Disulfide-Rearranged Molten Globule State of α-Lactalbumin

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ABSTRACT: A three-disulfide form of human α-lactalbumin, with free thiols on Cys6 and Cys120, can adopt the molten globule conformation. It then spontaneously rearranges its three disulfide bonds to many isomers that tend to maintain the molten globule conformation. The distribution of free thiol groups within the rearranged species has been determined quantitatively by chemical modification and peptide mapping. The protein's eight cysteine residues were modified with nearly equal frequencies, although there were significant departures from randomness. The results confirm that the molten globule state of α-lactalbumin does not maintain the nativelike topology of the polypeptide backbone but is more like a collapsed form of an unfolded protein.

Small single-domain proteins, as a result of the cooperativity of the folding process, generally adopt only two conformational states: the folded, native state and the unfolded state (Privalov, 1989; Creighton, 1993). Exceptions are usually due to the occurrence of the so-called molten globule (MG)[1] state, which is neither fully folded nor fully unfolded (Kuwajima, 1989; Ptitsyn, 1992). This third conformational state is the most stable under certain conditions in some proteins, for reasons that are not understood.

The MG state is important for protein folding, both *in vitro* (Kuwajima, 1989; Ptitsyn, 1992) and *in vivo* (Martin et al., 1991). Many unfolded proteins adopt the MG state rapidly when placed under refolding conditions, and it is populated transiently until refolding is completed (Ikeguchi et al., 1986; Ptitsyn et al., 1990; Baker et al., 1992). The MG state can be observed kinetically even with proteins in which it is not stable at equilibrium (Kuwajima et al., 1985; Ikeguchi et al., 1986). The MG state may be the energetically preferred conformational state of unfolded proteins under refolding conditions.

The physical nature of the MG state is very uncertain. Equilibrium MG states have been concluded to have a backbone topology close to that of the native fold (Ptitsyn, 1992), and specific elements of nativelike conformation are detectable in the case of α-lactalbumin (αLA) (Baum et al., 1989). Transient kinetic MG states of several proteins are believed to have substantial amounts of nativelike secondary and tertiary structure (Jeng et al., 1990; Udgaonkar & Baldwin, 1990; Serrano et al., 1992; Radford et al., 1992), perhaps like the major one-disulfide intermediate in the folding of bovine pancreatic trypsin inhibitor (van Mierlo et al., 1993). The MG state of αLA, however, has a tendency to rearrange the native disulfide bonds rapidly, indicating that it is more like a collapsed form of an unfolded protein (Ewbank & Creighton, 1991, 1993a,b).

The tendency of cysteine residues to form disulfide bonds is a very useful experimental probe of polypeptide conformation (Creighton, 1978, 1986). Under appropriate conditions, which if any disulfide bonds are present reflects the conformational properties of the polypeptide chain, particularly its tendency to bring pairs of cysteine residues into the close proximity required for disulfide bonding. The disulfide bonds present at any time can be trapped, and their pairings of cysteine residues can be determined by chemical means.

The best-characterized MG state is that of αLA, primarily due to the work of Kuwajima (1989). It tends to be populated when the native state is destabilized under only moderately unfolding conditions. The native state of αLA is stabilized by a single bound Ca^{2+} ion and by four disulfide bonds, Cys6–120, Cys28–111, Cys61–77, and Cys73–91 (Acharya et al., 1989, 1991). In the native states of both bovine and human αLA, the Cys6–120 disulfide bond can be reduced specifically (Iyer & Klee, 1973; Shechter et al., 1973; Kuwajima et al., 1990), followed by Cys28–111, to generate the three- and two-disulfide species 3SS and 2SS, respectively (Ewbank & Creighton, 1991, 1993a,b). Both 3SS and 2SS adopted primarily the MG state when the single Ca^{2+} ion was removed, and both tended to rearrange their remaining native disulfide bonds by thiol–disulfide interchange to very many alternative pairings (Ewbank & Creighton, 1991, 1993a,b). The disulfide rearrangements occurred at the same rate as in the presence of 8 M urea, but the products tended to retain the MG conformation.

There were too many different disulfide isomers in the rearranged 3SS and 2SS species for them to be individually identified; there are 420 and 210 possible isomers, respectively. The disulfide bonds present appeared not to be a totally random set, however, but were inferred to be predominantly between cysteine residues distant in the primary structure and presumably brought close in space by the compact MG conformation (Ewbank & Creighton, 1991, 1993a,b). A practical method to test this inference further and to characterize the distribution of species present is to measure the tendency of the various cysteine residues *not* to be present in disulfide bonds but to exist in the form with free thiol groups; this requires only that the cysteine thiol groups be labeled chemically and the distribution of label over the eight cysteine residues be measured. This has now been carried out for the rearranged 3SS species of human αLA, which has a relatively stable MG conformation.

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[1] Abbreviations: AEDANS, *N*-acetyl-*N'*-(5-sulfo-1-naphthyl)ethylenediamine group; αLA, α-lactalbumin; 3SS, human αLA with the Cys6–120 disulfide bond reduced; DTT, dithiothreitol; HPLC, high-pressure liquid chromatography; I-AEDANS, *N*-(iodoacetyl)-*N'*-(5-sulfo-1-naphthyl)ethylenediamine; MG, molten globule; R-AEDANS, fully reduced αLA with the cysteine residues blocked with AEDANS groups; TFA, trifluoroacetic acid.

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MATERIALS AND METHODS

Human αLA was obtained from Sigma. All experiments were performed at 25 °C in a Tris–KCl buffer, consisting of 0.1 M Tris-HCl (pH 8.7) and 0.2 M KCl, supplemented with either $CaCl_2$ or EDTA.

The three-disulfide form of αLA, 3SS, was generated from native αLA (0.4 mg/mL) in Tris–KCl buffer containing 0.1 mM $CaCl_2$ by reduction of the Cys6–120 disulfide bond upon adding $^1/_3$ volume of 0.4 mM DTT in the same buffer. After 2 min, conversion to 3SS is quantitative. For the spontaneous disulfide rearrangement, the 3SS was diluted with 4 volumes of Tris–KCl buffer containing 1 mM EDTA. Under these conditions, aggregation is not apparent and the disulfide rearrangements are primarily intramolecular (Ewbank & Creighton, 1991, 1993a,b). The disulfide rearrangements were quenched after various times by addition of $^1/_5$ volume of 0.2 M I-AEDANS in the same buffer. In some experiments, the I-AEDANS added was varied from $^1/_{10}$ to 1 volume. For the zero-time control, the I-AEDANS was added in the Tris–KCl–EDTA buffer used to initiate rearrangements. After a 10-min reaction, the protein was recovered by gel filtration on Sephadex G25 in 10 mM NH_4HCO_3 and lyophilized.

The resulting AEDANS-blocked proteins were dissolved to a concentration of about 3 mg/mL in 6 M guanidinium chloride, 0.1 M Tris-HCl (pH 8.7), 1 mM EDTA, and 8 mM DTT. After 40 min to fully reduce the protein, all free thiols were blocked by addition of $^1/_5$ volume of 0.6 M iodoacetamide in 0.5 M Tris-HCl (pH 8.0). After 2 min, the protein was isolated by gel filtration and lyophilization, as above.

The reduced, blocked proteins were dissolved to a concentration of about 1 mg/mL in Tris–NaCl buffer containing 10 mM $CaCl_2$. An equal volume of 0.15 mg/mL bovine trypsin (modified, sequencing grade, Boehringer Mannheim) dissolved in the same buffer was added, and digestion was allowed to proceed for 10 h. Digestion was terminated by the addition of at least 3 volumes of 0.1% TFA and cooling to 0 °C. The tryptic peptides were separated by reverse-phase HPLC on a Dynamax C18 5-μm column at a flow rate of 1 mL/min, using 0.1% TFA containing linear gradients of acetonitrile: 0% (v/v) at the start, 18% after 36 min, 25% after 64 min, 40% after 98 min, and 55% after 113 min. Elution of the AEDANS-labeled peptides was monitored by their absorbance at 340 nm. N-Terminal sequencing and mass spectrometric identification of the purified peptides were performed by M. Mann and R. Kellner of the EMBL peptide sequencing service.

The elution profiles were compared to that generated from αLA that had been fully reduced as above, trapped after 40 min by adding $^1/_5$ volume of 0.2 M I-AEDANS in 0.5 M Tris-HCl (pH 8.0), recovered by gel filtration and lyophilization, and digested as above.

RESULTS

The 3SS form of human αLA, with free thiols on Cys6 and Cys120, remains in the native conformation when Ca^{2+} is bound (Kuwajima et al., 1990; Ewbank & Creighton, 1991), and the three remaining disulfide bonds are stable. Adding excess EDTA removes the Ca^{2+} ion and changes the conformation to that of a MG, with a half-time of 1.5 s (Ewbank & Creighton, 1991). The three remaining disulfide bonds rearrange spontaneously, by intramolecular thiol–disulfide interchange with the two free thiols, and the original 3SS disappears with a half-time of about 3 min at pH 7 and within a few seconds at pH 8.7. The product is an apparently equilibrium mixture in which very many isomers with three

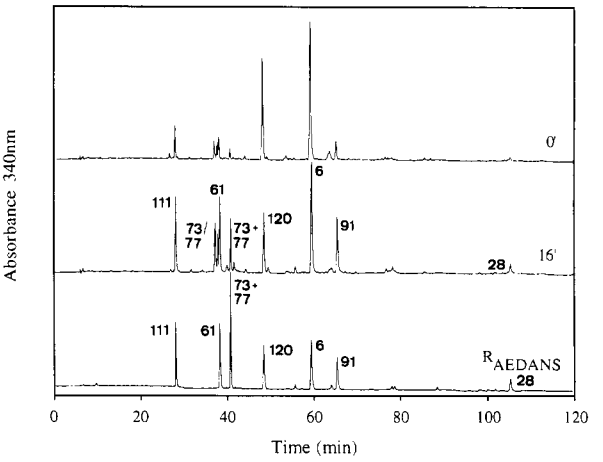

FIGURE 1: Reverse-phase HPLC separation of AEDANS-labeled tryptic peptides of αLA. At the bottom are the elution profiles of the peptides derived from R-AEDANS, in which all eight cysteine residues are completely blocked with AEDANS groups; the numbers of the cysteine residues present in the peptides are indicated. The peptides were identified, from their amino acid compositions and molecular weights determined by mass spectrometry, to contain residues 109–114, 59–62, 71–79, 115–122, 6–13, 80–93, and 14–58, from left to right. The peptides were obtained in similar quantities, except for that containing Cys28; Cys73 and Cys77 were present on the same peptide. At the top is the spectrum of peptides obtained from 3SS αLA trapped by I-AEDANS at the same time as rearrangement was initiated by addition of EDTA. The AEDANS groups are primarily on Cys6 and Cys120, as expected, although their presence on the other residues indicates that some disulfide rearrangements took place during trapping. The middle panel shows the spectrum observed after extensive rearrangement for 16 min, when no original 3SS remained. The two peptides eluting just before that containing Cys61-AEDANS, labeled 73/77, comprise residues 71–79 in which only one of the residues Cys73 or Cys77 is blocked with AEDANS.

disulfide bonds are present. The present goal was to characterize this mixture by measuring the distribution of the two free thiol groups on the eight cysteine residues. To do this, the two cysteine thiol groups present in each of the rearranged species were reacted with a spectrally distinct reagent, I-AEDANS (Kenyon & Bruice, 1977). The disulfide bonds between the other cysteine residues were subsequently reduced, and the cysteine thiols were blocked by reaction with iodoacetamide, a nonchromophoric reagent. The resulting αLA molecules have no disulfide bonds and differ only in the distribution of AEDANS groups on the eight cysteine residues. This distribution was measured by cleaving the polypeptide chain into peptides, then separating and quantifying specifically the AEDANS-containing peptides, using their unique absorbance at 340 nm.

Trypsin was found to digest R-AEDANS into the expected seven AEDANS-containing peptides (Figure 1, bottom), in comparable yields and each with one cysteine residue, except for a low recovery of the Cys28 peptide and the presence of both Cys73 and Cys77 in one peptide. No protease was found to cleave reproducibly between Cys73 and Cys77, so they were quantified together. They could be distinguished in the rearranged 3SS samples when only one was blocked with AEDANS, for these two peptides then eluted earlier than that with two AEDANS groups, as expected, just before the Cys61-AEDANS peptide (Figure 1, top and middle). The identities of these two peptides were confirmed by measurement of their molecular weights by mass spectrometry, but assignment of the AEDANS groups to Cys73 or Cys77 was not attempted. With the rearranged species, the two peptides with single AEDANS groups were present in roughly equal amounts and usually in somewhat greater amounts than the

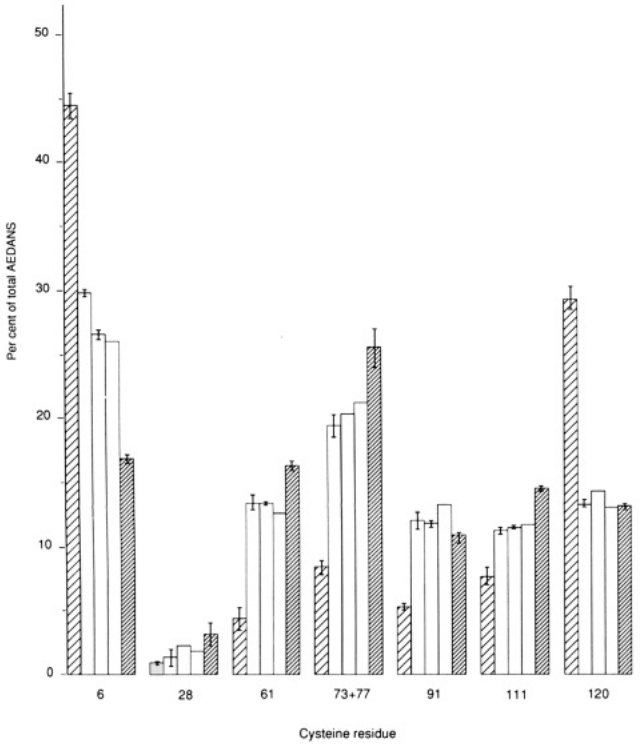

FIGURE 2: Quantification of the fraction of AEDANS on each cysteine residue during rearrangement of 3SS αLA. The absorbance at 340 nm present in each peptide, relative to that of all the peptides of each species, was determined by integration of the peak areas from HPLC profiles like those in Figure 1. The value for Cys73 + Cys77 is from the total for the two peptides with one residue blocked and the one peptide with both residues blocked with AEDANS groups. The first column (lightly cross-hatched) for each cysteine residue is of the 3SS trapped at the same time as rearrangement was initiated by addition of EDTA. The following three columns are for disulfide rearrangement times of 4, 8, and 16 min, respectively. The final column (closely cross-hatched) is for the peptides from R-AEDANS. The error bars give the mean deviation of 2–5 independent measurements, except for the 16-min sample, which was of a single measurement. Where no error bar is visible in other instances, the difference between multiple measurements was negligible.

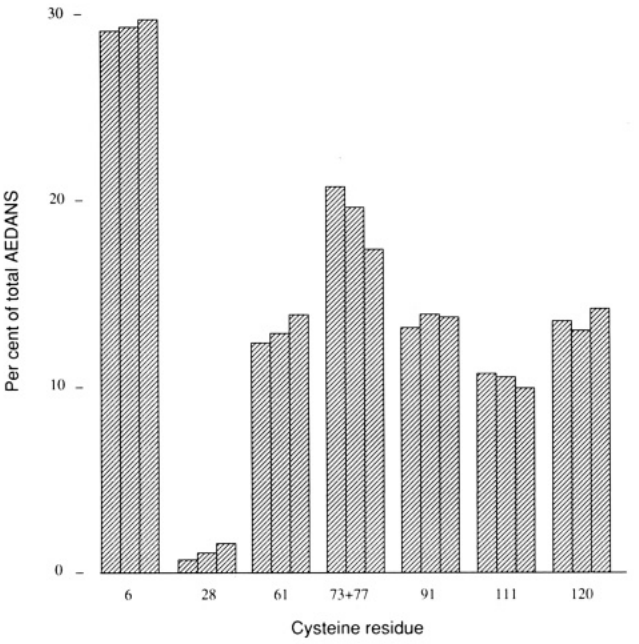

FIGURE 3: Distribution of AEDANS on the cysteine residues of 3SS αLA rearranged for 2 min and trapped with different concentrations of I-AEDANS. The first, second, and third columns are for 18, 33, and 100 mM I-AEDANS, respectively. The AEDANS distribution was measured as in Figure 2.

double AEDANS peptide, as would be expected if the two cysteine residues had similar and independent probabilities of reacting with I-AEDANS. Therefore, the results for all three peptides were combined to give a single value for both Cys73 and Cys77. This average value would be expected to be close to the values for the individual cysteine residues.

Native Ca^{2+}-bound 3SS trapped with I-AEDANS gave almost exclusively the expected Cys6-AEDANS and Cys120-AEDANS peptides (not shown). Likewise, the subsequent intermediate in reduction of the four disulfide bonds of αLA, 2SS, gave the expected pattern of AEDANS groups on the peptides containing Cys6, 28, 111, and 120 (not shown). When 3SS was trapped with I-AEDANS at the same time as EDTA was added, some disulfide rearrangement occurred during trapping, for small but significant amounts of AEDANS were present on the other cysteine residues (Figure 1, top).

When disulfide rearrangements in 3SS were permitted for varying lengths of time after addition of EDTA and before trapping with I-AEDANS, the AEDANS groups were found to be present on all the cysteine residues in substantial amounts (Figure 1, middle). There was very little change in the distribution after 2–16-min rearrangement (Figure 2), suggesting that the disulfide rearrangements had reached equilibrium.

When there is a rapid equilibrium between different isomers, the trapping reaction can pull the equilibrium if the thiol groups of some isomers react more rapidly than those of others (Creighton, 1974; Creighton & Goldenberg, 1984). This possibility was investigated by varying the I-AEDANS concentration from 0.02 to 0.1 M (Figure 3); if the equilibrium is being altered, the trapped profile should vary with the I-AEDANS concentration. The variation in the results was about the same as that observed in duplicate experiments, although the most extreme, and opposite, variations in the cases of Cys28 and Cys73 + Cys77 may be significant. This could indicate that Cys73 and Cys77 are generally more reactive toward I-AEDANS than the others, and Cys28 less so; alternatively, the variation could be due to experimental uncertainty, resulting from the poor recovery of the Cys28 peptide and the occurrence of Cys73 and Cys77 among three peptides, with one and two AEDANS groups (Figure 1). In any case, the results are unlikely to be greatly affected by differential reactivities of the cysteine thiol groups. In agreement, similar mixtures of rearranged disulfide species from 3SS were trapped with varying concentrations of iodoacetamide (Ewbank & Creighton, 1993b).

To obtain a quantitative measure of the relative tendencies of each cysteine residue to react with I-AEDANS, the results must be corrected for the recovery of each Cys-AEDANS-containing peptide. This was taken from the relative amounts of the peptides isolated from R-AEDANS (Figure 1, bottom); this comparison assumes that the tryptic digestion of this polypeptide chain, with eight AEDANS groups, was the same as those in which there were only two AEDANS groups and the remaining six cysteine residues were blocked by reaction with iodoacetamide. This assumption is likely to be valid in most instances, for only Cys6 was adjacent to a tryptic cleavage site; whether the Cys6 thiol group had been reacted with iodoacetamide or I-AEDANS might affect the cleavage by trypsin of the adjacent peptide bond, but there were no indications that this cleavage occurred partially in either case. All the AEDANS-labeled peptides were found in similar quantities, except for that containing Cys28; this is the largest peptide and Cys28 is located 14 residues away from the nearest

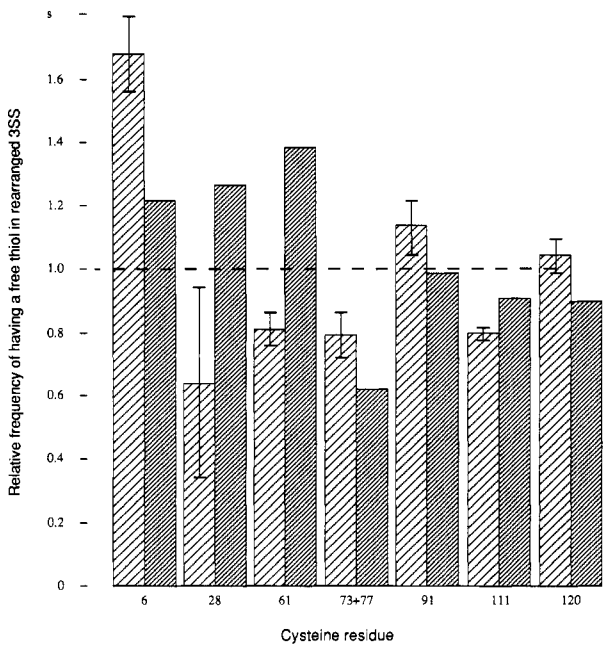

FIGURE 4: Relative frequencies of the cysteine residues of rearranged 3SS αLA being in the thiol form and reacting with I-AEDANS. The relative amounts of the AEDANS-containing peptides measured in five experiments on 3SS rearranged for 4, 8, or 16 min were divided by those obtained with five measurements on R-AEDANS (Figure 1) to give the results on the left. The error bars give the most extreme results expected with the most extreme combinations of the mean deviations of the two measurements. The right bars are the results of a single experiment with 3SS that had rearranged for 16 min in the same buffer, but also containing 8 M urea, before being trapped with 0.2 M I-AEDANS.

tryptic cleavage site, so its modification would not be expected to affect the cleavage of the peptide.

Comparing the amount of each AEDANS peptide isolated from the rearranged 3SS to that from R-AEDANS gives the distribution profile of the relative tendency of each cysteine residue in the rearranged 3SS αLA not to be involved in a disulfide bond but to be present as a thiol group (Figure 4). The results vary somewhat from a uniform distribution: Cys6 had a substantially greater tendency to exist as the thiol, Cys91 a slightly greater tendency, while Cys28, Cys61, Cys73, Cys77, and Cys111 had significantly lower tendencies, although that for Cys28 was not very accurate. The spectrum was significantly different from that obtained with 3SS that had been rearranged in 8 M urea (Figure 4).

DISCUSSION

The 3SS form of αLA, with the Cys6–120 disulfide bond reduced, adopts a MG-like conformation when Ca^{2+} is removed (Ewbank & Creighton, 1991). The results presented here confirm the previous conclusion (Ewbank & Creighton, 1991, 1993a,b) that the MG state of apo 3SS does not stabilize the three remaining native disulfide bonds but allows them to rearrange spontaneously to many alternative disulfide pairings, by intramolecular thiol–disulfide interchange. Not only are very many different disulfide bonds generated by the rearrangements, but the two free thiol groups originally on Cys6 and Cys120 in 3SS are interchanged to all the other cysteine residues, to nearly comparable extents (Figure 4). These alternative disulfide and thiol isomers are energetically preferred over the original native set of 3SS, which is not present at substantial levels in the mixtures of rearranged species. Therefore, the MG state of αLA does not retain a nativelike overall topology of the polypeptide chain and must be stabilized by relatively nonspecific interactions.

The disulfide bonds of the αLA MG state behave much more like those in unfolded proteins than in folded proteins (Ewbank & Creighton, 1993a). The disulfide rearrangements in apo 3SS occurred too rapidly to be taking place only through the very small amount of fully unfolded conformation present in apo 3SS and must have occurred in the predominant MG conformation; the rearranged products also tended to retain the MG conformation. Human αLA was used for this study because it has a relatively stable MG state (Nozaka et al., 1978; Ptitsyn, 1992).

If the MG state does not stabilize greatly any of the disulfide bonds, then the disulfide bonds cannot stabilize the MG state to any greater extent. This is confirmed by the experimental observations (Ikeguchi *et al.*, 1992; Ewbank & Creighton, 1993b), for the MG state is not destabilized to a great extent by reduction of the disulfide bonds, whereas the native state is. The MG state is still populated substantially in fully reduced αLA (Ikeguchi & Sugai, 1989; Ewbank & Creighton, 1993b).

The MG state is also largely populated in the rearranged 3SS: the molecules are very compact, have substantial amounts of secondary structure but little tertiary structure, and bind the hydrophobic dye 1-anilinonaphthalene-8-sulfonate but not Ca^{2+} (Ewbank & Creighton, 1991, 1993a,b). The disulfide bonds tend to be between cysteine residues distant in the primary structure but presumably brought close by the compact MG conformation. Too many disulfide bond isomers are present in the rearranged 3SS population for them to be identified, but the present study showed that which cysteine residues were present as thiol groups was largely, although not entirely, uniform (Figure 4).

The nonuniform distribution of AEDANS labels in the rearranged 3SS species appears to be significant, for a different profile was obtained when the rearrangement was carried out in 8 M urea (Figure 4). Under denaturing conditions, the disulfide bonds are expected to be primarily between cysteine residues close in the primary structure, to maximize the conformational entropy of the polypeptide chain, whereas the compact MG conformation tends to keep the cysteine residues distant in the primary structure in close proximity and disulfide-bonded. These expectations were confirmed by the hydrodynamic volumes of the unfolded, but disulfide-bonded, molecules generated by rearrangement of 3SS under different conditions (Ewbank & Creighton, 1993b). The nonuniform distribution of AEDANS groups in the rearranged species is unlikely to be due to differential recovery of the trapped rearranged species or of the AEDANS-labeled peptides. The rearranged species were manipulated only by one gel filtration step, before being reduced and trapped, and a different distribution was observed with the species rearranged in 8 M urea. The AEDANS peptides were recovered from R-AEDANS in very similar quantities (Figure 2), except for the longest peptide, that containing Cys28. Except for those containing one or both of Cys73 and Cys77, the AEDANS peptides were identical in each species and should have been isolated in the same yield in each instance. The results were corrected for any differences in recovery of the peptides by comparing them to the case of R-AEDANS, where all seven Cys-AEDANS peptides are present initially in the same molar amounts.

The very many disulfide isomers present in rearranged 3SS probably do not require that the polypeptide chain be totally disordered, for significant amounts of secondary structure

are present in the MG conformation. It would be surprising if this secondary structure were randomly distributed throughout the polypeptide chain, for different amino acid sequences clearly have different secondary structure propensities that are often incorporated into the final folded native conformation [e.g., Segawa et al. (1991)]. A MG is most likely, therefore, to consist of relatively constant segments of secondary structure elements in the interior of the compact MG structure, but without fixed relative positions. In the case of rearranged 3SS, the preferred disulfide bonds present might be expected to be primarily in the interior of the MG, with the cysteine thiols primarily on the surface. The disulfide rearrangements took place at pH 8.7, where protein thiols are normally ionized about half the time.

The present results do not lead to a simple model of the MG conformation, but they do place constraints on what models can be proposed.

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