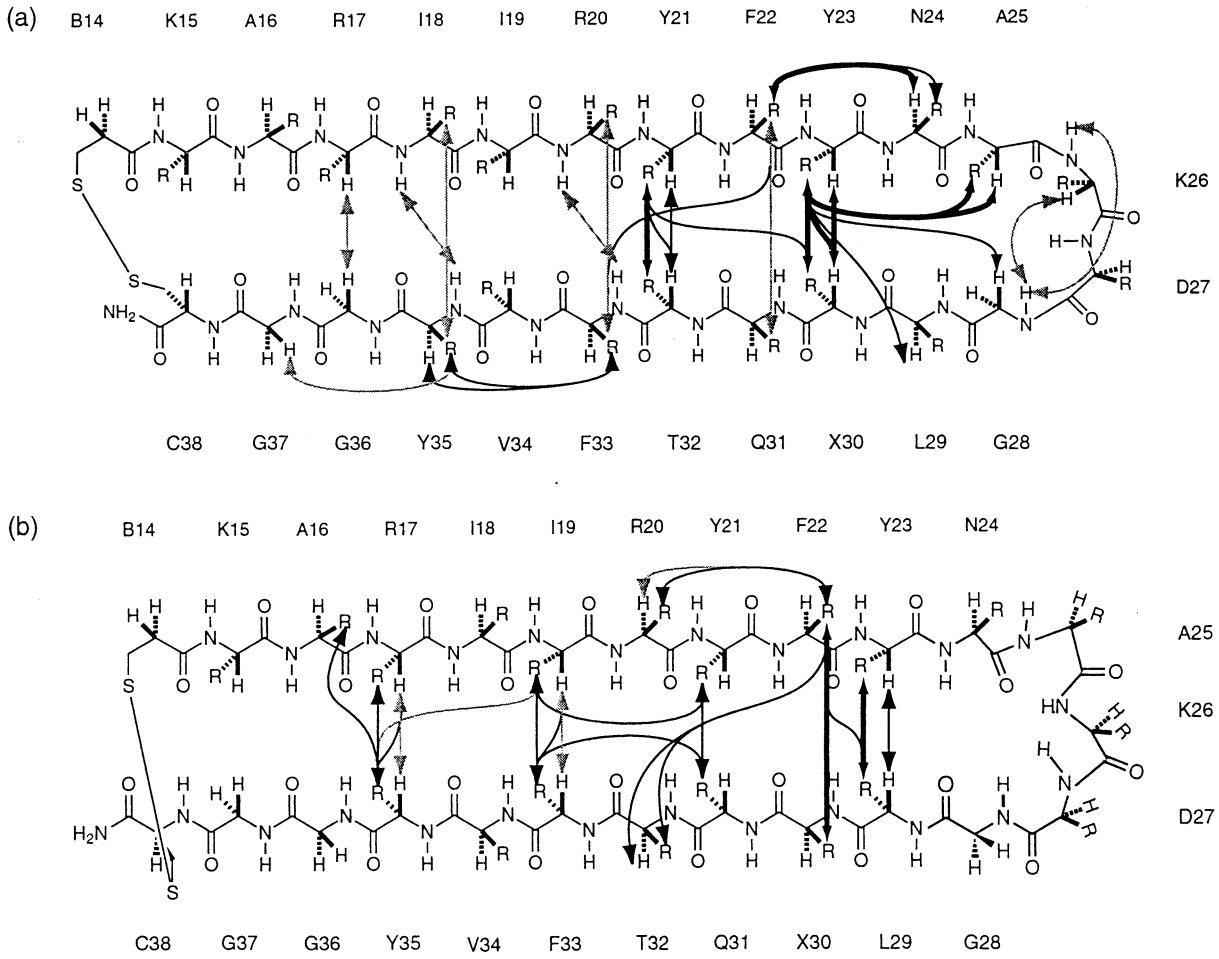

Figure 4. Schematic representation of long-range NOEs for the oxidized core module. NOEs observed in the oxidized module and in the 4:4 β-hairpin of native BPTI are shown in (a) as arrows superimposed on a 4:4 conformation. The other observed long-range NOEs in the oxidized module are consistent with a 3:5 β-hairpin, and are shown in (b) by arrows superimposed on a 3:5 β-conformation. The thickness and color of the arrow indicates NOE strength: strong ≡ black and thick; medium ≡ black and thin; weak ≡ grey and thin. To reduce visual clutter, only long-range aromatic-aliphatic, CαH—CαH, and NH—CαH NOEs are included. Reprinted with permission from Carulla et al.[18]

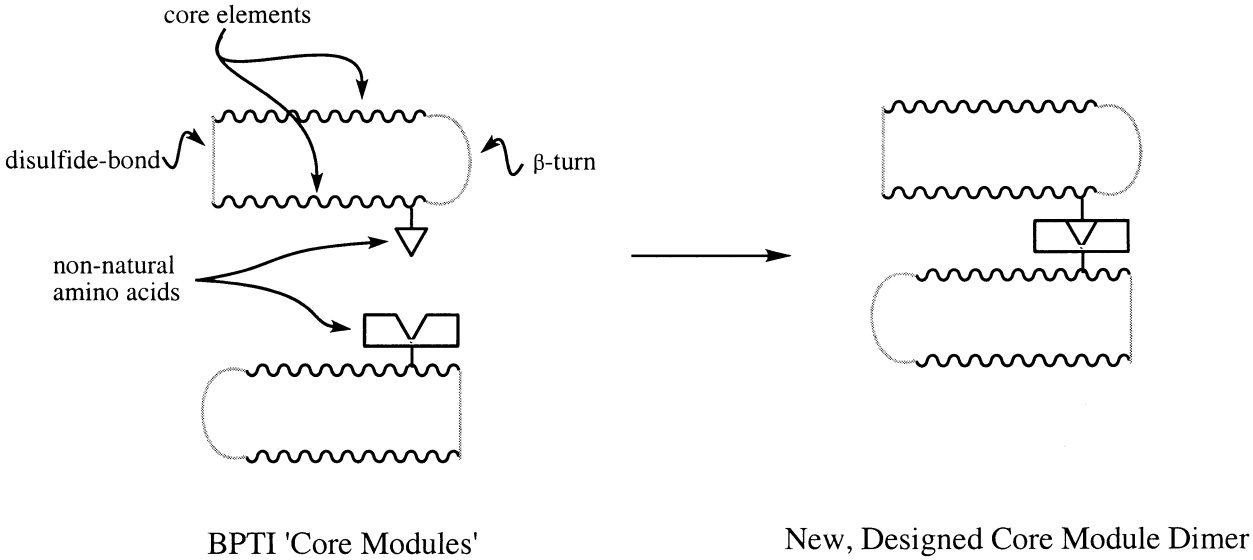

Figure 5. Schematic, general representation of our approach to a core module dimer. The triangle and the grooved rectangle represent the groups designed to react with each other to form a stable cross link.

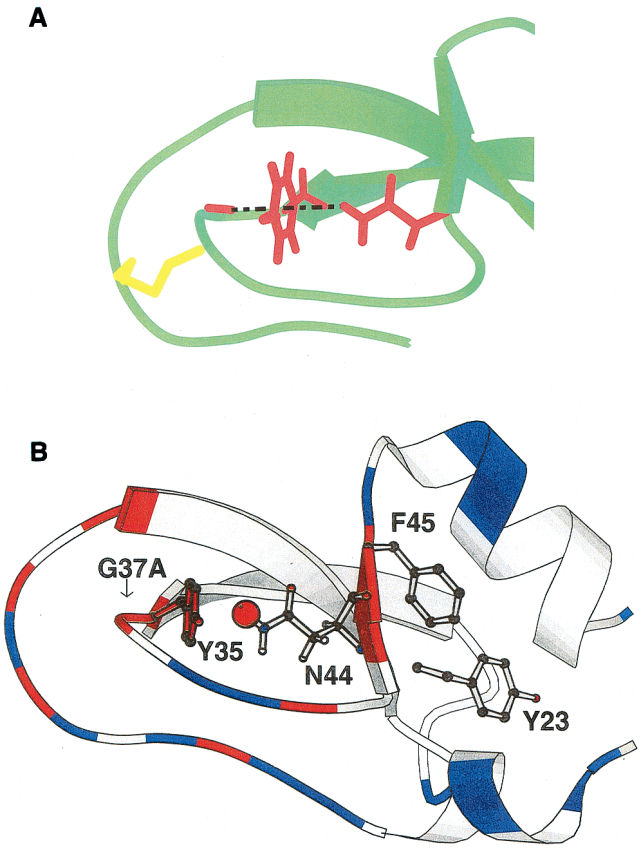

Figure 6. BPTI structures highlighting the site of the glycine to alanine replacement in G37A. (A) The loop region of wild type, showing the orientation of the groups involved in the NH-aromatic-NH interaction of Gly37 backbone NH, Tyr 35 side chain, and Asn 44 side chain NH (red), and the disulfide bond 14-38 between the loops (yellow). (B) Ribbon diagram showing the positions of NH groups that exchange significantly more rapidly in G37A than in wild type . Blue indicates positions that exchange too rapidly in WT to be quantified. Heavy atoms of aromatic rings whose flip rates have been measured are shown (red indicates acceleration of flip rate upon mutation). The large red CPK sphere on N44 indicates the side chain NH proton whose H/D exchange is accelerated upon mutation. Secondary structure elements are: helix 2–7, 47–56, beta-sheet 18–24, 29–35, 45 (beta-bridge), and loops/turns 8–17, 25–28, 36–44.

type BPTI; NOEs and the large upfield ring current shift (~4 ppm) of A37 HN clearly indicates that the average 37NH–35 ring geometry in G37A is similar to WT. Although the average G37A three-dimensional structure is indistinguishable from WT, the dynamics are altered significantly. In the region of the mutation increased flexibility is indicated by a >100-fold increase of Y35 ring flip rates and by a 10- to 1,000-fold increase of hydrogen exchange rates. Apparently, the majority of the 5-kcal/mol destabilization arises from local changes in the loop region of 37, most likely the combined energy penalties for maintaining an unfavorable backbone configuration and for disturbing the NH-aromatic interaction by increased flexibility in the region.

In summary, G37A is a case in which a single methyl group dramatically changes overall stability and local dynamics, but not average three-dimensional structure. The close similarity of WT and G37A structure, along with the increased local mobility of the mutant suggests that, on average, G37A contains the intact HN–aromatic interaction along with a strained backbone configuration of residues 36–37, but also has increased fluctuatations from the average to other conformations that relieve the strain and disrupt the HN–aromatic interaction. This is a particularly instructive example of the exquisite balance of stabilizing and destabilizing forces that shape proteins, and of the difficulty of surmising energetics from average structure.

Since the G37A mutation is outside the core of BPTI and the destabilization is so large, the location of perturbed amide groups and aromatic rings is of interest. For amides exchanging by the "folded state mechanism"[1] in G37A, there is no effect on exchange rates in the core, but there is a large increase in exchange rates in and around the mutation loop. Hydrogen isotope exchange rates for residue 37NH are 3 orders of magnitude faster in G37A than in WT. All amides accelerated in G37A are involved in hydrogen bonds to protein groups or to buried waters. The position of HN groups whose exchange by the folded state mechanism is at least an order of magnitude faster in G37A than in WT is superimposed on the protein backbone in Figure 6B A clear correlation between more rapid exchange in G37A and proximity to the mutation site is evident. Folded-state exchange rates of core backbone amides are not altered. In particular, amides of the β-sheet and helices in and near the core are not affected.

Similarly, flip rates of aromatic side chains outside the mutation region are not altered, while the ring within the core has highly accelerated flip rates. BPTI has three buried aromatic rings in the slow-exchange NMR regime for the symmetric protons at δ- and ϵ-positions of tyrosine and phenylalanine (Y23, Y35, and F45; Figure 6B). The presence of two distinct peaks for δ-protons indicates that the ring is motionally restricted and does not undergo free 180° rotations or "flips" about $\chi 2$. Not all tightly packed aromatic rings have slow exchange peaks for symmetric protons, but rather a single averaged peak, indicating intrinsic protein flexibility that allows rotations or flips faster than the millisecond time scale. Like hydrogen exchange, ring flip rates provide a qualitative measure of protein flexibility or dynamics in the vicinity of the ring. In WT BPTI, the flip rate for Y35 is one order of magnitude less than Y23 and two orders of magniture less than F45. In G37A, the Y35 flip rate is at least two orders of magnitude more than in WT, while Y23 and F45 flip rates are the same as WT. This indicates significantly increased internal motions in the region of the mutation but not in the rest of the molecule.

Both amide exchange rates and ring flip rates demonstrate the presence of motional subdomains, that is, molecular subdomains with different motional properties.[30] In the sense that we use the phrase, a motional subdomain is not *an* element of secondary structure, but rather a three-dimensional region that includes tertiary contacts of residues not close in primary sequence. Possible mechanisms for protein motions that give rise to hydrogen exchange from the folded state is a long standing debate (see Li and Woodward[1] and references therein). The effect of the G37A mutation on folded state exchange can be explained by a 'penetration' mechanism of small, noncooperative motions without involvement of locally coopera-

tive unfolding. Although mutations in the core have large effects on the stability of BPTI, they have minimal effects on folded-state exchange rates.[30] The mutants F22A, Y23A, N43G, and F45A all produce crevices with little rearrangement of surrounding atoms and little or no effect on the flexibility of the core subdomain, as monitored by folded-state hydrogen exchange.

Involvement of the G37–Y35 Interaction in Folding The polar NH–aromatic interaction between G37 and Y35 is implicated in BPTI folding due to the presence of an unusual upfield shift of G37 NH in small peptides[31] or reduced BPTI.[8] Also, in partially folded $[14\text{-}38]_{Abu}$, a nativelike aromatic interaction of 37 NH, with the hallmark upfield shift to ~4 ppm, is one of three conformations in slow exchange.[11] We have suggested, therefore, that the G37–Y35 interaction seeds the loop structure during folding. This possibility is supported by circular dichroism stopped flow studies, which show that G37A perturbs the folding kinetics of BPTI, causing a "rollover" (leveling out) in the Chevron plot of log (folding rate) versus denaturant.[32] Rollovers are not observed for WT or any other BPTI mutants we have studied. Rollovers are unusual and are proposed to be the result of a kinetic trap.[33] It would be unlikely for G37A to form nativelike 35–37 interactions during folding, since there is a large energy barrier for placing A37 backbone in a strained conformation, which is probably stabilized only after a larger set of cooperative interactions are formed. In the later stages of folding, after core conformations are mostly nativelike, ensembles favoring nonnative loop interactions may be formed, leading to a kinetic trap. The molecule would then have to 'climb' out of the trap before completing folding. This suggests a unique role of noncore loops in the folding process.

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