Disulfide content of reduced hen egg white and human milk lysozymes during the folding process

Jean-Paul Perraudin, Roger Guillard, Jean-Paul Prieels, Timothy Torchia[+] and Thierry Dubois

Laboratoire de Chimie Générale I, Université Libre de Bruxelles, Av. Franklin Roosevelt, 50, 1050 Bruxelles, Belgium and [+]*Department of Biological Chemistry, Milton S. Hershey Medical Center, Pennsylvania State University, Hershey, PA 17033, USA*

Received 21 January 1983

In order to obtain a better understanding of the possible influence of the primary sequence of a protein on its folding pathway, renaturation of reduced human milk lysozyme was compared to that of reduced hen egg white lysozyme. Following disulfide bond formation, under identical conditions, similar products were found during the folding of both lysozymes, but the kinetics of appearance and disappearance of these intermediates as well as the appearance of the native conformation were different.

Folding *Disulfide* *Kinetic intermediate* *Human lysozyme* *Hen egg white lysozyme* *Renaturation*

1. INTRODUCTION

In the search to determine how a protein acquires its biologically active three-dimensional structure, many experiments have attempted to establish the process by which the reduced, denatured polypeptide chain regains its native structure through observation of disulfide bond formation [1]. Most of these experiments were performed in vitro, with protein refolding being induced by reoxidation conditions supposedly close to those observed in the cell [2]. However, the cell's oxidizing agents have not yet been identified definitively, and the environment in which the polypeptide chain is generated in vivo is still open to speculation. In conjunction with the hypothesis of non-enzymatic catalysis, a mixture of reduced and oxidized glutathione has been proposed as one of several reoxidation systems favoring thiol–disulfide interchange. Glutathione is indeed present in most tissues in greater quantities than other thiol and disulfide compounds of low molecular mass [3]. Nevertheless, in such systems not all proteins acquire their native structures as rapidly as they do in vivo; such is the case for ribonuclease which led some investigators to support a theory involving enzymatic catalysis [4].

Until now the folding of various proteins under identical reoxidizing environments [5] and the folding of one protein under several reoxidation systems [6] have been compared. The object of this paper is to complete such studies by comparing the folding of two homologous proteins, hen egg white lysozyme (HEWL) and human milk lysozyme (HML) performed under identical conditions.

2. MATERIALS AND METHODS

Human lysozyme was reduced with dithiothreitol at room temperature for 2 h at pH 8.6 as in [7].

In this system [8] the regeneration mixture contained 1 mM reduced glutathione, 0.1 mM oxidized glutathione and 1 mM ethylene diaminetetraacetic acid (EDTA) in 0.1 M Tris (hydroxymethyl) aminomethane acetate buffer, at pH 8.0. Regeneration was initiated by mixing 1 vol. reduced lysozyme in 0.1 M acetic acid with 1000 vol. regeneration mixture. Lysozyme concentration in the regeneration mixture was 2.4×10^{-6} M and reoxidation was allowed to proceed at 37°C.

Published by Elsevier Biomedical Press
00145793/83/0000–0000/$3.00

Regeneration was stopped by converting the remaining free thiols into *S*-carboxymethylcysteine. Solid urea and iodoacetic acid were added to the regeneration mixture, until final concentrations of 8 M and 0.1 M, respectively, were reached and pH was immediately readjusted to 7 by addition of 6 M NaOH. The *S*-carboxymethylation was then allowed to proceed at room temperature for 15 min and glacial acetic acid was added to stop the reaction by lowering the pH to 3.

The *S*-carboxymethylcysteine content of each sample was determined by amino acid analysis after acid hydrolysis in 5.6 N HCl at 105°C for 24 h.

Resolution of densitometer tracings allowed determination of the relative amounts of each intermediate for each reoxidation time. The stained polyacrylamide electrophoresis gels were scanned spectrophotometrically but as the scanning showed poorly resolved bands for most of the densitometer tracings, a numerical analysis of data was performed as in [7].

3. RESULTS AND DISCUSSION

As shown in [7], HEWL refolds much more rapidly than HML, this difference had been explained by the more highly organized structure and more hydrophobic nature of HML. However, to obtain a better comparison of the two protein-folding mechanisms, one must study the behaviour of their various intermediates as a function of the total free thiol content.

The experimental conditions were using concentrations of reduced and oxidized glutathione corresponding to optimal refolding conditions for HEWL but not for HML [8]. These concentrations were yet used to induce HML regeneration as the objective was to compare the refolding mechanisms of HML and HEWL under identical reoxidation conditions.

After induction of disulfide bond formation associated with the refolding process of reduced HML, the molecules being formed were trapped in stable configurations by addition of iodoacetic acid at different reoxidation times. Each population of molecules trapped under those conditions was subjected to polyacrylamide gel electrophoresis, which enabled us to separate intermediates on the basis of their disulfide bond

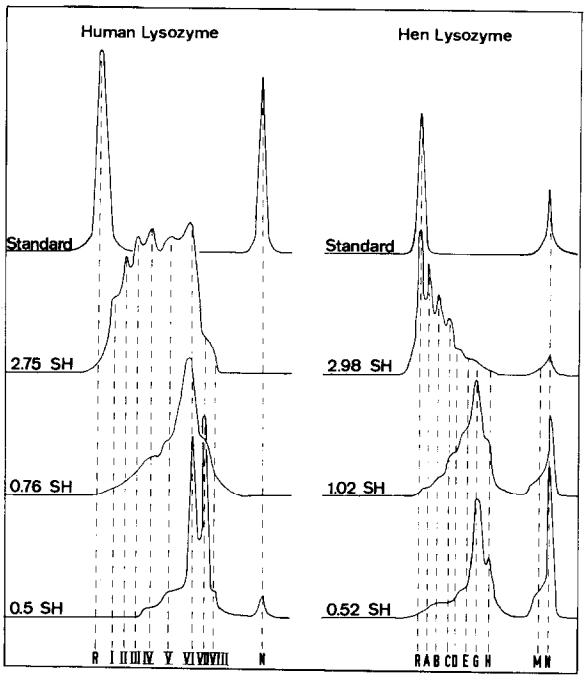

Fig.1. Comparison of electrophoretic pattern densitometer tracings of human milk lysozyme and hen egg white lysozyme [7] reoxidized at comparable residual free thiol contents.

contents and their polypeptide chain's average volume.

Electrophoretic patterns describing the reoxidation of HML and HEWL at identical free thiol contents are displayed in fig.1, there are visible differences between the two protein's electrophoretic patterns. Nevertheless, these differences are smaller than those observed [9] during comparisons of HEWL refolding in different reoxidation systems.

Fig.2a–c and 3a,b show the relative amounts of the various intermediates produced during renaturation of each lysozyme as a function of the total number of free thiols present in the reaction mixture. Only species II–V in the case of HML and species B–E in the case of HEWL behaved similarly and if HEWL's enzymatic activity appeared much more rapidly than that of HML [7], the disappearance of reduced HEWL (R) corresponded to a lower level of free thiols (2 to 0 SH) than reduced HML (3 to 2 SH). The curves representing the amount species I and A exhibit the

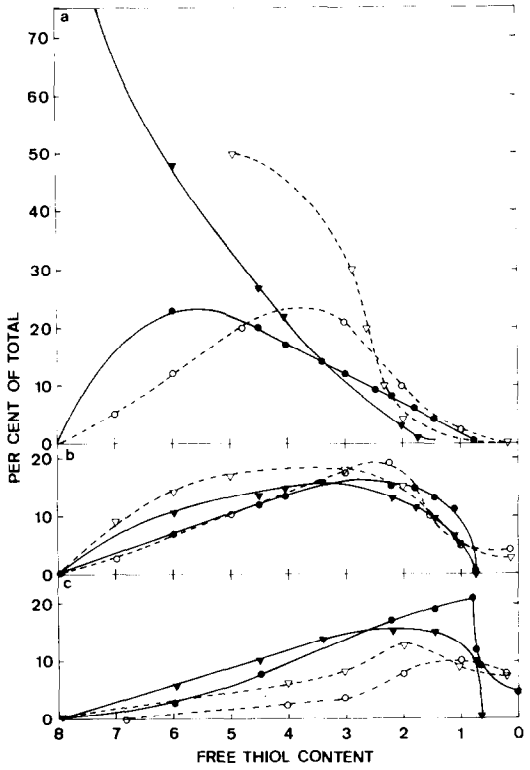

Fig.2. Study of the population of the various species (trapped during the renaturation process) as a function of the overall residual free thiol content of the reoxidation mixture: (a) HML, R (▼), I (●); HEWL, R (▽), A (○); (b) HML, II (▼), III (●); HEWL, B (▽), C (○); (c) HML, IV (▼), V (●); HEWL, D (▽), E (○).

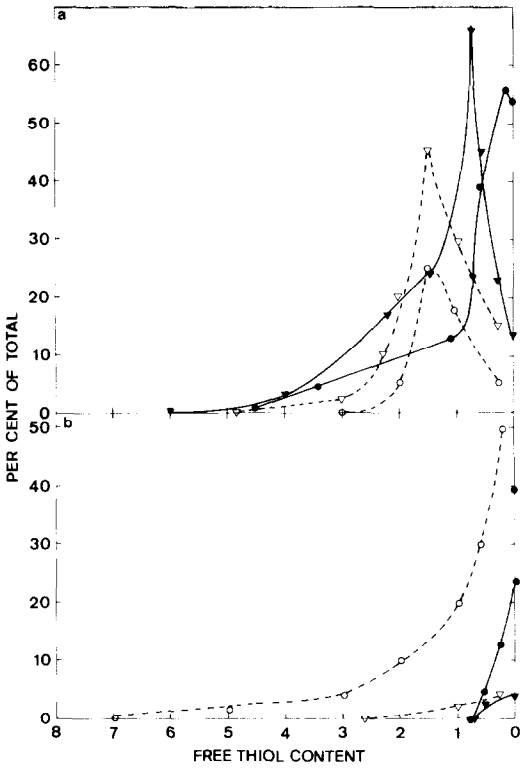

Fig.3. Study of the population of the various species (trapped during the renaturation process) as a function of the overall residual free thiol content of the reoxidation mixture: (a) HML, VI (▼), VII (●); HEWL, G (▽), H (○); (b) HML, VIII (▼), N (●); HEWL, M (▽), N (○).

same shape but reach maximum concentrations at different SH levels (6 to 5 SH for HML; 4 to 3 SH for HEWL). Their concentrations were of the same order as those of the reduced species, which indicates that species I and A are generated from the reduced proteins. The slowness of HLM reoxidation is explained mainly by the behaviour of intermediates VI and VII, which contain 4 disulfide bonds; indeed, if HEWL intermediates G and H appear simultaneously, with respective maximum concentrations of 45% and 25%, intermediates VI and VII appear consecutively, with maximum concentrations of 66% and 56%, respectively. In HEWL, intermediates G and H are not particularly stable, and the energy barrier between the native and the other conformations of HEWL are probably not high, a conclusion borne out by the appearance of a completely renatured protein almost immediately after initiation of reoxidation. On the other hand, a high energy barrier, which would constitute a limiting step, must exist in the case of HML. Thus, foldings of both lysozymes involve intermediates similar in distribution and type, that is to say the same number of species respectively containing the same number of disulfide bonds [7] but the kinetic behaviours of the two series of intermediates are different.

In conclusion, although it is not known why concentrations of catalysts have to be different to obtain optimal conditions of regeneration for each homologous protein, it is clearly observed that a modification in the primary sequence has a lesser effect on the folding pathway than a modification of the reoxidation conditions.

REFERENCES

[1] Freedman, R.B. and Hillson, D.A. (1980) in: The Enzymology of Post-Translational Modifications of Proteins (Freedman, R.B. and Hawkins, H.C. eds) pp.157–212, Academic Press, New York.
[2] Wetlaufer, D.B., Saxena, V.P., Ahmed, A.K., Schaffer, S.W., Pick, P.W., Oh, K.-J. and Peterson, J.D. (1977) in: Protein Crosslinking, pt A, pp.43–50, Plenum, New York.
[3] Isaacs, J. and Binkley, F. (1977) Biochim. Biophys. Acta 497, 192–204.
[4] Anfinsen, C.B. (1973) Science 181, 223–230.
[5] Creighton, T.E. (1978) Prog. Biophys. Mol. Biol. 33, 231–297.
[6] Perraudin, J.P., Oh-Johanson, K.H., Berga, S.E., Torchia, T., Voet, J.G. and Wetlaufer, D.B. (1978) Fed. Proc. FASEB 37, 1275.
[7] Dubois, T., Guillard, R., Prieels, J.P. and Perraudin, J.P. (1982) Biochemistry 21, 6516–6523.
[8] Wetlaufer, D.B., Johnson, E.R. and Lorraine, M.C. (1974) in: Lysozyme (Osserman, E.F. et al. eds) pp.269–286, Academic Press, New York.
[9] Perraudin, J.P. (1982) Ph.D. Thesis, Free University of Brussels.