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Oxidative Refolding of Amyloidogenic Variants of Human Lysozyme

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The oxidative refolding of human lysozyme and its two best characterised amyloidogenic variants, Ile56Thr and Asp67His, has been investigated *in vitro* by means of the concerted application of a range of biophysical techniques. The results show that in each case the ensemble of reduced denatured conformers initially collapses into a large number of unstructured intermediates with one or two disulphide bonds, the majority of which then fold to form the native-like three-disulphide intermediate, des-[77–95]. The slow step in the overall folding reaction involves the rearrangement of the latter to the fully oxidised native protein containing four disulphide bonds. The Ile56Thr and Asp67His variants were found to fold faster than the wild-type protein by a factor of 2 and 3 respectively, an observation that can be attributed primarily to the reduction in the barriers to conformational rearrangements that results from both the mutations. The efficient folding of these variants despite their enhanced propensities to aggregate when compared to the wild-type protein is consistent with their ability to be secreted in sufficient quantities to give rise to the systemic amyloidoses with which they are associated.

Keywords: amyloidogenic disease; disulphide bonds; human lysozyme; protein refolding

Introduction

Four single point mutations of human lysozyme have been identified as being involved in a familial systemic amyloid disease in which up to kilogram quantities of protein can be deposited in vital organs including the liver spleen and kidneys.[1–3] The two point mutations studied in greatest detail so far involve the non-conservative substitutions, Asp67His and Ile56Thr.[1] The two variant proteins are enzymatically active and X-ray crystallography has shown that the structures of the native states of both variants resemble closely that of the wild-type protein. There are, however, subtle differences at the interface between the two structural lobes of the protein (denoted the α and β domains), which could be significant in the context of the amyloidogenic behaviour of the two proteins.[4] Indeed, detailed studies of the mechanism of aggregation of the normally soluble protein into amyloid fibrils of the type associated with systemic disease have indicated that the reduced stability induced by the amyloidogenic mutation, and particularly the loss of cooperativity between the two domains of the protein, are of crucial importance.[4–8] Such findings have contributed significantly to our present level of understanding of the amyloidogenic behaviour of proteins in general.[9,10]

Lysozyme has been widely studied in order to enhance our understanding of the process of protein folding and its relationship to misfolding, aggregation and disease. The non-oxidative folding of the wild-type protein has been investigated in particular detail,[5,11] and recently the refolding behaviour of the two major amyloidogenic variants of lysozyme has also been characterised and the kinetics compared to those of the wild-type protein. As with the wild-type protein, both variants fold with kinetics that show three distinct phases.[5,12] The fast phase occurs within the dead-time of the stopped-flow experiments (ca 3.5 ms) and involves ~10% of the population of molecules for all three proteins. The origin of this phase is unclear but is

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Abbreviations used: ANS, 8-anilino-1-naphthalene sulfonic acid; ER, endoplasmic reticulum; DNTB, dinitrothiobenzolate; NTB, nitrothiobenzolate.

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likely to be indicative of a population of molecules able to form the native state more efficiently than the remainder of the molecules. The intermediate phase in the folding kinetics is associated with the development of native-like protection of amide hydrogen atoms *via* two intermediates that involve the formation of structure within the two structural domains. The kinetics of this phase are similar to those of the wild-type protein for the Asp67His variant but are significantly (sixfold) slower for the Ile56Thr protein. This finding suggests that residue 56 is in a region of the protein that becomes an at least partially structured transition state for folding, a view consistent with the fact that this residue is located at the interface of the two structural domains. The third slow phase has been tentatively attributed to the docking of the α and β domains and the concomitant close-packing of all the side-chains, processes that are slower for both mutants than for the wild-type protein.[5]

An important finding from studies of the amyloid deposits formed by the lysozyme variants *in vivo* is that they are extracellular and all four native disulphide bonds are intact.[1,4] Therefore, it can be assumed that oxidative folding of the mutant proteins is able to occur relatively efficiently *in vivo* such that they escape to some extent at least the quality control processes that result in the degradation of many proteins with destabilising mutations within the cells in which they are produced.[13] Indeed, it is known that in individuals who are heterozygotic for the single base change responsible for the amino acid substitution the variant protein is secreted in a correctly folded form along with the wild-type protein.[1] It is therefore of considerable interest to examine the refolding of human lysozyme and its amyloidogenic mutants from their reduced states (i.e. with no disulphide bonds present) to their fully oxidised states (i.e. all four native-like disulphide bonds formed). As it is an extracellular protein, oxidative folding of lysozyme occurs *in vivo* within the endoplasmic reticulum (ER) and must be completed efficiently in order for the protein to be targeted for secretion from the cell.[13]

The oxidative refolding of hen lysozyme, which shares 40% sequence identity with the human protein, has recently been investigated by a variety of biophysical techniques. Conditions were found in which reduced hen lysozyme can refold in high yield at protein concentrations sufficiently high (ca 25 μM) for the successful application of the various biophysical techniques.[14,15] Refolding was monitored by the recovery of enzymatic activity, by optical spectroscopy (fluorescence and CD) and by monitoring the distributions of species formed at different stages of the folding reaction using HPLC.[14,15] In addition, the overall formation of the four disulphide bonds was followed by monitoring the disappearance of the free thiol groups. The protein molecules were found to fold by two distinct tracks: a fast track, in which the native structure forms efficiently, and a slower track in which the three disulphide intermediate des-[76–94] accumulates. This intermediate lacks the disulphide bond that links the α and β domains, and it is the structural reorganisation required for formation of this bond that is the rate-limiting step in the folding process. Here, we describe the study of human lysozyme and its amyloidogenic variants in a similar manner to that used to study hen lysozyme. We show that both disease-associated variants in fact achieve their native structures more rapidly than the wild-type protein, a result that can be attributed to the more facile reorganisation of the folding intermediates in the destabilised proteins.

Results

Oxidative folding of wild-type human lysozyme

Wild-type lysozyme was fully reduced by incubation in a buffer solution containing 8 M urea, 100 mM Tris, and a 25-fold excess of DTT over protein disulphide bonds, using procedures described for hen lysozme.[15,16] The CD spectrum of the protein is indicative of a random coil conformation and the protein gave a single elution peak when analysed by reverse phase HPLC. The number of free thiol groups was measured as 7.98(±0.03) by Ellman's reagent confirming that the protein was fully reduced.[17]

The formation of disulphide bonds during oxidative refolding was followed by monitoring the change in the concentration of free thiols as a

Table 1. Refolding of wild-type and amyloidogenic variants of human lysozyme followed by various biophysical techniques by fitting the data to single, double or triple exponential functions

	Wild-type (min)			Asp67His (min)			Ile56Thr (min)		
Method	τ_1	τ_2	τ_3	τ_1	τ_2	τ_3	τ_1	τ_2	τ_3
Enzymatic activity	–	12.2±1.1	125±8	–	4.2±1.4	47.7±2.3	–	5.25±1.0	65.0±5.2
Intrinsic fluorescence	0.67±0.15	12.4±0.5	120±4	–	4.21±1.35	44.7±3.5	–	5.75±1.2	59.5±3.8
Far-UV CD	–	12.1±0.9	–	–	3.47±0.9	–	–	5.19±0.4	–
HPLC	–	12.5±1.1	131±14	–	4.8±5.2	48.0±5.2	–	5.5±0.8	62.1±4.2
SS bond formation	1.8±0.3	15.3±1.2	[a]	0.5±0.1	5.0±0.4	[a]	0.7±0.2	6.7±0.5	[a]

The time constants given here are an average of at least four experiments.

[a] The data in the experiments detailing free thiol groups could not be fitted to the third phase, although the presence of the refolding of the fraction of the protein that occurs through this route may be reflected in a larger time constant for τ_2. A similar situation was found with the hen protein.[14]

function of time after diluting aliquots of the solution containing the reduced protein into refolding buffer. The kinetics were found to be biphasic (Table 1, Figure 1(f)). During the initial phase, with a time constant of 1.8(±0.3) minutes, an average of two disulphide bonds per protein molecule are formed. The remaining two disulphide bonds were observed to form during a second slower phase, with a time constant measured as 15.3(±1.2) minutes. The recovery of enzymatic activity was also followed during the refolding process by assaying aliquots of the refolding mixture removed at different time points during the reaction. An initial lag phase of ~2 minutes (Figure 1(a)), was observed, in accord with that seen previously during the oxidative refolding of the hen protein,[15,18] reflecting the requirement of a highly native-like structure for activity. Although the refolding process is clearly complex, the kinetics could be approximated by a double exponential curve with time constants, $\tau_1 \sim 12$ minutes and $\tau_2 \sim 120$ minutes (Figure 1(a)). The first time constant is comparable to that associated with the formation of the fully native disulphide bonds, as discussed above. The second time constant represents a very slow phase, that can be attributed to folding *via* pathways that involve the population of native-like intermediate states with lower enzymatic activities than the native protein; a similar phase is seen for the hen protein. The slow conversion of the various three disulphide intermediates to the native protein was not resolvable as a separate kinetic phase associated with disulphide bond formation, an observation that may be attributable at least in part to the location of the free thiols within the partially folded proteins as well as to the sensitivity of the assay.[19,20] In addition, the degree of recovery of the fully native protein after refolding was found to be in the region of 80%; the remaining 20% of the protein is present as insoluble aggregates. These aggregates are evident soon after the initiation of refolding and can be attributed to the insolubility of the initial highly unfolded species formed during the refolding reaction.

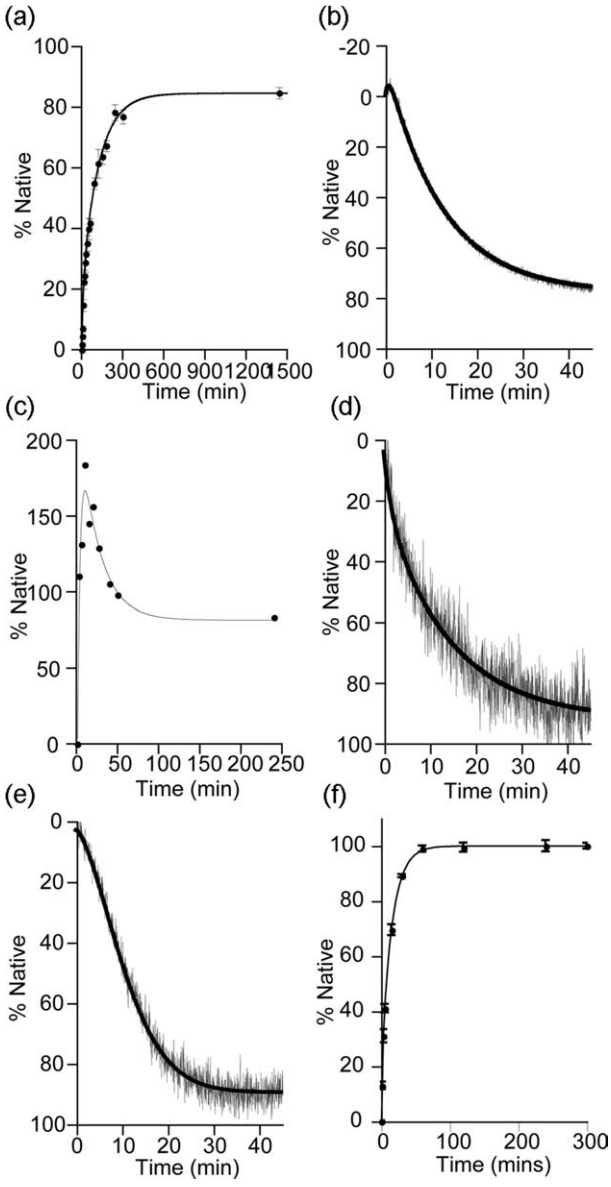

Figure 1. Oxidative refolding of 15 μM wild-type human lysozyme at pH 8.5 monitored by (a) recovery of enzymatic activity, (b) intrinsic fluorescence, (c) ANS binding, (d) far UV-CD, (e) near-UV CD, (f) disulphide bond formation.

The oxidative refolding of the proteins was also followed by a variety of different optical techniques (Table 1). Fluorescence spectroscopy was used to examine the evolution of the local environments of the five tryptophan residues in the protein (Figure 1(b)). A very fast initial phase (τ 0.67(±0.15) minute) is observed, and is likely to result from the burial of the tryptophan residues during a rapid hydrophobic collapse process. A second slower phase (τ 12.4(±0.5) minutes) reflects subsequent changes in the environment around the tryptophan residues (Figure 1(b)) and is comparable in rate to the faster phase observed by measurement of enzymatic activity. When the intrinsic fluorescence was followed over a period of four hours a third phase became evident with a time constant of 120(±4) minutes, comparable to the slower phase observed in the activity measurements. The kinetics of the change in ANS fluorescence show an initial fast phase (τ 3.2(±0.5) minutes) that involves a significant enhancement of intensity that can be attributed to the formation of hydrophobic clusters accessible to ANS (Figure 1(c)). The fluorescence enhancement is, however, much less than that seen with well-characterised stable molten globule states of proteins,[21] indicating that the intermediate or intermediates detected in the present study of human lysozyme have a significantly smaller fraction of exposed hydrophobic surface. The second slow phase (τ 23.3(±0.8) minutes) involves a decrease in ANS fluorescence and can be associated with the formation of a tightly packed native-like structure.

The refolding kinetics were also followed by CD spectroscopy, in the far UV (Figure 1(d)) to probe secondary structure formation, and in the near UV (Figure 1(e)), to measure the development of persistent tertiary structure. The kinetics observed using far-UV CD (225 nm) (Figure 1(d)) follow single exponential behaviour with a time constant of ~12 minutes, consistent with the second time constant found by intrinsic fluorescence and the first by enzymatic recovery. The kinetics measured by the near -UV CD (270 nm) signal show a similar time constant, although the curve can be better fitted to a stretched exponential, indicating that multiple events are occurring during this time period prior to the establishment of a fully native-like tertiary structure. These results, however, indicate that the stable secondary and tertiary structure of the protein are formed essentially simultaneously during the second phase of refolding.

The existence of the different folding phases revealed by the spectroscopic and activity measurements implies that persistent partially structured intermediates are formed during the folding reaction. To observe their presence directly, the oxidative refolding reaction was followed by reverse phase HPLC (Figure 2). The reaction was quenched at various time points by diluting into buffer at pH 2, under which conditions disulphide bond formation and reorganisation are very slow.[14,22] Samples injected immediately after acidification were found to give identical elution profiles to those of samples that had been stored at 4 °C for several hours, confirming that intramolecular disulphide rearrangements do not occur at a detectable rate under these conditions. In the HPLC analysis the peak corresponding to the reduced protein disappears within ten minutes of the initiation of refolding (Figures 2(a) and 3(b)). A variety of intermediates, each of which is populated only to a low level, are then evident from the transient appearance of several peaks of low intensity. By comparison with the experiments in which disulphide bond formation was monitored, these peaks can be attributed to short-lived species having one or two disulphide bonds. As the folding progresses, two major peaks become visible on the HPLC elution profiles, one corresponding to the fully native protein and the other to an intermediate (N*), which elutes at a position close to the native protein. The intermediate (N*) is visible after about three minutes of refolding, reaching a maximum population after about 30 minutes and decreasing almost to zero after approximately 24 hours of refolding (Figure 2). The appearance of the native peak (N) can be fitted to a biphasic curve, with time constants comparable to those for the recovery of enzymatic activity (τ_1 12.5($\pm$1.1) minutes, τ_2 131($\pm$14) minutes; Figure 3). The similarity between the kinetics of formation of the native protein and of the formation and disappearance of N* indicate that the conversion of N* into the native state is responsible for the slow refolding phase.

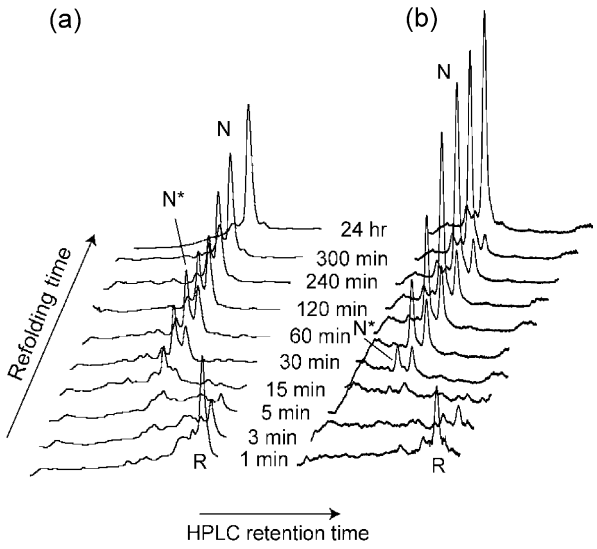

Figure 2. Oxidative refolding of (a) human lysozyme and (b) hen lysozyme followed by analytical HPLC. The major species present during refolding are labelled: R, reduced protein; N, native protein; and N*, major refolding intermediate.

The substantial accumulation of N* during refolding makes it feasible to isolate this species and hence to discover its identity. N* elutes close to the native protein, indicating that it is highly native-like; the slightly lower retention time suggests, however, that it is somewhat more hydrophilic. N* was purified by reverse phase HPLC and was found to have two (2.1$\pm$0.2) free thiol groups by Ellman's assay, indicating that it is a three disulphide species. N* has an activity of 68($\pm$8)%, confirming that it

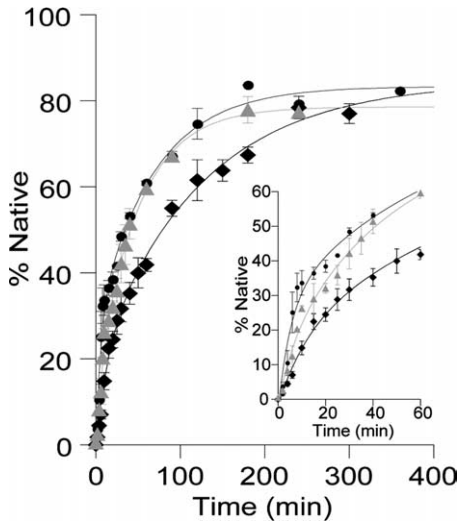

Figure 3. Refolding kinetics followed by monitoring the recovery of enzymatic activity of wild-type (diamonds), D67H (circles) and I56T (triangles) human lysozyme. The inset shows a close-up of the activity data for the initial stages of refolding.

Table 2. Major peptide fragments produced in the digestion of the dominant intermediate (N*) in the oxidative folding of wild-type human lysozyme analysed by mass spectrometry

Peptide	M/Z_{obs}	M/Z_{calc}	Assignment	Disulphide bond present
1	1037.40	1037.02	Lys1-Thr11, Tyr124-Val130	6–128
2	889.40	889.49	Leu12-Gly19	–
3	982.40	982.16	Lys1-Gly19, Tyr124-Val130	6–128
4	707.31	708.40	Tyr20-Leu25	–
5	116.90	116.54	Trp 28-Leu31, Trp109-Val121	30–116
6	1212.20	1211.57	Ala32-Arg41	–
7	1315.30	1315.58	Asn46-Phe57	–
8	1454.50	1453.80	Gln58-Leu84	65–81
9	859.21	858.41	Leu85-Ala108	–
10	870.40	870.00	Val93-Ala108	–
11	641.28	641.37	Pro103-Ala108	–

Assignments were made using the BioLynx rountine within the MassLynx program (Micromass Ltd.).

has a highly native-like conformation. To provide greater insight into the identity of this species, N* was subjected to peptic digestion at pH 2 coupled with mass spectrometric analysis of samples taken at various times after the initiation of the digestion. Spectra were analysed using the BioLynx routine within the MassLynx program (Micromass, UK) to identify the resulting peptides (Table 2). This analysis reveals the presence of the peptides Gln58-Leu84 and Val93-Ala108 in the digestion mixture (Table 2), indicating that the disulphide bond, 77–95 is not present in N*. Moreover, the assignments of the peptides formed during the digestion are consistent with those detected following a peptic digest of Cys77Ala, a mutant of human lysozyme lacking the 77–95 disulphide bond.[23] It can therefore be concluded that N* is a three disulphide intermediate of human lysozyme lacking the 77–95 disulphide bond, denoted des-[77–95]. This result is consistent with the finding that the analogous species des-[76–94] is the major intermediate populated during the oxidative refolding of hen lysozyme.[14] Interestingly, during the secretion of human lysozyme by *Saccharomyces cerevisiae*, it has been found that the protein sometimes did not contain the 77–95 disulphide bond, suggesting that a significant fraction of this species is a stable intermediate formed during the *in vivo* folding of human lysozyme.[24]

The crystal structure of the C77A/C95A mutant of human lysozyme has been described and shows that the removal of the 77–95 disulphide bond does not affect significantly the overall structure of the molecule.[20] This observation explains why the elution peak of des-[77–95] is so close to that of the native protein. The crystal structures of the C77A/C95A variant and wild-type human lysozyme also reveal that Cys77 is solvent-accessible whereas Cys95 is buried within the core of the protein.[20,25] These results indicate that des-[77–95] needs to unfold at least partially and then rearrange in order for the fourth disulphide bond to form, providing an explanation as to how this species could act as a kinetic trap on the refolding pathway. This general idea is further supported by the more detailed analysis of the kinetics of refolding of des-[76–94] during the oxidative refolding of the hen protein.[14] Highly native-like folding intermediates have also been found during the refolding of BPTI;[22] these species have buried disulphides that have been shown to slow the refolding rate of the protein except under denaturing conditions. In both hen lysozyme and BPTI the rates of refolding were found to be increased in the presence of moderate concentrations of denaturant or at elevated temperatures, where the folded state is destabilised.

Oxidative folding of the amyloidogenic variants

The oxidative refolding of the two amyloidogenic variants of human lysozyme studied here, Ile56Thr and Asp67His, was carried out using conditions identical with those used for the wild-type protein and the same set of techniques. The overall character of the refolding kinetics was found to be closely similar to those of the wild-type protein although the corresponding time constants are significantly shorter, see Table 1. As with wild-type lysozyme, approximately 20% of the protein aggregated in the early stages of refolding. Unlike the aggregates found during the refolding of the wild-type protein, however, those formed in the present study generated a film on the side of the cuvette rather than a white solid; similar deposits have been observed in other systems in which amyloid fibrils are present,[26] suggesting that such species could have formed during the early stages of refolding of the variants.

The enzymatic activity of native Asp67His and Ile56Thr was found to be 95($\pm$2)% and 77($\pm$2)% that of the wild-type protein, respectively; the value for the Ile56Thr variant has been reported as 76%,[12] a value in excellent agreement with the present data. The refolding kinetics of both amyloidogenic variants showed similar time constants to each other, although shorter by a factor of between 2 and 3 than those of the wild-type protein (Table 2). In addition, the lag phase seen with the wild-type protein is not evident in the kinetics of either variant, as enzymatic activity is detected immediately after the initiation of folding. This

difference is likely to result simply from the faster folding of the amyloidogenic variants.

Analysis of the effective time constants derived from the various biophysical techniques again shows that Ile56Thr and Asp67His fold faster than the wild-type protein by a factor of about 2 and 3, respectively (Table 1). A fast (τ_1) phase can only be observed in the two variants of human lysozyme when monitoring the number of free thiol groups because of the short dead-time in these experiments. As a result of the increase in overall folding rate in both cases the slowest phase in the folding process can be observed clearly by fluorescence spectroscopy for Asp67His and Ile56Thr. The refolding elution profiles measured using reverse phase HPLC are also similar to those of wild-type human lysozyme, both showing a well-defined native-like refolding intermediate. The kinetic profiles of the disappearance of the reduced protein, the appearance and disappearance of the intermediate N*, and the formation of the native protein for the two variants compared to wild-type lysozyme are shown in Figure 4. The formation of the native protein in the HPLC traces follows the same kinetics as those seen for the recovery of enzymatic activity, and shows again that the two mutational variant lysozymes attain their native states faster than does wild-type lysozyme (Table 2).

During the refolding of both amyloidogenic variants the dominant intermediate, N*, forms more rapidly than in the case of the wild-type protein; it accumulates to its maximum population after ca 30 minutes of refolding and has decreased to undetectable levels by 240 minutes (Figure 5). This behaviour is qualitatively similar to that of the wild-type protein, except that for the latter protein N* reaches its highest population after ca 40 minutes and is still detectable after 400 minutes. The levels to which N* accumulates in solution also differ somewhat for the different proteins; the maximum proportion of N* in the refolding mixture is up to 35% of the total protein present in the amyloidogenic variants compared to ~25% for wild-type lysozyme (Table 3). The kinetics of the disappearance of N* are consistent with the proposal that N* slowly converts into the native protein, and the rates of the disappearance of N* and of the formation of the native state are similar for both variants. The similarity of the refolding profiles of the two mutant variants and the wild-type protein strongly suggests that the intermediate in the folding of both variants is again the three disulphide species des-[77–95].

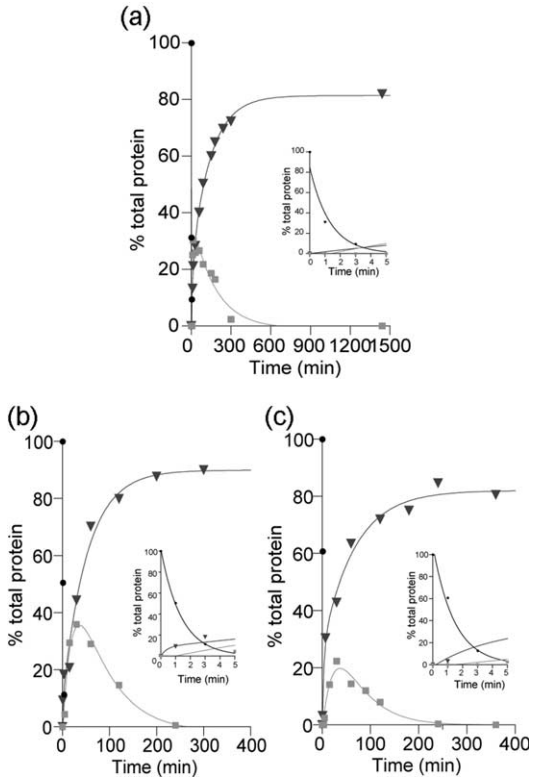

Figure 5. Relative concentrations of reduced protein (circles), N* (squares) and native protein (triangles) determined by reverse phase HPLC during the refolding of human (a) wild-type, (b) D67H and (c) I56T lysozyme. Relative protein concentrations were determined by the integration of the peak areas in reverse phase HPLC chromatographic traces.

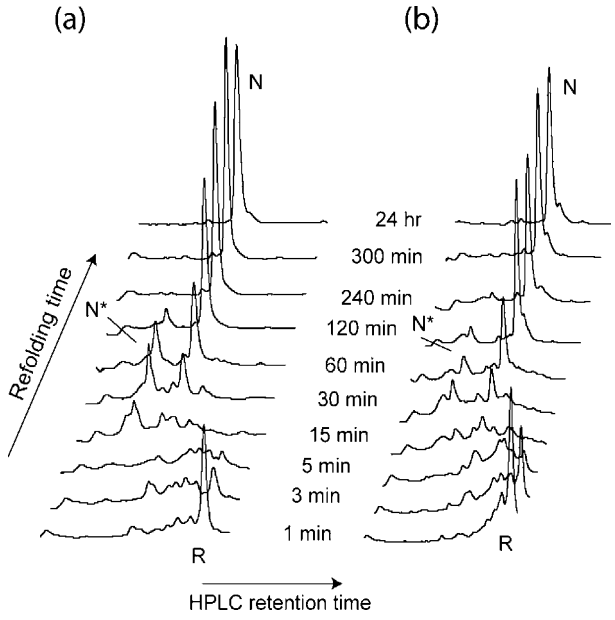

Figure 4. HPLC elution profiles depicting the oxidative refolding of (a) D67H and (b) I56T lysozyme. The major species present during refolding are labelled: R, reduced protein; N, native protein; and N*, major refolding intermediate.

Discussion

Although the oxidative refolding of the c-type lysozyme is manifestly complex, with a multitude of different species forming and disappearing at different rates, it has proved possible to define

Table 3. The kinetics of the formation of the native protein (N), the formation and disappearance of the major intermediate N*, and the disappearance of the reduced protein (R), during the oxidative refolding of wild-type lysozyme and the Asp67His and Ile56Thr variants

	WT		Asp67His		Ile56Thr	
Species	τ_1 (min)	τ_2 (min)	τ_1 (min)	τ_2 (min)	τ_1 (min)	τ_2 (min)
R	1.31±0.04	–	1.37±0.06	–	1.46±0.10	–
N*	11.3±1.3	144±17	2.3±0.8	51.6±6.8	6.2±1.2	57.2±5.7
N	12.5±1.1	131±14	4.8±0.6	48.0±5.2	5.5±0.8	62.1±4.2

The quantities of each species were determined by integration of the reverse phase HPLC peaks.

apparent rate constants describing several different stages of the process. The results from such analysis of the time dependence of the various biophysical and biochemical parameters monitored here indicate that, like the oxidative refolding of hen lysozyme,[14] the oxidative folding of wild-type human protein and its amyloidogenic variants take place in two major stages. The reduced protein, which corresponds to a highly denatured ensemble of unfolded conformers,[27,28] initially collapses rapidly into a large number of relatively unstructured intermediates. This collapse process has an apparent time constant (τ_1) that can be derived from fluorescence measurements. The species formed at this stage of the folding reaction do not contain any detectable quantity of secondary structure by CD, and do not show the strongly enhanced ANS fluorescence typical of a molten globule-like state.[29] The stabilisation of extensive native-like structure takes place after this initial phase. Formation of species containing three disulphide bonds follows this initial phase. The majority of molecules during this phase have formed the intermediate des-[77–95] with the minority passing through other three disulphide species on the way to the fully native state.

From the HPLC elution profiles it is clear that the native protein is formed by both fast and slow tracks within this overall reaction framework (Figure 3). As with hen lysozyme, the slow track can be attributed to folding *via* the intermediate, des-[77–95] whilst the fast track probably involves folding *via* the intermediates des-[6–128] and des-[65–81].[15] The slow conversion of des-[77–95] into the native protein is then reflected in the slow time constant τ_3, observed by HPLC analysis and measurements of the recovery of enzymatic activity. Overall, the amyloidogenic variants Asp67His and Ile56Thr of human lysozymes refold oxidatively in the same qualitative manner as the wild-type protein, although it is significant that they both fold faster than the wild-type protein, by factors of 3 and 2, respectively. This observation can be attributed to the lower stability of the native-like intermediates of the variant proteins compared with those of the wild-type protein; a reduction in stability will result in the faster rearrangement of des-[77–95] as this process is limited by the need to unfold significantly the protein prior to the final oxidative step.[15]

It is particularly fascinating in this regard that the region of the structure that is transiently unfolded under somewhat destabilising conditions in the fully oxidised protein, and ultimately leads to aggregation into amyloid fibrils, involves a large part of the β domain along with the C-helix that forms part of the α domain.[6,7] These regions of structure are linked by the 77–95 disulphide bond, whose formation represents the rate-limiting step in oxidative folding. It is interesting to speculate from such an observation that the prevalence of disulphide bonds in secreted extracellular proteins may be an evolutionary development that not only stabilises proteins but also limits the conditions under which they are liable to unfold into aggregation-prone intermediate states. Thus, cross-linking of the polypeptide chain by intramolecular disulphide bonds is likely to hinder its ability to incorporate a large proportion of its sequence into the characteristic cross-β-structure of amyloid fibrils, and hence to reduce the propensity of the latter to form under at least some conditions.

The two amyloidogenic variants of human lysozyme studied here are each associated with the accumulation of large extracellular deposits of amyloid fibrils in systemic amyloidosis.[4] These variants must therefore refold relatively efficiently *in vivo* in order to escape degradation prior to secretion from the endoplasmic reticulum (ER). The lower stability of the native state of Asp67His and Ile56Thr results in a higher population of partially unfolded states under given conditions than is the case for the wild-type protein.[4] Thus, the amyloidogenic variants have a greater probability of being targeted in the cell and of aggregation following their secretion and hence of fibril formation in an extracellular environment.[5,30] Within the ER, however, the reduced stability of the amyloidogenic variants may well cause them to fold more rapidly than the wild-type protein, as has been found here to occur during *in vitro* refolding. Faster folding will not only enhance the efficiency of the folding process itself, but also reduce the time that highly aggregation-prone and incompletely folded species are present within the cell. It seems likely, therefore that the amyloidosis associated with these lysozyme variants results from an unfortunate combination of circumstances, in which folding *in vivo* is not impeded, and may even be assisted, by the reduced stability of the intermediates in the variant proteins. Nevertheless,

once the variants are secreted, they are less able to resist aggregation and conversion to amyloid fibrils than the wild-type protein, resulting in the accumulation of misfolded protein and the onset of disease.

Materials and Methods

Protein expression and purification

Wild-type human lysozyme and its amyloidogenic mutational variants, Ile56Thr and Asp67His, were expressed in *Aspergillus niger* as described.[31] Individual colonies were grown in 100 ml of polyvinyl pyrrolidone medium and assayed for lysozyme activity. Expression was carried out in white soya milk medium and the secreted protein purified initially by ion-exchange chromatography and gel filtration. A further purification step was carried out using reverse phase HPLC with an elution gradient consisting of five minutes at 30% CH_3CN followed by eight minutes at 50% CH_3CN. The protein was then lyophilised and stored at −20 °C.

Lysozyme reduction and denaturation

Lysozyme (10–20 mg/ml) was dissolved in a denaturation buffer (buffer A: 8 M urea, 100 mM Tris, 1 mM EDTA, pH 8.5). Reduction of disulphide bonds and subsequent denaturation of the protein was achieved by incubation for three hours at 40 °C in the presence of a 25-fold excess of dithiothrietol (DTT) over the number of disulphide bonds. The process was quenched by the addition of 2 M HCl to reduce the pH to 2, where oxidation of free thiol groups is very slow. The reduced protein was purified by reverse phase HPLC, lyophilised and stored at −20 °C. Samples were frequently checked after storage by analytical reverse phase HPLC to ensure that the protein was still fully reduced and that no aggregates were present.

Lysozyme renaturation

Aliquots of lyophilised, reduced lysozyme were dissolved in buffer A. Renaturation was achieved by rapid fourfold dilution into buffer B (100 mM Tris, 133 mM NaCl, 1 mM EDTA, 133 mM GSH, 0.27 mM GSSG) to give final folding conditions of 2 M urea, and 1.0 mM/0.2 mM GSH/GSSG. The final protein concentrations were 15 μM. These conditions gave a maximum refolding yield of 70–80% and were carried out at 20 °C at pH 8.5.

Lysozyme activity assay

Enzymatic activity was measured by the decrease in absorbance at 450 nm over a period of 30 s when aliquots of the refolding solutions were rapidly mixed with 1 ml of *Micrococcus lysodeikticus* cell wall suspension ($A_{450\ nm}$ 0.6–1) in 50 mM phosphate buffer, 0.1% (w/v) glucose at pH 7.4.[32] Activities were normalised against a solution of native lysozyme of known concentration.

Free thiol group assay

Ellman's reagent, dinitrothiobenzolate (DNTB), was used to determine the number of free thiol groups in the reduced protein and in aliquots of protein extracted from the refolding mixture.[17] The release of nitrothiobezolate (NTB) was measured at 412 nm on the addition of DNTB to a solution of reduced lysozyme of known concentration. A calibration curve was obtained using solutions of GSH of known concentration. For determination of the kinetics of disulphide bond formation, refolding aliquots were taken during the reaction, desalted and lyophilized. Samples were then dissolved in buffer containing 8 M urea and immediately diluted to 2 M urea. The number of free thiols was determined by absorbance at 412 nm.

Fluorescence spectroscopy

Changes in intrinsic fluorescence during refolding were measured in real-time using a Perkin Elmer LS 50B fluorimeter, with the excitation/emission wavelength set at 280 nm/350 nm and using slit widths of 2.5 nm. ANS binding experiments were performed by the addition of 200 μM ANS to aliquots of refolding lysozyme at various times after the initiation of refolding. Excitation/emission wavelengths of 394 nm/478 nm were used with a slit width of 5 nm.

CD spectroscopy

Kinetic CD experiments were carried out at 255 nm (far-UV CD) and 289 nm (near-UV CD) using a Jasco J720 spectropolarimeter with cells having path lengths of 1 mm and 5 mm, respectively. All kinetic data were fitted to double-exponential curves with KaleidaGraph™ (Synergy Software).

Reverse-phase HPLC

Aliquots were extracted at various stages of the refolding process and the reaction quenched by the addition of 1/20 vol. of 2.5 M HCl. The various species present were separated by reverse phase HPLC using an analytical Phenomenex Jupiter 10 μ C18 300 Å column (Phenomenex, CA, USA) with a flow rate of 1 ml/minute. Absorbance was recorded at 280 nm or 220/280 nm. The HPLC solvent A was H_2O/0.1% (v/v) trifluoroacetic acid (TFA) and solvent B was CH_3CN/0.1% TFA for hen lysozyme or 10% H_2O/90% CH_3CN/0.1% TFA for human lysozyme. Refolding kinetics were determined by measuring the peak areas of the major species in the chromatograph using Gilson Unipoint or Varian Star analysis software. The refolding intermediate, des-[77–95], was purified using a semi-preparative Phenomenex Jupiter 10 μ C5 300 Å column (250 mm × 10 mm) at 5 ml/minute with a gradient of 40%–46% CH_3CN over 25 minutes. The protein obtained in this way was lyophilised and stored at −20 °C.

Peptic digest of the N* intermediate

Samples (~50 μM) of the intermediate were dissolved in formic acid (pH 2) and digested with 2% pepsin at 37 °C. A further 2% pepsin was added after one hour and then another 2% after a further three hours. Aliquots were taken at various time points throughout the reaction, frozen rapidly in liquid nitrogen to quench the reaction and stored at −80 °C prior to analysis.

Analysis of peptic digest by mass spectrometry

Nanoflow electrospray capillaries were prepared in-house from borosilicate glass capillaries on a model P-97

Flaming/Brown micropipette puller (Sutter Instruments, CA,USA), and gold coated with a Polaron SEM coating system. Protein samples were introduced directly into a quadruple time-of-flight (Q-TOF) mass spectrometer (Micromass, UK) equipped with a nanoflow z-spray ionisation source. All spectra were acquired in the positive ion mode and calibrated using CsI. Capillary voltages were typically 900–1100 kV with the skimmer set at 80 V. The temperature in the ESI source was set to 20 °C and no heating was applied. Spectra were processed using MassLynx 3.1 and analysed using BioLynx, (Micromass Ltd., UK).

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