Transglutaminase catalyses the modification of glutamine side chains in the C-terminal region of bovine β -lactoglobulin

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The transglutaminase-catalysed incorporation of primary amines (putrescine and monodansylcadaverine) into bovine β -lactoglobulin has been studied. In the presence of 1 mM-dithiothreitol between 1 and 2 mol of amine can be incorporated per mol of β -lactoglobulin subunit. There is very little incorporation of amines in the absence of reducing agent. By isolating and sequencing the modified peptides, the sites of modification have been identified as Gln-159 (preferred) and Gln-155. C.d. has been used to study the structure of β -lactoglobulin over a range of pH values and in the presence or absence of dithiothreitol. The results are discussed in terms of the X-ray-crystallographically determined structure of β -lactoglobulin.

INTRODUCTION

Transglutaminase (EC 2.3.1.13) catalyses the aminolysis of the γ -carboxamide group of the glutamine side chains of peptide and protein substrates. The reaction proceeds via an acyl-transfer mechanism in which the γ -carboxamide group acts as an acyl donor and suitably unbranched primary amines act as acyl acceptors. *In vivo* the acyl acceptor is the ϵ -amino group of a lysine side chain, whereas in model reactions [14 C]putrescine or monodansylcadaverine are frequently employed as acceptors, since the reactions are readily monitored by the incorporation of radioactivity or fluorescence respectively.

The reaction catalysed by transglutaminase offers a potential method for selective introduction of functional groups into proteins under mild conditions. From the relatively limited studies undertaken with model peptide and protein substrates it is clear that both the amino acid sequence and the local conformation around a glutamine side chain can contribute to the specificity shown by the enzyme (Gorman & Folk, 1980, 1984; Berbers *et al.*, 1983; Wold, 1985). As part of a programme in which we have been studying the transglutaminase-catalysed modification of proteins of known structure (Coussons *et al.*, 1991), we have examined the modification of bovine β -lactoglobulin, which had been previously shown to act as a substrate for the enzyme (Lorand & Campbell, 1971).

Here we report that the preferred sites of labelling are the two glutamine side chains (Gln-155 and Gln-159) in the C-terminal portion of the polypeptide chain. The findings are discussed in terms of the known X-ray-crystallographically determined structure of β -lactoglobulin (Sawyer *et al.*, 1985; Papiz *et al.*, 1986). We also report c.d. data obtained under conditions similar to those employed in the modification reaction showing the effect of variation of pH and the presence of reducing agent (dithiothreitol) on the structure of β -lactoglobulin. In a Biochemical Journal Letter the specificity of transglutaminase for peptide and protein substrates was discussed in the light of available data (Coussons *et al.*, 1992). A preliminary account of some of this work has been given (Coussons *et al.*, 1990).

EXPERIMENTAL

The sources of guinea-pig liver transglutaminase and other enzymes and reagents for the determination of stoichiometry of

incorporation and isolation of modified peptides have been given previously (Coussons *et al.*, 1991). Bovine β -lactoglobulin (A and B variants and the mixture containing both A and B) was purchased from Sigma Chemical Co., Poole, Dorset, U.K. In our experiments we found no detectable difference between the behaviour of the A and B forms or the mixture. The data reported here refer to the mixture. Concentrations of solutions of β -lactoglobulin were determined spectrophotometrically at 278 nm by using an absorption coefficient of $0.96 \text{ ml} \cdot \text{mg}^{-1} \cdot \text{cm}^{-1}$ (Kella & Kinsella, 1988).

Modification of β -lactoglobulin was carried out with the following concentrations of reagents in 0.1 M-triethanolamine/HCl buffer, pH 7.6, at 25 °C (or when the variation with pH was studied, in 50 mM-Mes plus 50 mM-Tris adjusted to the appropriate pH with HCl or NaOH): primary amine (putrescine or monodansylcadaverine), 5 mM; dithiothreitol, 0, 1 or 5 mM; CaCl_2 , 5 mM; β -lactoglobulin, 2 mg/ml; transglutaminase, 0.05 mg/ml. The results obtained with the different buffers at the same pH were identical within experimental error. From SDS/PAGE analysis (Laemmli, 1970) of the modified protein on 10% acrylamide gels there was no evidence of transglutaminase-catalysed cross-linking of the protein.

Sequence analysis of modified β -lactoglobulin was undertaken according to Coussons *et al.* (1991). After reduction and carboxymethylation the protein was digested with trypsin and the peptides separated by reverse-phase h.p.l.c. (using a propan-2-ol gradient) on a Vydac C_{18} (end-capped) column. Modified peptides were detected either by fluorescence or by scintillation counting when monodansylcadaverine or [14 C]putrescine was used as the amino acceptor respectively (Coussons *et al.*, 1991). Peptides were further purified by h.p.l.c. using an acetonitrile gradient (Coussons *et al.*, 1991), before being applied to an Applied Biosystems model 470 gas-phase sequencer with on-line 120A phenylthiohydantoin (PTH) analyser (Coussons *et al.*, 1991). The repetitive yields during Edman degradation were $\geq 85\%$. Portions of the PTH derivatives obtained at each cycle were analysed for radioactivity or fluorescence (Coussons *et al.*, 1991) in order to confirm that any unidentified derivative corresponded to a site of incorporation of putrescine or monodansylcadaverine respectively. C.d. spectra were recorded at 25 °C using a JASCO J-600 spectropolarimeter. Molar-ellipticity values were calculated using a value of 113.6 for the mean residue weight (Braunitzer *et al.*, 1973). The secondary

Abbreviations used: Cbz, benzyloxycarbonyl; PTH, phenylthiohydantoin.

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structure content was derived by using the CONTIN program (Provencher & Glöckner, 1981). In addition, the α -helical content was derived using the procedure of Siegel *et al.* (1980).

RESULTS

Modification of β -lactoglobulin

The time course of incorporation of [14 C]putrescine into β -lactoglobulin is shown in Fig. 1, which shows that, over a 24 h period, approx. 1.5 mol of primary amine can be incorporated per mol of β -lactoglobulin subunit. The level of incorporation of monodansylcadaverine (determined after removal of excess reagent by gel filtration) is somewhat lower (1.2 mol/mol after 24 h) and is not increased by extending the period of reaction. A comparable extent of incorporation of monodansylcadaverine (1.25 mol/mol) was observed by Lorand & Campbell (1971) under similar conditions. Fig. 1 shows that significant incorporation of amine only occurs in the presence of the reducing agent. In the absence of dithiothreitol, the incorporation is < 0.2 mol/mol after 24 h. The effect of inclusion of the reducing agent does not arise from an effect on the transglutaminase itself, since various studies (Folk & Cole, 1965; Brenner & Wold, 1978) have shown that inclusion or omission of reducing agent has only a small effect on the activity with peptide or protein substrates. We have also shown in preliminary experiments with Cbz-Gln-Gly (Cbz is benzyloxycarbonyl) as substrate that there is less than 25% difference between the activity in the absence or presence of 1 mM-dithiothreitol.

We have previously noted (Coussons *et al.*, 1990) that, as the pH is raised towards 9.0, the extent of incorporation of primary amines after 24 h increases to 3 mol/mol of β -lactoglobulin subunit (in the presence of 1 mM-dithiothreitol).

Identification of sites of labelling

β -Lactoglobulin that had been allowed to react for 24 h at pH 7.6 in the presence of 1 mM-dithiothreitol with either monodansylcadaverine or [14 C]putrescine as the primary amine was reduced and carboxymethylated and digested by trypsin (1:40, w/w) as described previously (Coussons *et al.*, 1991). The modified peptides were detected by fluorescence or scintillation counting of fractions from the h.p.l.c. column. Fig. 2 shows the

Fig. 1. Time course of incorporation of [14 C]putrescine into β -lactoglobulin

The reaction was carried out at 25 °C in 0.1 M-triethanolamine/HCl buffer at pH 7.6 as described in the Experimental section. Reactions carried out in the presence (Δ) and absence ($\blacktriangle$) of 1 mM-dithiothreitol.

Fig. 2. Purification by h.p.l.c. of peptides produced by tryptic digestion of β -lactoglobulin

The modification reaction with monodansylcadaverine was performed as described in the Experimental section. (a) and (b) refer to separations of peptides on a Vydac C_{18} column for modified and unmodified protein respectively. In each case approx. 0.2 mg of digested protein was applied. Solvent A consisted of aqueous 0.4% triethylamine, adjusted to pH 2.5 with H_3PO_4 . Solvent B was solvent A containing 60% (v/v) propan-2-ol. In (a) the rectangles show the relative fluorescence of the fractions (excitation and emission wavelengths 320 nm and 520 nm respectively). The relative amounts of P1 and P2, as judged by measurements of A_{220} , were 1.4:1.

separation of the modified peptides produced after reaction with monodansylcadaverine. In the case of the reaction with [14 C]putrescine, there were again two new peaks (both radioactive) observed in the digest, although the resolution was somewhat poorer than with monodansylcadaverine. (In the latter case, the presence of the large hydrophobic dansyl moieties aids separation of the peptides.) In both cases, the sequences of the two modified peptides (after further purification as described in the Experimental section) could be determined unambiguously as:

Peptide 1	Leu-Ser-Phe-Asn-Pro-Thr-Gln-
	Leu-Glu-Glu-Xaa-CmCys-His-Ile
Peptide 2	Leu-Ser-Phe-Asn-Pro-Thr-Xaa-
	Leu-Glu-Glu-Xaa-CmCys-His-Ile

These peptides correspond to the sequence 149–162 of β -lactoglobulin, with Xaa corresponding to Gln-159 in peptide 1 and to Gln-155 and Gln-159 in peptide 2. From this it can be deduced that Gln-159 represents the preferred site of incorporation of amine, with Gln-155 representing the second site.

We have not performed any structural studies on β -lactoglobulin modified at high pH (9.0), when a third molecule of primary amine can be incorporated (Coussons *et al.*, 1990).

C.d. studies of β -lactoglobulin

The far-u.v. c.d. spectrum of β -lactoglobulin at pH 7.6 is shown in Fig. 3(a). This spectrum was recorded in 20 mM-triethanolamine buffer, since in the more concentrated buffer (0.1 M) used for the modification reaction it was not possible to obtain data below 200 nm, because of the high absorbance. For the same reason, data could only be collected above 200 nm in the Mes/Tris buffers used for the modification reactions at different pH values. Analysis of the spectrum (Fig. 3a) from 240 to 190 nm, according to the CONTIN procedure (Provencher & Glöckner, 1981), gave the following estimates of secondary structure: $17 \pm 1.1\%$ α -helix; $45 \pm 1.5\%$ β -sheet; $38 \pm 1.8\%$ remainder. These values are very similar to those reported in earlier c.d. studies [15% α -helix and 50% β -sheet (Creamer *et al.*, 1983); 17% α -helix and 41% β -sheet (Takeda and Moriyama, 1989)]. The results of X-ray crystallography (Papiz *et al.*, 1986) indicate that there are 11 and 83 out of the total of 162 amino acids in the α -helix and β -sheet respectively (i.e. 7% and 51% respectively). It is noteworthy that, by using the method of Siegel *et al.* (1980), which analyses the c.d. spectrum over a smaller range of wavelengths (240 nm–210 nm), the α -helix content is estimated to be $11 \pm 2\%$, which is closer to the value obtained by X-ray crystallography.

The far-u.v. c.d. spectra of β -lactoglobulin were recorded at pH values of 5.5, 7.6 and 9.0 in 50 mM-Mes plus 50 mM-Tris buffers [i.e. over the pH range used in our earlier studies of the modification reaction (Coussons *et al.*, 1990)]; the spectra are shown in Fig. 3(b). There are no significant differences between the spectra obtained in the two different buffers at pH 7.6. There are small differences between the spectra obtained at pH 5.5 and pH 7.6, which are reflected in a small decrease in α -helical content (Siegel *et al.*, 1980) from 13% at pH 5.5 to 11% at pH 7.6. The α -helical content at pH 9.0 is 10% . The structural changes occurring between pH 5.5 and 7.6 corresponds to the well-documented Tanford transition (Tanford & Taggart, 1961; Lyster, 1972).

In the near-u.v. the c.d. spectra (Fig. 3c) show signals in the 280–300 nm region characteristic of Trp and Tyr side chains. The diminution in the signals between pH 5.5 and 7.6 reflects changes in the tertiary structure which affect the environment of aromatic side chains. The Tanford transition has been previously proposed to involve the change in environment of one Tyr side chain (Lyster, 1972).

When β -lactoglobulin was incubated in the buffers at pH 5.5

Fig. 3. C.d. spectra of β -lactoglobulin

Spectra were recorded at 25°C at a protein concentration of 0.9 mg/ml in cells of path length 0.02 cm (far u.v.) or 0.5 cm (near u.v.). (a) Far-u.v. spectrum in 20 mM-triethanolamine/HCl buffer, pH 7.6. (b) Far-u.v. spectra in 50 mM-Mes plus 50 mM-Tris buffers, (---), pH 5.5; (—), pH 7.6; (---), pH 9.0. (c) Near-u.v. spectra in the same buffers as in (b). The spectrum at pH 7.6 is identical with that recorded in 20 mM-triethanolamine/HCl buffer at this pH. (d) Far-u.v. spectra in 50 mM-Mes plus 50 mM-Tris buffer, pH 9.0, in the presence of 1 mM-dithiothreitol. —, --- and --- refer to spectra recorded after incubation for 1 min, 4 h and 24 h respectively. (e) Near-u.v. spectra recorded under the same conditions as in (d).

and pH 7.6 for 24 h at 25 °C (i.e. the period over which the transglutaminase-catalysed modification reactions were conducted), there were no significant changes in either the far-u.v. or near-u.v. c.d. spectra over this time. When incubated at pH 9.0, there was no significant change in the far-u.v. spectrum, although the amplitude of the near-u.v. spectrum decreased by approx. 20% over this time, suggesting some loss of native tertiary structure. Instability of the protein above pH 8.0 has been previously commented on (Groves *et al.*, 1951; Akroyd, 1965; McKenzie & Sawyer, 1966; Lyster, 1972).

The various spectra were also recorded in the presence of 1 mM dithiothreitol, which, as shown in Fig. 1, is necessary for significant incorporation of primary amines in the transglutaminase-catalysed reaction. The far-u.v. spectra indicate that, immediately after addition of the reducing agent, there are only small effects on the secondary structure of the protein (results not shown). In the near u.v. the amplitude of the spectrum at pH 5.5 is about 15% less than that in the absence of dithiothreitol, although this effect does not occur at pH 7.6 or pH 9.0. The most striking result, however, is that at pH 9.0 there are considerable time-dependent structural changes observed in both the far-u.v. and near-u.v. c.d. spectra (Figs. 3d and 3e). This time-dependence is not observed at the lower pH values.

DISCUSSION

The structure of the β -lactoglobulin molecule determined by X-ray crystallography shows that each subunit consists of an anti-parallel β -sheet, formed by nine strands wrapped round to form a flattened cone or calyx, with a core consisting of an eight-stranded anti-parallel β -barrel (Papiz *et al.*, 1986). There are two disulphide bonds (Cys-66–Cys-160 and Cys-106–Cys-119) with a free thiol group (Cys-121). In each subunit there are nine glutamine side chains. A detailed examination of the X-ray structure shows that, of these, the side chains of Gln-13, Gln-35, Gln-68 and Gln-120 are not significantly exposed to the solvent and would not therefore be likely to act as potential candidates for the transglutaminase-catalysed reaction. The side chains of Gln-5, Gln-59 and Gln-115 appear to be reasonably exposed. Little is known about the location of the side chains of Gln-155 and Gln-159, since these are in a C-terminal loop whose electron density, apart from the Cys-66–Cys-160 disulphide bond, remains poorly defined.

In the absence of dithiothreitol there is little if any incorporation of primary amines into β -lactoglobulin (Fig. 1), and thus it appears that of the glutamine side chains which appear to be exposed in the X-ray structure (i.e. Gln-5, Gln-59 and Gln-115) none can act as potential substrates for the enzyme. It is noteworthy that, in the case of Gln-5 and Gln-59, there are positively charged side chains in close proximity on the C-terminal side (Lys-8 and Lys-60 respectively); previously (Coussons *et al.*, 1991) we had noted that this would discourage the transglutaminase-catalysed reaction, possibly by repulsion of the incoming primary amine substrate. This point is discussed in more detail in the Biochemical Journal Letter (Coussons *et al.*, 1992).

In the presence of dithiothreitol, one or both of the disulphide bonds are likely to be broken, and this would lead to exposure of potential new sites for the transglutaminase reaction. Since the sites of modification at pH 7.6 are confined to Gln-155 and Gln-159, and since the c.d. spectra show that, at this pH, 1 mM-dithiothreitol has little effect on the overall secondary and tertiary structure, it seems reasonable to postulate that only the Cys-

66–Cys-160 disulphide bond is cleaved. This would lead to the exposure of the C-terminal loop (from residues 151 to 162) so that Gln-155 and Gln-159 are available for modification. The c.d. spectra show that, at higher pH, particularly in the presence of dithiothreitol, there are pronounced time-dependent structural changes in the protein, and it is probable that the second disulphide bond (Cys-106–Cys-119) is cleaved. This bond connects strands G and H in the β -barrel, so it is likely that there would be considerable loss of tertiary structure under these conditions: a conclusion confirmed by the near-u.v. c.d. spectrum. Even so, there is evidence from the far-u.v. c.d. spectrum that some secondary structure persists after 24 h at pH 9.0 in the presence of dithiothreitol, and this is consistent with the exposure of at most only one additional glutamine side chain, which can be modified by transglutaminase under these conditions (Coussons *et al.*, 1990). The proposed cleavages of disulphide bonds could be confirmed by the 'trapping' procedure involving rapid reaction with iodoacetate and subsequent analysis of peptide fragments (Creighton, 1978). It is noteworthy that incubation of lysozyme with dithiothreitol leads to selective cleavage of one disulphide bond, permitting conjugation to ubiquitin (Dunten *et al.*, 1991), a situation analogous to that proposed here.

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