Structural and Folding Dynamic Properties of the T70N Variant of Human Lysozyme* [S]

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Definition of the transition mechanism from the native globular protein into fibrillar polymers was greatly improved by the biochemical and biophysical studies carried out on the two amyloidogenic variants of human lysozyme, B56T and D67H. Here we report thermodynamic and kinetic data on folding as well as structural features of a naturally occurring variant of human lysozyme, T70N, which is present in the British population at an allele frequency of 5% and, according to clinical and histopathological data, is not amyloidogenic. This variant is less stable than the wild-type protein by 3.7 kcal/mol, but more stable than the pathological, amyloidogenic variants. Unfolding kinetics in guanidine are six times faster than in the wild-type, but three and twenty times slower than in the amyloidogenic variants. Enzyme catalytic parameters, such as maximal velocity and affinity, are reduced in comparison to the wild-type. The solution structure, determined by 1H NMR and modeling calculations, exhibits a more compact arrangement at the interface between the β-sheet domain and the subsequent loop on one side and part of the α domain on the other side, compared with the wild-type protein. This is the opposite of the conformational variation shown by the amyloidogenic variant D67H, but it accounts for the reduced stability and catalytic performance of T70N.

Amyloidosis is an emerging category of diseases characterized by the extracellular accumulation of protein aggregates that share a common fibrillar conformation. The 20 proteins that can generate amyloid deposits in humans are extremely heterogeneous in function and structure, but, along the pathological transformation leading to aggregation and precipitation, all of them exhibit the same peculiar conformational pattern named cross-β structure, irrespective of the parent-starting arrangement (1). The lack of any sequence similarity and folding analogy among the amyloid-forming proteins led Dobson to conclude that the ability to form cross-β structure, wherein hydrogen bonds are formed between polypeptide chains in directions parallel to the fiber axis, is a generic property of polypeptide chains (2). Investigations of structure (3–4), folding dynamics (5–7), and fibrillogenesis (3, 8) of the initially reported amyloidogenic variants of lysozyme have made important contributions to a better understanding of the process involved in the conversion of globular proteins into amyloid fibrils. Amyloidogenic lysozymes represent probably the most convenient and informative model of fibrillogenesis from a globular protein. Besides being, in fact, one of the best characterized enzymes, its fibrillogenic mechanism is not influenced by protein fragmentation; nor, to our knowledge, does the wild-type species generate amyloid deposits in vivo, even in the elderly. Thorough analysis of several biochemical properties of the amyloidogenic variants in comparison to the wild-type species showed that pathogenic lysozymes are less stable than wild-type (3–8). This thermodynamic destabilization correlates with an increased concentration of partly unfolded intermediates that self-aggregate into fibrillar polymers. In this study we present the biochemical and structural characterization of a new natural variant of human lysozyme, T70N (9), that displays the general properties of a less stable and less efficient enzyme in comparison to wild-type but does not undergo pathological fibrillar conversion in vivo. The T70N variant is present in the British population with an allele frequency of 5% (9). The comparison of the biochemical characteristics of this variant with those of the amyloidogenic species can highlight the role of some of the folding abnormalities identified in the pathogenic species.

EXPERIMENTAL PROCEDURES

Clinical Studies—Genotyping for lysozyme T70N was performed as described previously (9) in 110 patients with systemic amyloidosis referred to the United Kingdom Centre for Amyloidosis, whose amyloid fibril type was initially uncharacterized but in whom the clinical phe-
not only was consistent with lysozyme amyloidosis. The variant was also sought in 23 patients with amyloidosis of unknown type but not in 110 controls. The proportion of individuals with I56T and D67H lysozyme amyloidosis. In these 55 patients, the T70N allele frequency was 0.08 (9/110 alleles, including one homozygote). The amyloid fibril type was subsequently identified as the T70N lysozyme variant. This has been the case despite investigations designed to identify such individuals. Amyloid fibril type is ultimately identified in all patients presenting at the United Kingdom Centre for Amyloidosis. Prior to fibril identification, the prevalence of the T70N lysozyme allele was determined in patients with systemic amyloidosis, the clinical phenotype associated with I56T and D67H lysozyme amyloidosis. In these 55 patients, the T70N allele frequency was 0.08 (9/110 alleles, including one homozygote), similar to the allele frequency in the normal population. In all other patients with amyloidosis seen at the clinic, the fibril type had been characterized (except one, see below). In these eight patients carrying at least one T70N allele, the amyloid fibril type was subsequently identified as serum amyloid A (two patients), AL amyloid (four patients), not amyloidosis (one patient, the homozygote), and hereditary amyloidosis of unknown fibril type (one patient). In the one patient with hereditary systemic amyloidosis, the fibril protein has yet to be characterized. It is not lysozyme, and an individual in the kindred has the T70N lysozyme but not amyloid. These data are consistent with the hypothesis that T70N variant does not cause systemic amyloidosis in the British population.

**RESULTS**

**T70N Lysozyme Is Not Amyloidogenic in Vivo**—Several clinical and biochemical findings suggest that T70N lysozyme is not amyloidogenic. The allele frequency of T70N is relatively high in the normal population (5/100) (9). Both I56T and D67H are very rare and have only been identified in kindreds with amyloidosis wherein there is 100% penetrance (25). If T70N was as amyloidogenic as these variants, 5% of the British population might be expected to have amyloidosis. However, no individual with T70N lysozyme amyloidosis has ever been identified. This has been the case despite investigations designed to identify such individuals. Amyloid fibril type is ultimately identified in all patients presenting at the United Kingdom Centre for Amyloidosis. Prior to fibril identification, the prevalence of the T70N lysozyme allele was determined in patients with systemic amyloidosis, the clinical phenotype associated with I56T and D67H lysozyme amyloidosis. In these 55 patients, the T70N allele frequency was 0.08 (9/110 alleles, including one homozygote), similar to the allele frequency in the normal population. In all other patients with amyloidosis seen at the clinic, the fibril type had been characterized (except one, see below). In these eight patients carrying at least one T70N allele, the amyloid fibril type was subsequently identified as serum amyloid A (two patients), AL amyloid (four patients), not amyloidosis (one patient, the homozygote), and hereditary amyloidosis of unknown fibril type (one patient). In the one patient with hereditary systemic amyloidosis, the fibril protein has yet to be characterized. It is not lysozyme, and an individual in the kindred has the T70N lysozyme but not amyloid. These data are consistent with the hypothesis that T70N variant does not cause systemic amyloidosis in the British population.

**Non-amyloidogenic Natural Variant of Human Lysozyme**

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1 The abbreviations used are: AL, immunoglobulin light chain amyloidosis; CD, circular dichroism; DQF-COSY, double quantum-filtered correlated spectroscopy; GdnHCl, guanidine hydrochloride; MD, molecular dynamic; (NAG)3, β-p1,4-linked trimer of N-acetyl-β-glucosamine; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; TOCSY, total correlation spectroscopy.
To test whether there was co-deposition of lysozyme within the amyloid fibrils created by other proteins, we have isolated the natural amyloid fibrils from amyloid deposits of one heterozygous T70N heterozygote patient clearly affected by AL amyloidosis. No lysozyme was detected in the fibrils by immunoblot. To test whether T70N lysozyme was interacting with other fibril types, the allele frequency in rheumatoid arthritis patients (2 T70N from 56 alleles) and AL (4/120) amyloidosis groups was compared. Asn-70 was not over- or under-represented, thus neither promoting nor inhibiting fibril formation. Finally, in the one subject homozygous for the mutation, serum lysozyme concentration was determined, and, according to the lysoplate method, the circulating protein was 12 mg/liter (nv 4–13 mg/liter.)

Equilibrium Denaturation of T70N Lysozyme—The unfolding of the T70N variant was monitored at equilibrium by intrinsic fluorescence emission at 340 nm as a function of denaturant concentration at pH 6.5 and 20 °C. The unfolding curves for the wild-type, T70N, I56T, and D67H, normalized to the fraction of unfolded protein (f_u), are shown in Fig. 1. All transitions are characterized by the presence of a single sharp change in the fluorescence intensity that is typical of cooperative transition in a two-state system. The transition midpoints are reduced, as compared with that of the wild-type protein, by 0.5, 0.9, and 1.3 denaturant concentration units for the T70N, D67H, and I56T, respectively. The data for the I56T and D67H variants are in good agreement with previous data of Takano et al. (6). The values of ΔG^H2O of unfolding, calculated according to Santoro and Bolen (10), indicate that the three variants, T70N, D67H and I56T, are destabilized, in comparison to the wild-type, by 3.7, 4.7, and 7.2 kcal/mol, respectively.

Thermal Unfolding—We have used circular dichroism to monitor T70N lysozyme unfolding behavior upon heating from 20 to 90 °C. The measurements were performed in the far and near UV regions (Fig. 2, a and b) to estimate the equilibrium thermal unfolding of the protein at pH 5. The coincidence of the two transition curves by the CD data at 222 and 270 nm, normalized to the apparent fraction of unfolded species (Fig. 2c), was observed over the entire temperature range studied. Such behavior is quite similar to the cooperative two-state unfolding displayed by the wild-type protein under these conditions (3), even if the midpoint of thermal denaturation of T70N variant is 3 °C below that of the wild-type lysozyme. From the analysis of CD measurements, there is no evidence of the existence of an intermediate state with a helical secondary structure but lacking tertiary interactions as was previously documented in the amyloidogenic species (3).

Enzyme Catalysis Performance—The enzyme kinetics data (Table I) suggest that T70N lysozyme has a reduced enzymatic activity with respect to wild-type, comparable with that reported for the D67H variant. This comparatively poor performance is the combined result of both a lower substrate affinity and a less efficient turnover. The reduced affinity of T70N for the chitotriose (NAG)_3, the trisaccharide inhibitor of lysozyme, which can be inferred from the dissociation constant (K_d) value of Table I, is also confirmed in D67H and I56T, but the T70N

![Fig. 1. Equilibrium GdnHCl denaturation curve.](http://www.jbc.org/)

![Fig. 2. Thermal denaturation of T70N lysozyme.](http://www.jbc.org/)

FIG. 1. Equilibrium GdnHCl denaturation curve. GdnHCl unfolding curves for the wild-type (●), T70N (○), D67H (▲), and I56T lysozymes (△) at pH 6.5 and 20 °C are shown. The solid lines represent the results of the fitting procedure.

FIG. 2. Thermal denaturation of T70N lysozyme. CD spectra in the far UV (a) and near UV (b) of the protein in water at pH 5 and recorded at 20 (○), 60 (△), 70 (▲), and 90 °C (●) are shown. Unfolded fraction of T70N lysozyme (c) was estimated from the ellipticities at 222 nm (■) and 270 nm (△) as a function of temperature. The midpoint of thermal denaturation is 71 °C.
variant has the lowest affinity for \((\text{NAG})_3\) of the all natural human lysozyme variants.

**Unfolding-Refolding Kinetics of Mutant Human Lysozymes**—To assess the effects of the T70N substitution on the folding and unfolding kinetics, comparative stopped-flow kinetic studies of the reversible unfolding-refolding process were performed. The unfolding-refolding reactions were monitored by fluorescence intensity. The unfolding kinetics of T70N are described by a single exponential function, as reported previously (5–6). The refolding reaction from the guanidine-denatured protein consists of two phases in which the fast phase is predominant, in amplitude, over the slow phase as shown previously (5–6). No significant differences were found between T70N variant and the wild-type protein in the rates of the refolding phases (data not shown). On the contrary, the unfolding process of the T70N is 3–4 times faster than that of the wild-type protein. Fig. 3 depicts the unfolding kinetics of wild-type, T70N, D67H, and I56T. The unfolding rates for the amyloidogenic mutants are consistent with the data reported previously by Canet et al. (5) and Takano et al. (6) and are, respectively, three and twenty times faster than that of the T70N variant.

**\(^1\)H NMR Chemical Shift Changes**—Based on the assignments of wild-type human lysozyme (23) and amyloidogenic variants (4), the \(^1\)H chemical shifts for all residues of the T70N lysozyme were carefully controlled (at least for the backbone resonances) by standard methodology (27). The deviations of the \(^1\)H chemical shift values with respect to the wild-type protein are shown in Fig. 4a. Comparison with the corresponding histograms reported for the I56T and D67H mutants (4) suggests that the T70N variant has a \(\Delta \delta \text{HN}^2\) pattern somewhat intermediate, i.e. whereas the extent of \(\delta \text{HN}^2\) deviations is generally limited, similar to I56T, a number of \(\Delta \delta \text{HN}^2\) above the average are observed, as with D67H, for residues 52, 63, 64, 67, 72, and 77 (upfield) and residues 68, 69, 73, 76, and 79 (downfield). The interpretation of amide chemical shift changes may be complex and tricky, but there is no doubt that all of the variations seen with T70N are spread over the corresponding locations of the \(\beta\)-sheet, the subsequent loop, and the following \(3_1\) helix segment of the wild-type structure. For proteins, \(^1\)H NMR chemical shifts exhibit an established correlation with secondary structure only for \(^1\)H resonances (28). When compared with D67H, the \(\Delta \delta \text{HN}^0\) of T70N show meaningful differences only at positions 51, 61, 66, 68, 73, and 80 (Fig. 4b). In general, the accepted threshold for meaningful difference, i.e. \(\Delta \delta \text{HN}^0 \pm 0.1\) ppm, refers to comparison with peptides in a statistically disordered conformation. Thus, to ascertain meaningfulness, the deviations of Fig. 4b were analyzed against the corresponding parameters obtained from the comparison with the wild-type protein data, which, in turn, were compared with the basic peptide shifts. In particular, on moving from the D67H to the T70N mutant, the downfield shifts at residues 51, 61, and 66 suggest that the conformation and extension of the \(\beta\) strands of the wild-type tend to be restored. Similarly, a recovery of the wild-type geometry at the end of the turn-like segment 70–73 is likely to be responsible of the opposite shift observed for residue 73. An analogous interpretation applies also to the upfield shift of residue 68 and the downfield shift of residue 80.

**Restrained Modeling**—To test the structural inference obtained from the assignment and analysis of chemical shift changes in mutant T70N with respect to D67H, I56T, and wild-type proteins, a number of unambiguously classified NOEs, connecting hydrogens in the region 41–92 of the investigated mutant, were quantified to extract the relative internuclear separations. The substantial invariance of the chemical shifts of T70N with respect to the wild-type protein in the fragments that flank the region 41–92 should grossly reflect a persistence of the local spatial arrangement of the canonical structure. It appears therefore conceivable to adopt the geometry of the original structure outside the fragment 41–92. To constrain part of the structure in a fixed arrangement, tethered MD simulations were performed using the standard tools of Discover software (Accelrys) (24). Because of the high degree of signal overlap in the lysozyme spectrum, the collection of the NOEY cross-peaks for quantitative purposes was necessarily limited to resolved correlations of the selected region resonances. Overall, 125 internuclear distances, mostly medium

### Table I

| Protein   | \(C_m\) \(\text{kcal/mol}^{-1}\) | \(\Delta \Delta G_{m}^{0}\) \(\text{kcal/mol}^{-1}\) | \(K_m\) \(\mu\text{M}\) | \(k_{\text{cat}}\) \(\text{min}^{-1}\) | \(K_d\) \(\mu\text{M}\) | \(k_{\text{cat}}/k_d\) |
|-----------|-------------------------------|---------------------------------|----------------|----------------|----------------|----------------|
| Wild type | 3.4                           | 9.99                            | 3.71           | 30             | 0.095          | 0.014          |
| T70N      | 2.4                           | 6.47                            | 7.23           | 18.5           | 0.34           | 0.03           |
| I56T      | 2.8                           | 8.99                            | 4.71           | 38             | 0.34           | 0.03           |
| D67H      | 2.8                           | 8.99                            | 4.71           | 38             | 0.34           | 0.03           |

a: GdnHCl concentration of the midpoint of equilibrium denaturation; experimental error is \(\pm 0.1\) M.

b: Free energy change in the absence of denaturant; experimental error is \(\pm 10\)%.  

\(\Delta \Delta G_{m}^{0} = \Delta \Delta G_{m}^{0}\) (mutant) – \(\Delta \Delta G_{m}^{0}\) (wild type).

d: Enzyme activities determined with PNP-(GlcNAc) \(_3\); experimental errors are \(\pm 20\%\) for \(K_m\) and \(\pm 5\%\) for \(k_{\text{cat}}/k_d\).

e: Dissociation constants for (NAG), obtained at pH 7.2 and 30 °C; values are mean \(\pm\) standard deviation.

f: Unfolding rate constants determined at pH 5 with 5.4 M GdnHCl, and 20 °C; values are mean \(\pm\) standard deviation.
Conformation of Relevant Side Chains—Because a drastic geometry change of a few side chains around the mutation site of D67H accompanies the loss of the wild-type H-bond network that has been considered to affect significantly the stability of the lysozyme β-domain (3), a detailed conformational analysis of some relevant side chains in variant T70N was attempted. Additional independent evidence was necessary to characterize in detail these structural features because of the resolution limits of the tethered MD results. In the wild-type structure, the side chain of residue 67 is involved in one or possibly two H-bonds to residue 70 (T70N-D67O$^{\delta1}$ and T70O$^{\nu L}$-D67O$^{\delta1}$) (Fig. 6a). The number of H-bonds of residue 70 is conserved in the D67H mutant, although none of the original side chain H-bonds survives for the mutated residue (Fig. 6b). The examination of the NOESY and DQF-COSY pattern of T70N and wild-type lysozymes in H$_2$O and D$_2$O enabled us to conclude that both molecules possess the same conformation of the Asp-67 side chain with $\chi_1 \approx 60^\circ$. This geometry is consistent with all coordinate sets available for the wild-type lysozyme from x-ray data and, because it can accommodate the mentioned H-bonds to residue 70, the question arises whether the mutation of threonyl into asparaginyl in T70N still supports those H-bonds. Unfortunately the spectra of the T70N lysozyme showed resonance degeneration for the two prochiral H$^\beta$s of Asn-70, which hinders extracting their diastereotopic assignments and the local conformation, as was done with Asp-67. The chemical equivalence of Asn-70 H$^\beta$ resonances may be only fortuitous rather than due to rotational averaging that would not support an H-bond involvement of Asn-70 side-chain carboxamide. High resolution one-dimensional NOESY measurements enabled us to measure precisely the chemical shifts of a few side-chain amides in the range 33–40 $^\circ$C. The calculated chemical shift temperature coefficients of Asn-70 side-chain amides were $-3$ ppb/deg and $-6$ ppb/deg for the anti (H$^{\delta1}$) and syn (H$^{\delta2}$) amide resonances, respectively. Values between $-6$ and $-8$ ppb/deg were obtained also for other primary amide pairs of the T70N lysozyme. Because $|\Delta \delta/\Delta T| > 4-5$ ppb/deg is typically observed for solvent-exposed primary and secondary amides when not involved in intramolecular H-bonds, our result is consistent with the occurrence of an H-bond at the Asn-70 anti-amide hydrogen. According to the structure obtained from the tethered MD calculation described previously, Asn-70 H$^{\delta2}$ could form two simultaneous H-bonds with Asp-67 and Ser-61 side-chain oxygen atoms, with the Asp-67 side chain adopting a $\chi_1$ value close to 60 $^\circ$. Thus two independent lines of evidence converge toward the same conclusion, namely that in the T70N lysozyme the spatial arrangement of the Asp-67 side chain is preserved along with some H-bonds that are part of the interaction network within the large loop of the β-domain in the wild-type lysozyme.

Isotope Exchange Measurements—To further confirm the previous structural conclusions, $^1$H-$^2$H amide exchange rate determinations were performed on the mutant T70N. The protection factors were calculated from the experimental apparent kinetic constants. The values should be compared with the analogous data obtained for the wild-type and the mutant D67H (7), as shown in Fig. 7, for a specific selection. Apart from a few limited local discrepancies, which are likely to arise from the specific experimental conditions of each determination, the most important difference among our and previous data sets is observed in the segment 65–90, comprising the last stretch of the four-stranded sheet, the loop, the $\beta_{10}$ helix, and the connection turn to helix C (according to the wild-type protein geometry). In particular, in T70N the NH of Cys-65 recovers the extremely high protection factor that is lost in mutant D67H.
whereas for residue 66 a protection factor \( > 10^4 \) is obtained. The \( \beta \) helix appears more protected in mutant T70N than even in the wild-type protein. Four residues in T70N (83, 84, 85, and 86) exhibit slow or moderately slow amide exchange, with protection factors ranging between 200 and 5000, against only two residues (84 and 85) in the wild-type protein. A similar degree of protection is observed also for the amides of residues 74, 77, 79, and 90 of T70N. The same amides, instead, were reported to exhibit no exchange protection in the D67H variant in the comparative study with the wild-type species (7).

Previous qualitative \(^1\)H-\(^2\)H exchange data on human lysozyme at pH 3.8 had shown slow amide exchange rates for residues 65, 66, 76, and 77 and intermediate rates for residues 79 and 86 (23). Therefore the amide exchange pattern of the T70N lysozyme can be considered quite related to that of the wild-type protein.

**DISCUSSION**

**Linking Functional and Structural Properties of T70N Variant**—Compared with the structure of wild-type lysozyme, the T70N replacement generates a more compact arrangement at the interface between the \( \beta \)-sheet and subsequent loop from one side and part of the \( \alpha \)-domain from the other side. In particular, the large loop 66–74 moves toward helix C, followed by \( \beta \)-sheet 42–55 that extends in a plane nearly perpendicular to the former loop and thus shifts away from helix D. This is the opposite of the conformational variation observed in mutant D67H (3). Based on the deuterium exchange results, some
additional structural deviations of T70N lysozyme should occur in regions further apart from the mutation site. The differences should concern the Ser-80 capping role, the actual extension of the 310 helix, and the angle between the latter and helix C as imposed by the intervening turn. However, the extent of divergence of T70N from the wild-type structure is much more limited than the deviations observed with the D67H mutant, and, overall, when compared with the latter, T70N resembles the wild-type species much more closely (Fig. 5). The network of H-bonds that stabilizes the β-domain of lysozyme (3) appears preserved in the variant containing asparagine at position 70, as demonstrated by direct determination of conformation and H-bond involvement for the side chains of Asp-67 and Asn-70, respectively (Fig. 6). In the wild-type structure, that network connects Thr-52, Tyr-54, Ser-61, Asp-67, and Thr-70, and its rupture in the D67H mutant determines extensive structural modifications and pathological destabilization (3). Similar effects have been documented also for mutation at residue 70 with Ala and Val (29). The T70A substitution results in local disorder that prevents structure determination. The analogous mutation into Val, however, induces a significant rearrangement in the region 68–78 that preserves the local geometry and packing through the involvement of the Lys-69 side chain for the H-bonds and that of Arg-62 and Tyr-63 for interaction with the isopropyl group. Indeed, at 64.9 °C and pH 2.7, mutant T70V is destabilized by 2.9 kJ/mol with respect to wild-type, whereas T70A loses 6.2 kJ/mol of stability (29). The structural elements obtained by our 1H NMR study qualitatively account for the stability properties of T70N. The wild-type packing geometry between the β and α-domains is largely preserved along with the H-bonding pattern within the β-domain loop. Thus, the corresponding contributions to the folding free energy should be conserved as well. Based on the analogy of the comparison between T70V and T70A or the T70N and D67H mutants, one may be tempted to link, in any lysozyme, the conservation of the H-bond network of the β-domain or the whole structure with folding stability. The significant destabilization of mutant I56T, despite substantial invariance with respect to wild-type fold (3), suggests that such a generalization may be too simplistic and risky. The features of the H-bond network of the β-domain should be explicitly addressed. In addition, the surface hydration structure should also be considered (30) before drawing stability conclusions from structural invariance.

The 1H NMR results qualitatively account for the activity loss of T70N also (Table I). Because of the shift of β-sheet 42–55, the width of the catalytic cleft is increased compared with that of the wild-type protein (Fig. 8), and, hence, a decrease of affinity should ensue that is expected to become more evident the smaller the substrates or inhibitors are. A decreased affinity should be responsible for the significant in-

**Fig. 7. Comparison of the protection factors.** Protection factors within the fragment 65–90 of T70N (black bars), wild-type (gray bars), and D67H (white bars) lysozyme. The bar is not reported on the scale when the corresponding protection factor value was below 10^2. The values for wild-type and D67H are taken from Canet et al. (7).

**Fig. 8. Best fit superposition of wild-type lysozyme-NAG complex (PDB ID 1LZR) and the T70N lysozyme in the region of the catalytic site.** The wild-type protein structures with and without substrate do not show any major difference in practice. A single ribbon trace is drawn outside the region 42–90. The Cα traces are red for wild-type and blue for the T70N variant. The presence of the substrate, shown as a green space-filling model, highlights the increased width of the catalytic cleft in the variant protein.
crease of the equilibrium dissociation constant of T70N and the (NAG)₃ inhibitor. Along the same lines, one can also explain the data obtained with the chitin oligosaccharide used for testing the catalytic activities. The Kᵣ of T70N increases and reaches a similar value to that seen for the pathogenic mutant D67H, for which the activity loss results from a more complex conformational rearrangement. Overall however, the affinity loss is reduced with a substrate larger than the (NAG)₃ inhibitor as argued from comparison of Kᵣ and Kᵢ values (Table I).

The T70N Variant Is Less Destabilized Compared with the I56T and D67H Amyloidogenic Variants—The equilibrium unfolding experiments show that the T70N variant is less stable than the wild-type lysozyme. Nevertheless, the free energy destabilization resulting from mutation is less pronounced than that resulting from the previously characterized amyloidogenic variants I56T and D67H. This “intermediate” behavior of the T70N variant is also observed in its unfolding rate, which is faster than that of the wild-type but slower than those of the two amyloidogenic variants. Finally, as discussed above, the structural perturbation derived from the T70N substitution is not as marked as that resulting from the two established amyloidogenic mutations. These three observations are all correlated. The minor structural perturbation of the T70N variant, compared with those of the amyloidogenic variants, indeed results in a lower free energy destabilization and a less important acceleration of the unfolding reaction. The ultimate result is that the partially denatured state that is thought to promote fibrillogenesis of lysozyme in vitro (3, 7) is not as significantly populated as in patients in which one of the two amyloidogenic variants is present. Fully consistent with these data are the results obtained by monitoring thermodenaturation in the near and far UV CD. By this analysis, one of the main differences in the unfolding pathways of normal and pathological species was previously highlighted (3). Temperature-dependent unfolding of the amyloidogenic variant appeared to be not cooperative, and the partially unfolded state was highly populated near the midpoint of unfolding. T70N, to the contrary, presents a cooperative unfolding transition in which the loss of tertiary and secondary structures are concomitant. These data prompt us to exclude the presence of the partially unfolded structure at the midpoint of denaturation for this new variant, and, even if the melting point is lower, the mode of denaturation appears to be quite similar to that reported for the wild-type lysozyme.

Concluding Remarks—Globular protein stability has been inversely correlated with the propensity to form amyloid fibrils in vitro (31–33). The T70N lysozyme variant represents, in the category of amyloidogenic proteins of medical interest, the first globular protein in which the mutation destabilizes the molecule but does not cause, in vitro, the pathological conversion of the globular protein into amyloid fibrils. The comparison of the properties shared by wild-type and T70N, on one side, with those shared by I56T and D67H, on the other, allows one to highlight the abnormalities involved in vitro in the genesis of amyloid disease. The biochemical effects of the mutation make this new lysozyme variant less active, less stable, and less protected than the wild-type form against a denaturing environment, but, notwithstanding the destabilizing mutation, the level of amide exchange protection is not affected or is even increased, as for helix 3–10. This means that the local stability, which contributes to the overall folding free energy, is preserved, i.e. structures, with unfolded deviation beyond a certain level to allow competitive formation of the amyloid aggregate, are poorly represented within the statistical ensemble of the native folded state (34). Therefore the conformational modifications induced by the replacement of Thr in position 70 with Asn minimize fluctuations from the folded to a partially folded or an unfolded state, which appears to be one of the key pathogenic properties of amyloidogenic variants (7). Thus the hypothesis that amyloid transition involves those fluctuations is consistent with our observations on T70N, wherein such fluctuations should be much less extensive than in the pathological variants. In the field of new therapeutic strategies against conformational diseases (35) in which pathogen point mutations of target proteins affect their stability, the discovery of destabilized variants devoid of pathological implication bears the relevance of a finely adjusted model. As a matter of fact, stabilization through synthetic ligands is becoming a promising approach against these diseases, and encouraging experimental results have been provided for the amyloidogenic variants of transthyretin (36) or, for a completely different type of disease, the oncogenic variants of the p53 protein (37). The demonstration of the existence of thermodynamic and kinetic thresholds that are lower than those of wild-type proteins but still adequate to protect from the pathological conversion suggests that conformational diseases could be prevented even with a partial stabilization of the pathological proteins.

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