KINETIC DETERMINISM OF LYSOZYME FOLDING AT HIGH TEMPERATURES

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Reduced, disordered hen egg lysozyme rapidly regains enzymic activity in a nonenzymic system previously reported from this laboratory. When such regenerations are carried out at high temperatures (where the native enzyme is unstable), native enzyme is formed as a transient intermediate. Thermal inactivation does not depend on irreversible changes in the protein, as shown by experiments wherein thermally inactivated regenerated lysozyme spontaneously reactivates at 37°. These findings are inconsistent with thermodynamic determinism of protein structure.

The mechanisms of formation of three dimensional structure in proteins are presently engaging much active investigation (1-3). A fundamental issue in this matter is whether native protein structures are determined solely by thermodynamic factors, or whether kinetic constraints also operate. Different consequences can be expected from thermodynamic and kinetic determinism in the matters of speed of protein self-assembly, stability or metastability of native structures, and metabolic turnover (3-5).

As part of our continuing studies (6-9) on the mechanism of protein folding, we have examined the glutathione-facilitated regeneration (6,9) of reduced hen egg lysozyme (HEL) at high temperatures. The results show a strong kinetic component in the self-assembly process.

Materials and Methods

Hen egg lysozyme (HEL) was obtained from Worthington Biochemical Corp. (lot OCC). It was purified chromatographically (see below) before use. Reduced glutathione (GSH), oxidized glutathione (GSSG), dried cells of *M. lysodeikticus*, and "Tris" base (Trizma quality) were obtained from Sigma Chemical Co. Urea of "Ultra-pure" grade was Schwarz-Mann. EDTA (Na_2 salt) was an AR product of Mallinckrodt Chem. Works. Other reagents were of reagent grade. Water was de-ionized, followed by glass distillation from 0.2 N H_2SO_4.

Ion-exchange chromatography of HEL was carried out according to Stevens and Bergstrom (10). The major peak was desalted on a 15 cm. x 50 cm. Seph-

adex G-25 (medium) column equilibrated with and eluted with 0.10 N acetic acid. This material was lyophilized and used in all subsequent experiments.

The reduction of HEL was carried out with dithiothreitol in 8 M urea, (1.0 mg. dithiothreitol/mg. protein) in 0.10 M Tris-acetate buffer. The apparent pH of the complete reduction solution was adjusted to 8.6 (with "Tris" base) and the reduction allowed to proceed 2 hr. at room temperature. The solution was acidified to pH 3.5 with acetic acid and passed through a 2.5 cm. x 70 cm. Sephadex G-25 (coarse) column equilibrated with and eluted with 0.10 N acetic acid. The reduced protein was immediately frozen and lyophilized. Sulfhydryl analysis (11) showed the reduced protein to contain 7.6-7.9 equiv./mole. Protein concentrations were determined photometrically (6,9).

The regeneration solutions contained 10^{-4} M EDTA, 1.0×10^{-4} M GSSG, 1.00×10^{-3} M GSH, and 0.10 M Tris-acetate buffer (pH = 8.0 at 25° after addition of reduced HEL). The regenerations were begun by adding 1.00 ml. reduced HEL (1.0 mg./ml. 0.10 N acetic acid) to 99.0 ml. of regeneration solution. After mixing, 1.00 ml. aliquots of regenerating lysozyme solution was added to 1.00 ml. 0.20 N acetic acid to quench the regeneration by bringing the pH to 4.0. The quenched aliquot was assayed within a few minutes. Control experiments have shown lysozyme activity to be independent of time in the quench solution up to 60 minutes.

Lysozyme was assayed at 37° using a modification (6,9) of the method of Jolles (12). The turbidity (A_{350}) of a suspension of killed cells of M. lysodeikticus at 37° is recorded continuously over a two minute interval. The apparent activity of a lysozyme standard is determined every third assay during a regeneration. Fresh suspensions of substrate cells are prepared every 90 minutes.

Results and Discussion

Fig. 1 shows the family of curves for regeneration of enzymic activity from reduced HEL at temperatures from 37° to 66°. Results at the three lower temperatures are similar in rate, in yield, and in the generation of a plateau of activity vs. time. The 66° regeneration, however, shows a clear maximum in yield at about 20 min., and subsequent decline until termination of the observations at 240 min. Regenerations carried out at still higher temperatures also show maxima in yield, with complete decay of enzymic activity at 150 min. or less. These results are shown in Table I. The difficulty of finding suitable coordinates for an undistorted graphical presentation of such data is apparent in the left half of Fig. 3.

Every regeneration depicted in the three figures and Table I represents a different experiment. Although we find only fair quantitative reproduci-

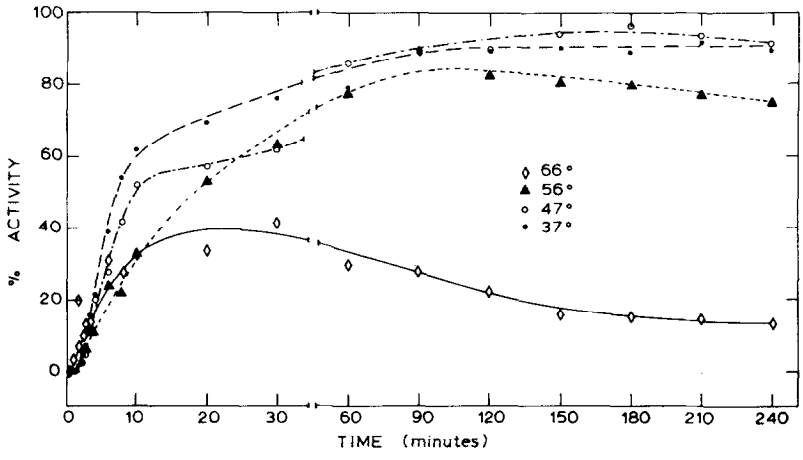

Fig. 1 Nonenzymic lysozyme (HEL) regenerations at elevated temperatures. The regeneration system is described in Materials and Methods. The regeneration is started at 0 time. Regenerations are carried out at the indicated temperatures, but aliquots are withdrawn, quenched in dilute acid and assayed at 37°.

bility in regenerations carried out above 60°, the qualitative features of a maximum yield followed by decay of activity are invariably found.

The general character of regenerations carried out between 60° and 90° qualitatively fits the oversimplified model: A→B→C, where A represents reduced, inactive HEL, B represents enzymically active protein, and C represents thermally inactivated HEL. Two consecutive processes are proposed: the first representing regeneration as seen at temperatures from 37° to 50° (Fig. 1), and the second representing thermal inactivation.

To test the validity of the proposed thermal inactivation (B→C), we carried out several paired regeneration and "degeneration" experiments at temperatures between 60° and 90°. In the "degeneration" member of the pair, the starting material was chromatographically purified HEL. The other components of the solution were the same as for the regeneration. A typical result is shown in Fig. 2, which displays the results of a regeneration, degeneration pair carried out at 84°. In the regeneration member we see a slow monotonic decay. Qualitatively the same results were seen in similar paired experiments at 60°, 65°, and 74°.

None of the foregoing evidence proves kinetic determinism of structure if the loss of activity is irreversible. A real possibility existed that the inactivation was due to hydrolysis of labile peptide or disulfide bonds, or to some other irreversible covalent change. It was therefore necessary

Table I

% Lysozyme Activity in High-Temp. Regenerations

Time (Min)	76° C	86° C	94° C	99° C
0.5	0	0	0	0
1.0	0	3	0	0
1.5	0	2	3	0
2.0	0	2	2	0
2.5	0	1	0	0
3.0	4	2	2	0
3.5	3	2	0	0
4.0	2	1	2	0
6	3	5	1	0
8	3	2	2	0
10	5	0	0	0
20	3	2	0	0
30	2	1	1	0
60	2	0	0	0
90	1	0	0	0
120	0	0	0	0
150	0	0	0	0
180	0	0	0	0
210	0	0	0	0
240	0	0	0	0

Table I Lysozyme (HEL) regenerations at elevated temperatures. Experimental protocol as for Fig. 1.

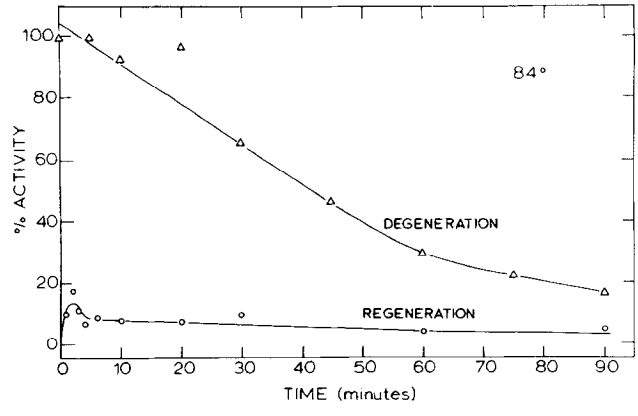

Fig. 2 Regeneration of reduced lysozyme (HEL) at 84°, and simultaneous "degeneration" of native lysozyme (HEL) at the same temperature. Experimental protocol as for Fig. 1.

to test the reversibility of the degeneration component of the high-temperature regenerations. Fig. 3 shows the results of three such tests.

Examination of Fig. 3 shows first a control 37° regeneration. The re-

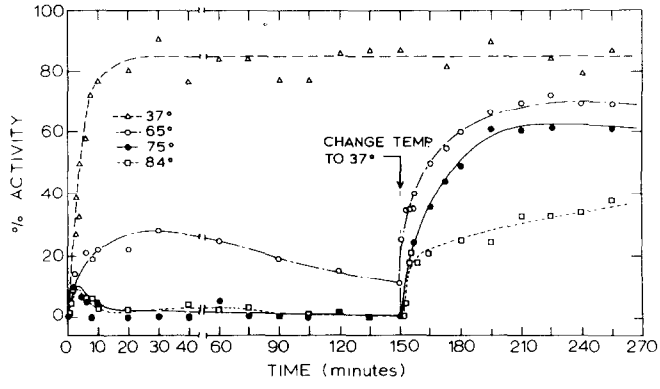

Fig. 3 Test of reversibility of inactivation of lysozyme (HEL) regenerated at elevated temperatures. Experimental protocol as for Fig. 1, but the vessels containing the regeneration solutions were transferred to a 37° water bath at 150 min.

generations carried out at 65°, 75°, and 84° are labeled on the figure. In each of these three regenerations a maximum in activity is found early in the regeneration, with decrease in activity (to zero activity at 75° and 84°) occurring up to 150 min. At that time, the regeneration vessel was transferred to a bath thermostatted at 37°, and aliquots were withdrawn for assay just as in the beginning of a regeneration. A rapid increase in enzyme activity was seen in all three regenerations. It is clear, however, that the yield after temperature-drop correlates inversely with the temperature before temperature-drop. This seems to indicate the existence of a (small) fraction of irreversible inactivation at 65° and 75°. This fraction is about 1/2 at 84° (but the yield was still increasing when the observations were terminated). Thus most of the inactivation processes at 75° and lower are reversible.

Without characterization of the active product of high-temperature regenerations, we cannot be sure of its identity with the native protein. However, it seems fair to say that demonstration of enzymic activity shows a very close structural relationship to native HEL.

We have therefore shown formation of active HEL as a transient intermediate under conditions such that the potential for regeneration is re-

tained. In other words, native lysozyme can form under conditions where it is thermodynamically unstable. In our view, this can only come about from the operation of kinetic factors in the folding process. If the native structure were determined wholly by thermodynamic factors, no activity would be observed in the attempts to regenerate HEL at elevated temperatures.

Strictly speaking, the above evidence only justifies the conclusion of kinetic determinism of structure for hen egg lysozyme at high temperatures. It does not prove kinetic determinism under physiological conditions of self-assembly. However, until more direct evidence becomes available, that extrapolation seems neither large nor unreasonable.

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