EVIDENCE FOR NUCLEATION IN THE FOLDING OF REDUCED HEN EGG LYSOZYME

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We have examined the early intermediate products of the regeneration of lysozyme by acidifying the regeneration solution containing the partially folded products, alkylating the free sulfhydryls and digesting the proteins sequentially with pepsin and chymotrypsin. Peptide maps were produced. Results indicate that a limited number of different disulfide bonds is formed early in the regeneration. From this we conclude that a limited number of 3-dimensional structures is formed in the early stages of the refolding process, and the process is not a random search.

Recently there has been great interest in the assembly of fundamental biological structures. Basic to this interest is the formation of three-dimensional structure in proteins. It has been generally assumed that all proteins have a conformation of lowest free energy which is a result of their covalent sequence and the solvent in which they are immersed (1-3). Several studies have appeared recently which suggest kinetic determinism of the folding process and of the resultant native structure (4-7). Argument has also been presented that it would be impossible in biological time for a protein to randomly search through all possible structures to arrive at a structure of lowest free energy (8). We show in the experiments described below that a limited number of folding pathways exist for lysozyme.

Materials and Methods

Reduction and storage of lysozyme. Hen egg lysozyme (Worthington, 3x cryst. was reduced with 0.065 M dithiothreitol in 10 M "Ultrapure" aqueous urea (Mann). This solution also contained 10^{-3} M EDTA, and had been adjusted to apparent (app.) pH 8.6 with Tris base. The reduction was allowed to proceed at room temperature for 2 hours, adjusted to app. pH 3 with acetic acid,

and applied to a column of Sephadex G-25 coarse which had been pre-equilibrated with 0.10 M acetic acid. The separated reduced lysozyme was lyophilized and stored at -10° in a desiccator. All glassware in these experiments was cleaned with a washing protocol which ended with a thorough rinse with 0.1 N HCl, followed by several rinses with water which had been glass-distilled following deionization.

Sulfhydryl analysis. These analyses were carried out according to Ellman's method (9), using a solvent whose final composition was 8 M "Ultrapure" urea, Tris buffer app. pH 8.6. Protein concentration was determined by measuring A_{280} and employing $A^{1\%}_{280} = 24.5$ for hen egg lysozyme (10).

Regeneration procedure. An air-oxidation with Cu^{++}-catalysis (11,12) was employed. Two liters of 0.01 N Tris acetate, pH = 8.0, containing 2 x 10^{-7} N $CuCl_2$ was prepared and warmed to 37° C. Forty mg lyophilized reduced lysozyme was dissolved in 6 ml 0.1 N acetic acid and was added to the warmed buffer. The sulfhydryl content was monitored by Ellman's method so that the oxidation could be stopped when the average number of disulfide bonds formed was between .25 and 1.5 bonds.

Stopping the regeneration reaction. The stop solution was two liters of 10 M urea (Baker analyzed grade) previously passed down a 2.5 x 40 cm column of Biorex AG-501-X8 mixed bed ion exchange resin (Biorad, Richmond, Calif.). The urea solution was 2 x 10^{-3} M in EDTA sodium salt and 3.2 x 10^{-2} M in N-ethylmaleimide (Pierce Chemicals, Rockford, Ill.) and adjusted to app. pH 4.0 with HCl. When mixed with an equal volume of regeneration solution the final app. pH was 5.2. This mixture was allowed to react 30 min. to insure complete alkylation of thiols.

Concentration of the regeneration products. After the alkylation reaction, the four liters of stopped regeneration solution were brought to pH 2.6 with HCl. The mixture was concentrated by ultrafiltration through a UM-2 membrane in a Diaflo apparatus (Amicon Corp., Lexington, Mass.) until the volume reached 50 ml. This volume of protein was applied to a 2.5 x 80 cm column of Biogel

P-6 (medium mesh) (Biorad Laboratories, Richmond, Calif.) and eluted with 0.05 N HCl. The column volume peak was collected and lyophilized twice, 6 ml of water being added between lyophilizations to remove HCl.

Check on disulfide content of product. One mg of the product was dissolved in 1 ml of 10 M urea (Mann Ultra Pure) containing 8 mg of dithiothreitol adjusted to pH 8.6 with 2 M Trizma base (Sigma Chemical Co.). After one hour of reduction the product was adjusted to pH 3 with acetic acid and was passed down a 1 x 25 cm column of Sephadex G-25 (medium mesh) eluted with 0.1 M acetic acid. The sulfhydryl determination with Ellman's reagent was repeated.

Visualizing disulfide containing peptides. The digestion, mapping, and staining procedures were those described by Maeda et al (11). The blocked regeneration products were digested by pepsin, followed by chymotrypsin, and them chromatographed with butanol:acetic acid:water 4:1:5 for 20 hours on Whatman 3MM sheets 46 x 57 cm. The chromatography was followed by an electrophoresis in formic acid (88%)/acetic acid/H_2O, 25/87/888, in a Michl apparatus cooled with Varsol. The dried chromatograms were sprayed with sodium borohydride solution, the excess borohydride neutralized with acid vapors (11), and the thiol-containing peptides visualized with Ellman's reagent. The papers were counterstained with ninhydrin by the method of Dreyer and Bynum (14). The paper was dipped in the ninhydrin stain only after the Ellman stain was thoroughly dry. After the ninhydrin stain had dried, the paper was wrapped in white paper and left in the dark for 2 or 3 days to develop color.

Results and Discussion

We routinely obtained a peptide map from peptic, chymotryptic digests of native lysozyme like that in Fig. 1 which contained 10 -S-S- spots. The resolution is similar to that obtained by Meienhofer's group (11). The three additional disulfide spots that we find appear to be closely related to peptides 5, 6, and 7 in Meienhofer's designation.

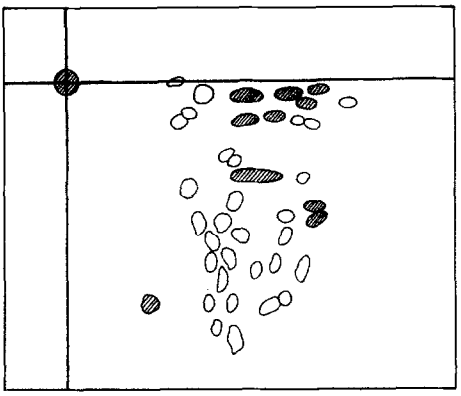

Fig. 1. Peptide map of a peptic, chymotryptic digestion of native lysozyme; 8 mg of digest was applied. Conditions for proteolytic digestion and mapping are described in the Materials and Methods section. Chromatography left to right, electrophoresis top to botton. Cross-hatched spots are disulfide-positive; unfilled spots are ninhydrin-stained.

To test whether any of the disulfide positive spots are derived from undigested lysozyme, a control in which native lysozyme was chromatographed and electrophoresis was performed. The result was a continuous smear along the origin line with no peptides or -S-S- stain visible off the smear.

Lysozyme whose sulfhydryls were completely blocked with N-ethylmalemide was digested with pepsin and chymotrypsin. This digest was mapped to test whether peptides containing derivatized cysteinyl residues produce a color with Ellman's reagent. They did not: the map showed a generous display of ninhydrin-staining spots, but Ellman-positive stain was found only at the origin.

Another control was performed by permitting the autolysis of 2 mg of pepsin in 10 ml of 0.05 N HCl and subsequently digesting the autolyzed pepsin by 2 mg chymotrypsin at pH 5, i.e., performing the standard digestion procedure without the substrate. The map of this digest showed a disulfide-containing spot at the origin (containing pepsin and chymotrypsin) and 3 ninhydrin positive spots near the center of the map. This control establishes that no cystine-containing peptides derived from pepsin and chymotrypsin appear except at the origin.

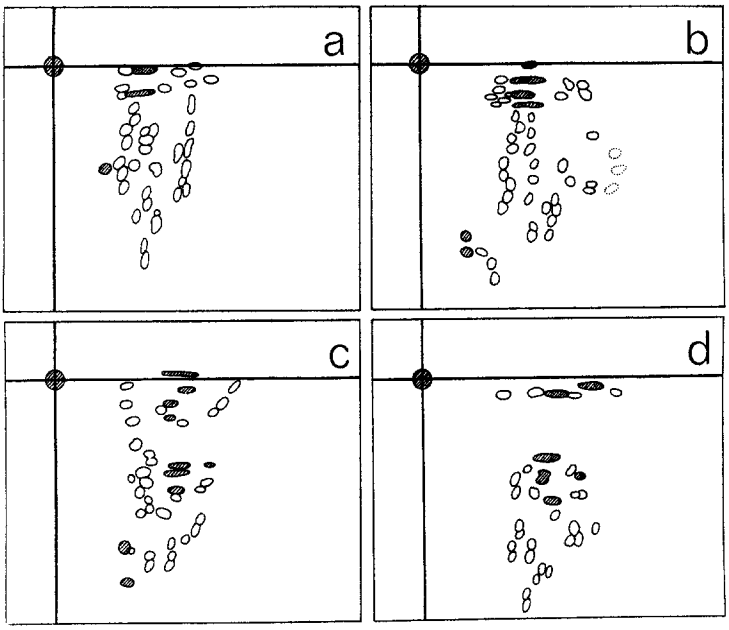

Fig. 2, a-d. Peptide maps of a peptic, chymotryptic digestion of lysozyme regenerated to varying extents. Conditions and designations are the same as in Fig. 1. The extent of regeneration is 1/4 disulfide bond in 2a, 1/2 disulfide in 2b, 1 disulfide in 2c, and 1 1/3 disulfide in 2d.

Figures 2 a-d are typical of many maps obtained of regenerations ranging from 1/4 to 1 1/2 stoichiometric disulfide bonds formed. Note that in map 2a (1/4 disulfide bond formed) only three disulfide spots are present. As the regeneration progresses to one half disulfide, six disulfide-positive spots are present, and at one disulfide regenerated the number has increased to ten. At 1 1/3 disulfides the number decreases to seven, and the pattern begins to resemble that of the native map, but some of the "native" spots have not yet appeared.

If one allows the lysozyme to oxidize to completion (<0.1 -SH) and then digests the product with the standard pepsin-chymotrypsin procedure one obtains a map like that in Fig. 3. One extra spot which does not correspond to those on the native map (Fig. 1) is found. This result is of interest since the highest yields of regenerated lysozyme obtained by this route approach only 85% of specific activity of native lysozyme.

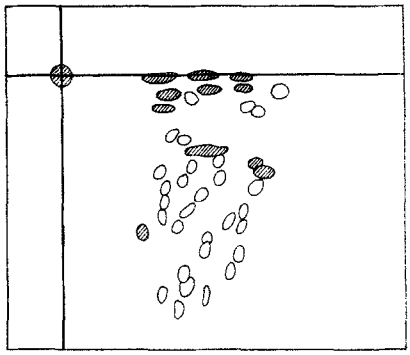

Fig. 3. Peptide map of peptic, chymotryptic digestion of lysozyme regenerated to maximum extent (lysozyme specific activity 80% that of native lysozyme, -SH titer < 0.1 -SH/mole lysozyme. Conditions and designations are the same as in Fig. 1.

Our attempts to identify the disulfide-containing peptides by amino acid analysis of hydrolyzed eluates have been given ambiguous results, presumably because of incomplete separation of peptides on the paper. We are proceeding to separate digests by column chromatographic methods for more certain identification.

There are 28 possible half-cystine pairs containing heterogeneous pairings of the sulfhydryls, plus 8 self pairings (which would occur if the same cysteinyl residue from two different molecules formed a disulfide). If the formation of structure in lysozyme is preceded by random searching over all possible structures, we would naturally expect random pairings of sulfhydryls. One should then see many different pairings occuring early, and thus many chromatographic spots. As the regeneration system begins to produce active molecules of lysozyme, the number and pattern of the disulfide peptides should be expected to approach that of the native map.

The fact that we find few, and not many disulfide peptides in the early stages of the regeneration, indicates a limited search of structures rather than an exhaustive search. The results obtained thus far leave open the possibility that "non-native" disulfide pairings form. Native lysozyme, with 4 disulfide bonds, gives rise to ten Ellman-positive spots in our system. This is probably

due to the heterogeneity of the lysozyme used; or to heterogeneity introduced by the proteolysis. We are tentatively interpreting the number of chromatographic spots as representing something like a general two-fold over-estimate of the number of disulfide pairings. In this perspective the number of disulfide pairings found is slightly larger than would be expected from a single, obligatory folding pathway, but still represents a high degree of restriction. The most plausible mechanism available to restrict the folding pathways is nucleation.

We believe that these results represent the first experimental evidence for a restricted search of structures in the formation of the three-dimensional structure of a protein.

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