Molecular mechanisms of the irreversible thermal denaturation of guinea-pig liver transglutaminase

Sylvie NURY and Jean-Claude MEUNIER*

Laboratoire de Chimie Biologique, Centre de Biotechnologie Agro-Industrielle (C.B.A.I.), 78850 Thiverval-Grignon, France

When transglutaminase is heated at temperatures above 40 °C, it loses its activity according to a two-step mechanism [Nury, Meunier & Mouranche (1989) *Eur. J. Biochem.* **180**, 161–166]:

However, the nature of the molecular events responsible for the irreversible denaturation is still unknown. Investigation of the effects of dithiothreitol and 5,5'-dithiobis-2-nitrobenzoate on the kinetics of inactivation, titrations of ammonia released by deamidation and of thiol groups on the native and denatured enzymes and SDS/PAGE rule out the involvement of covalent processes during the denaturation of transglutaminase at 55 °C and pH 7. Of the two possible kinds of non-covalent events, i.e. unfolding of the polypeptide chain and aggregation of enzyme molecules, we show that both occur, though only the former process is responsible for the denaturation. The latter process, aggregation, follows the unfolding of the molecules.

INTRODUCTION

The kinetics of the thermal irreversible denaturation of guinea-pig liver transglutaminase (protein-glutamine γ -glutamyltransferase, EC 2.3.2.13) is characterized by two steps:

where N represents the native form, X represents an intermediate form, the structure of which depends on the deactivation temperature T_D , and D is the completely denatured form of the enzyme [1]. Ca^{2+} is able to stabilize the enzyme against thermal inactivation. Up to now the molecular mechanisms involved in the irreversible thermal denaturation of transglutaminase have remained unknown. However, information is available concerning the thermal denaturation of some other enzymes, such as trypsin [2], ribonuclease [3], lysozyme [4], triose-phosphate isomerase [5] and α -amylases [6,7]. Deamidation of asparagine and glutamine residues, modification of cysteine residues and cuts in the polypeptide chain are the main covalent events of the irreversible thermal denaturation [8]. Conformational changes can also occur, sometimes leading to aggregation. Covalent modifications are reported to happen at very high temperatures (above 80 °C) and at extremes of pH (below 5 and above 8).

The aim of the present paper is to study the molecular mechanisms responsible for the irreversible thermal denaturation of guinea-pig liver transglutaminase.

MATERIALS AND METHODS

Purification of transglutaminase

Guinea-pig liver transglutaminase was obtained and purified to homogeneity as described by Brookhart *et al.* [9], slightly modified by Bercovici *et al.* [10].

Protein assay

Protein concentrations were determined with the Bio-Rad reagent [11].

Kinetic measurements

The esterase activity was monitored by using the *p*-nitrophenyl acetate assay described by Folk *et al.* [12]. The transferase activity was measured by the formation of hydroxamate, by a method established by Waelsch & Mycek [13] and Folk [14]. For both assays the reaction rates were linear for at least 10 min with 70 nm-enzyme.

Thermal inactivation process

Small test tubes containing enzyme were incubated at 55 °C. At intervals one of the assay tubes was taken out and quickly cooled on ice (15 min). The activity remaining was monitored at a constant temperature (25 °C). It was checked that the remaining activity reached a constant value immediately after cooling on ice, and remained constant for at least several hours. Precautions were taken to prevent changes in pH when the temperature of the sample was altered. The same protocol was used for experiments carried out to analyse structural modifications of the enzyme.

Ammonia release

The release of ammonia was measured enzymically by the reaction catalysed by glutamate dehydrogenase. The assay was performed in the standard conditions described by Levitzski [15].

Succinylation (3-carboxypropionylation) of transglutaminase

Guinea-pig liver transglutaminase was succinylated by following the general procedure of Riordan & Vallee [16]. This modification resulted in acylation of 32 out of 34 ϵ -amino groups of the lysine residues in the protein, as

* To whom correspondence should be addressed.

determined by a spectrophotometric titration with 2,4,6-trinitrobenzenesulphonate [17]. The specific activity of the succinylated enzyme was 38 % of that of the native enzyme.

Measurement of thiol groups

The assay was performed with 5,5'-dithiobis-2-nitrobenzoate as reactant, according to the method described by Habeeb [18]. To ensure measurement of the whole thiol content of transglutaminase, 2 % (w/v) SDS was included in the reaction mixture. The percentage of available thiol groups as a function of incubation time was determined by the following equation:

$$\%SH_{av.} = \frac{SH_{denat.} - SH}{SH_{denat.} - SH_{nat.}}$$

Oxidation of transglutaminase

By using the method described by Habeeb [18] and the protocol used by Connellan & Folk [19], we created a single disulphide bridge in the enzyme. The reactant 5,5'-dithiobis-2-nitrobenzoate was allowed to react with transglutaminase at the ratio of one reactant molecule per enzyme molecule.

PAGE

PAGE was performed in the presence of denaturant (SDS) and 2-mercaptoethanol or in native conditions according to the method of Laemmli [20]. The acrylamide concentration was 7.5 % (w/v). The proteins were stained with Coomassie Blue G-250.

RESULTS AND DISCUSSION

Since it has been reported [2–8] that deamidation of asparagine and glutamine residues might be responsible for the irreversible denaturation, we have performed

Fig. 1. Kinetics of the thermal inactivation of transglutaminase with respect to its redox state

Inactivation was carried out at 55 °C. □, In the presence of a reductant (80 mM-dithiothreitol); ●, without any additive; ▲, enzyme treated with 5,5'-dithiobis-2-nitrobenzoate, as described in the Materials and methods section. The initial activity was taken as 100 %. All the kinetic assays were performed at 25 °C with the *p*-nitrophenyl acetate method. Enzyme concentration was 3 μ M during the inactivation and 70 nM in the assay.

titration of ammonia on the native and the denatured transglutaminase (after 30 min at 55 °C). With an enzyme concentration of 4 μ M, one deamidation process per molecule should have produced an absorbance decrease at least equal to 0.03. The observed absorbance decrease was equal to 0.002, suggesting that no ammonia had been released. Hence this kind of covalent modification is not involved in the thermal denaturation.

Transglutaminase is known to possess 16 free thiol groups [21], but no disulphide bridge. The thermal inactivation of enzyme might depend on its redox state. The inactivation was carried out at 55 °C in the presence of a reductant (80 mM-dithiothreitol) as described in the Materials and methods section. The kinetics of the inactivation were not modified by the presence of dithiothreitol (Fig. 1). Since the inactivation rate constants were the same (within the experimental errors) with or without the presence of the reductant, the formation of disulphide bonds was not responsible for the loss of activity. Indeed, by fitting the data to the equation of a two-step inactivation model (cf. the Introduction and ref. [1]), the observed inactivation constants are $1.20 \pm 0.16 \text{ min}^{-1}$ (k_1) and $0.20 \pm 0.05 \text{ min}^{-1}$ (k_2) for the control and $1.42 \pm 0.40 \text{ min}^{-1}$ (k_1) and $0.32 \pm 0.10 \text{ min}^{-1}$ (k_2) for the reduced enzyme.

By treating the enzyme with 5,5'-dithiobis-2-nitrobenzoate, one disulphide bridge was created [19]. The oxidized enzyme was less active than the native one (only 70 % of its initial activity), but its thermostability was similar to that of the native one (Fig. 1). The observed inactivation constants are $1.20 \pm 0.16 \text{ min}^{-1}$ (k_1) and $0.20 \pm 0.05 \text{ min}^{-1}$ (k_2) for the control and $2.25 \pm 0.60 \text{ min}^{-1}$ (k_1) and $0.20 \pm 0.05 \text{ min}^{-1}$ (k_2) for the oxidized enzyme. The existence of a disulphide bridge in the molecule therefore did not enhance the enzyme thermostability. When more than one disulphide bridge was created in the protein, the enzyme lost its activity completely [19]. Both experiments (with dithiothreitol and 5,5'-dithiobis-2-nitrobenzoate) show that the thermal denaturation is not linked to the redox state of transglutaminase.

The number of thiol groups of the native and the inactivated transglutaminase was investigated. After

Fig. 2. Enzyme activity and availability of thiol groups during the heating of transglutaminase at 55 °C

●, Activity remaining after heating enzyme at 55 °C (*p*-nitrophenyl acetate was the substrate); data are normalized as percentages of initial activity. △, Availability of thiol groups after heating enzyme at 55 °C; data are normalized as percentages of total content of available thiol groups.

Fig. 3. Electrophoretic patterns of transglutaminase

Native PAGE pattern of enzyme heated at 55 °C for various incubation times: lane A, control; lane B, 30 s; lane C, 1 min; lane D, 10 min; lane E, 20 min.

heat treatment of transglutaminase the total number of thiol groups per molecule remained the same (16.2 ± 0.1 and 16.0 ± 0.1 for the native and the inactivated transglutaminase respectively). This confirmed that there was no disulphide bridge formation, and proved that no thiol groups had been destroyed (converted into sulphonic acid). This latter experiment shows that the thermal denaturation process is not linked to a modification of the thiol groups of transglutaminase.

On the native form of transglutaminase 6.9 ± 0.2 thiol groups were accessible to the solvent. During the inactivation process their number increased to 15.8 ± 0.4 . A good correlation occurred between the loss of activity and the availability of thiol groups (Fig. 2). The kinetics of appearance of the thiol groups was characterized by two steps (results not shown), as was the case in the kinetics of inactivation. Therefore the inactivation process seems to be linked to unfolding of the polypeptide chain.

When enzyme was heated at 55 °C for different incubation times between 0 and 20 min and then analysed by SDS/PAGE, the pattern showed a single band cor-

responding to the same molecular mass, namely 80000 Da (results not shown). These results proved that no cut in the polypeptide chain occurred during the heating. In view of the pH value, 7, during the experiment and the relatively low inactivation temperature, 55 °C, a cut in the polypeptide chain would have been very surprising. In contrast, PAGE analysis in native conditions showed that the transglutaminase molecular mass increased with incubation time at 55 °C (Fig. 3), showing that aggregates were being formed. Nevertheless this result does not mean that irreversible denaturation is linked to aggregation. Indeed, this process was ruled out by there being a first order of reaction for both inactivation steps [1], and by the independence (hydroxamate test; results not shown) or even the increase (*p*-nitrophenyl acetate test; results not shown) of the inactivation rate on enzyme concentration.

Aggregate formation is therefore not responsible for the enzyme inactivation. Aggregation follows the conformational changes that were revealed by titration of the thiol groups. This interpretation was confirmed by the finding that succinylated enzyme did not lead to aggregates when submitted to thermal inactivation, as demonstrated by analysis of the native PAGE pattern (results not shown). Further, in the same conditions this modified enzyme lost its activity (Fig. 4), with nearly the same inactivation rates as those for the native enzyme.

To take into account the fact that aggregate formation is not responsible for the enzyme inactivation, one can add a third step to the two-step scheme given in the Introduction, namely:

The unfolding of the chain leads to inactivation and to the exposure of hydrophobic regions to water. In order to avoid this unfavourable thermodynamic situation, molecules aggregate by means of hydrophobic interactions.

REFERENCES

1. Nury, S., Meunier, J. C. & Mouranche, A. (1989) *Eur. J. Biochem.* **180**, 161–166
2. Klibanov, A. M. (1978) *Biochem. Biophys. Res. Commun.* **83**, 1012–1017
3. Zale, S. E. & Klibanov, A. M. (1986) *Biochemistry* **25**, 5432–5444
4. Ahern, T. J. & Klibanov, A. M. (1985) *Science* **228**, 1280–1284
5. Ahern, T. J., Casal, J. I., Petsko, G. A. & Klibanov, A. M. (1987) *Proc. Natl. Acad. Sci. U.S.A.* **84**, 675–679
6. Tomazic, S. J. & Klibanov, A. M. (1988) *J. Biol. Chem.* **263**, 3092–3096
7. Tomazic, S. J. & Klibanov, A. M. (1988) *J. Biol. Chem.* **263**, 3086–3091
8. Klibanov, A. M. (1983) *Adv. Appl. Microbiol.* **29**, 1–28
9. Brookhart, P. P., McMahon, P. L. & Takahashi, M. (1983) *Anal. Biochem.* **128**, 202–205
10. Bercovici, D., Gaertner, H. F. & Puigserver, A. J. (1987) *J. Agric. Food Chem.* **35**, 301–304
11. Bradford, M. M. (1976) *Anal. Biochem.* **72**, 248–254
12. Folk, J. E., Cole, P. W. & Mullooly, J. P. (1967) *J. Biol. Chem.* **242**, 2615–2621
13. Waelsch, H. & Mycek, M. J. (1962) *Methods Enzymol.* **5**, 833–838
14. Folk, J. E. (1970) *Methods Enzymol.* **17**, 889–894

Fig. 4. Thermostability of succinylated transglutaminase

Kinetics of the thermal inactivation of succinylated transglutaminase. Native enzyme (■) and succinylated enzyme (□) were heated at 55 °C and assayed with respect to time with *p*-nitrophenyl acetate as substrate.

15. Levitzski, A. (1970) *Anal. Biochem.* **33**, 335–340
16. Riordan, J. F. & Vallee, B. L. (1967) *Methods Enzymol.* **11**, 565–570
17. Habeeb, A. F. S. A. (1966) *Anal. Biochem.* **14**, 328–336
18. Habeeb, A. F. S. A. (1972) *Methods Enzymol.* **25**, 457–464
19. Connellan, J. M. & Folk, J. E. (1969) *J. Biol. Chem.* **244**, 3173–3181
20. Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
21. Folk, J. E. & Cole, P. W. (1966) *J. Biol. Chem.* **241**, 5518–5525

Received 6 July 1989/19 September 1989; accepted 21 September 1989