A Dimer–Dimer Binding Region in β-Galactosidase†

Franco Celada and Irving Zabin*

ABSTRACT: α-Complementation in β-galactosidase is the restoration of enzyme activity by addition of the α donor CNBr2, from amino acid residues 3–92 of the polypeptide, to inactive M15 protein from the *lacZ* deletion mutant strain M15. M15 protein lacks residues 11–41 and is a dimer; the active complex, like native β-galactosidase, is tetrameric [Langley, K. E., & Zabin, I. (1976) *Biochemistry 15*, 4866–4875]. A dimer–dimer binding region in β-galactosidase has been identified by proteolytic and immunologic studies of α-complementation. Proteolytic experiments were carried out with trypsin. Treatment of native β-galactosidase with trypsin, followed by reaction of the mixture with cyanogen bromide, yields intact CNBr2 as measured by its ability to complement M15 protein. Active CNBr2 is not obtained when urea-denatured β-galactosidase is treated in the same way. Therefore the segment corresponding to CNBr2 is apparently buried within the folded protein. Immunologic experiments were carried out with antibodies against CNBr2, tryptic peptide T8 (residues 60–140), and CNBr3 (residues 93–187). Anti-CNBr2 and anti-T8 bind to M15 protein but not to β-galactosidase, indicating that this area is exposed in the dimer. Anti-CNBr2, but not anti-T8 or anti-CNBr3, inhibits the formation of α-complemented enzyme. These results indicate that an early part of the sequence, within the segment corresponding to CNBr2, is involved in dimer–dimer interaction.

β-Galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) of *Escherichia coli* is a tetramer of four identical polypeptide chains, each containing 1021 amino acid residues (Fowler & Zabin, 1978). Small amounts of hexamers, octamers, decamers, and higher aggregates are also often found in preparations of this protein (Appel et al., 1965; Marchesi et al., 1969). Apparently, β-galactosidase has isologous bonding and D_2 symmetry (Monod et al., 1965; Klotz et al., 1975).

The noncovalent forces which maintain β-galactosidase in its native conformation are clearly very specific and very strong. The native enzyme can be "nicked" quite considerably by proteolytic enzymes with no loss in biological activity or in overall structure (Givol et al., 1966). At least 30% of the enzymic activity of this molecule can be regained by dialysis after 10 min of boiling, and under certain conditions, quantitative recovery of activity is obtained after denaturation with urea or guanidine (Perrin & Monod, 1963; Ullmann & Monod, 1969). The specific nature of the forces involved in holding the molecule together is also illustrated by complementation experiments in which various inactive fragments of β-galactosidase associate together to produce enzymically active products (Ullmann & Perrin, 1970; Zabin & Villarejo, 1975).

Which segments of the polypeptide chain are involved in binding the subunits to form a tetramer? A clue is available from α-complementation studies. The *E. coli* deletion mutant strain M15 produces an enzymically inactive β-galactosidase, a dimer, lacking residues 11–41 of the normal protein (Langley et al., 1975a). When the cyanogen bromide peptide, CNBr2, from residues 3–92 of normal β-galactosidase (Langley et al., 1975b) is mixed with M15 protein, the two materials associate noncovalently and α-complemented enzyme is formed which now has essentially full enzyme activity. Since α-complemented enzyme contains CNBr2 and M15 protein in a ratio of 1:1 and is a tetramer (Langley & Zabin, 1976), it is possible that the residues supplied by CNBr2 and missing in M15 protein are necessary for conversion of dimer to tetramer.

In a previous paper we have shown that antibody prepared against peptide CNBr2 does not bind to β-galactosidase, and we have suggested that this region of the polypeptide chain is buried within the folded protein (Celada et al., 1978). In the present report we support this hypothesis and we suggest that part of this region contains dimer–dimer contact points.

Experimental Procedures

Materials. β-Galactosidase, M15 protein, and tryptic and cyanogen bromide peptides were isolated as previously described (Fowler, 1972; Langley et al., 1975a; Fowler et al., 1978; Fowler, 1978). Complemented enzyme was prepared by addition of CNBr2 to M15 protein (Langley & Zabin, 1976).

Treatment of β-Galactosidase with Trypsin. Native and urea-denatured β-galactosidase were each treated with trypsin. A sample of native protein, 2.3 mg, in 0.5 mL of 0.1 M sodium phosphate, pH 7.2, and 0.005 M mercaptoethanol was incubated with 0.2 mg of trypsin at 28 °C for 1 h. Another sample of 2.3 mg was allowed to stand at 28 °C under N_2 for 1 h in 0.3 mL of 0.1 M sodium phosphate, pH 7.2, 8 M urea, and 0.005 M mercaptoethanol. The solution was then diluted to 3 M urea by addition of buffer to 1 mL, 0.2 mg of trypsin was added immediately, and incubation was continued for 1 h. Control samples with and without urea were treated in the same way except that trypsin was omitted. Portions of each of the four solutions were treated with CNBr and assayed for α-donor activity as before.

Antisera. Antisera against the cyanogen bromide peptides, CNBr2 and CNBr3, and the tryptic peptide, T8, were prepared as described in a previous paper (Celada et al., 1978). Antisera against M15 protein was prepared by injecting the purified protein in complete Freund's adjuvant into rabbits (two doses of 0.2 mg at a 1-month interval into the footpads, followed by an intradermal challenge of 10 μg into several loci in the back; bleeding was done 10 days after this last injection).

Antigen–Antibody Assays. Antibody binding to native β-galactosidase and to complemented enzyme was tested by following the enzyme activity after incubation with antiserum

† From the Cattedra di Immunologia, Universita di Genova 16132, Genova, Italy (F.C.), and the Department of Biological Chemistry, UCLA School of Medicine and Molecular Biology Institute, University of California, Los Angeles, California 90024 (I.Z.). *Received September 1, 1978.* This work was supported in part by a grant of the Italian Research Council (CNR), Progetti Bilateriali to F.C. (United States–Italy Cooperative Science Program, Division of International Programs, National Science Foundation), and Grant AI 04181 from the U.S. Public Health Service to I.Z.

0006-2960/79/0418-0404$01.00/0

Table I: Effect of Trypsin on α-Donor Activity

β-galactosidase (mg)	urea (M)	trypsin (mg)	α-donor (enzyme units/mg)
2.3[a]	8	0	129 000
2.3[a]	8	0.2	350
2.3	0	0	148 000
2.3	0	0.2	141 000

[a] Preincubated in 8 M urea for 1 h and diluted to 3 M in urea. All samples were then incubated for 1 h at 28 °C and a portion of each was cleaved with CNBr in 70% formic acid. α-Donor activity was assayed by complementation with M15 protein. Enzyme units are expressed in terms of milligrams of β-galactosidase.

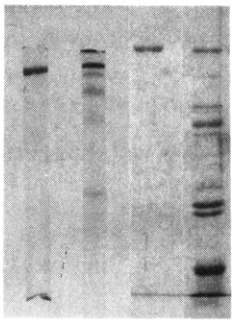

FIGURE 1: Polyacrylamide gel electrophoresis of β-galactosidase. (A) and (B), gels run in 8 M urea; (C) and (D), gels run in 0.1% $NaDodSO_4$. (A) and (C), native β-galactosidase (Table I); (B) and (D), native β-galactosidase treated with trypsin (Table I).

and precipitation with goat antirabbit γ-globulin antibody. Antibody binding to all other antigens was tested by radioimmunoassay. Both methods have been described in detail (Celada et al., 1978).

Results

Effect of Trypsin on α-Donor Complementation Activity. An experiment with trypsin shown in Table I was carried out to determine whether the segment 3–92 of the β-galactosidase polypeptide chain is available to proteolysis or is protected. Samples of protein were incubated with trypsin and treated with CNBr, and the digest was tested for α-donor activity with M15 protein. It can be seen that, when β-galactosidase is denatured with urea, trypsin destroys all but a trace of α-donor peptide. However, trypsin destroys none of the potential α-donor activity in the native molecule. This is the case even though the native molecule was hydrolyzed quite considerably by trypsin, as illustrated by gel patterns of the trypsin-treated material taken before cleavage with CNBr (Figure 1) . It is also of interest that this sample of β-galactosidase lost no enzyme activity under these conditions in agreement with earlier results (Givol et al., 1966). We conclude that the α-complementing activity of segment 3–92 is protected in the native enzyme.

Reactivity of Antibodies. Antibodies prepared with CNBr2 (residues 3–92), T8 (residues 60–140), CNBr3 (residues 93–187), and M15 protein (residues 11–41 absent) were tested against the antigens listed in Table II. Each of the four antisera reacted strongly with its homologous antigen. Anti-CNBr2 bound T8, M15 protein, and complemented enzyme as expected, and it also bound CNBr3. There was no reaction with β-galactosidase, as was reported previously (Celada et al., 1978). Anti-CNBr3 reacted only with CNBr3 but anti-T8 reacted with all antigens except for β-galactosidase.

Table II: Reactivity of Antibodies

antigen	antibody (nmol of antigen bound/mL of antiserum) anti-CNBr2	anti-T8	anti-CNBr3	anti-M15 protein
CNBr2	1.3	0.14	0	3.3
T8	0.9	1.0	0	0.10
CNBr3	0.68	1.0	2.26	1.45
M15 protein	0.37	0.14	0	5.7
complemented enzyme	1.2	0.20	0	5.0
β-galactosidase	0	0	0	4.0

Table III: Effect of Antibodies on α-Complementation

antibody	enzyme act. at molar ratio of M15/CNBr2[a] 30:1 units	30:1 % inhibn	0.3:1 units	0.3:1 % inhibn
normal serum	53.5	0	496	0
anti-CNBr2	9.8	82	29	94
anti-T8	65.0	0	599	0
anti-CNBr3			619	0
anti-M15 protein	53.5	0	5	99

[a] For the molar ratio of 30:1, 7 pmol of CNBr2 was mixed with 215 pmol of M15. For 0.3:1, 35 pmol of CNBr2 was mixed with 10.7 pmol of M15. Specified serum (10 μL) was added at time 0 in a final volume of 0.11 mL. Enzyme activity was determined after incubation for 2 h.

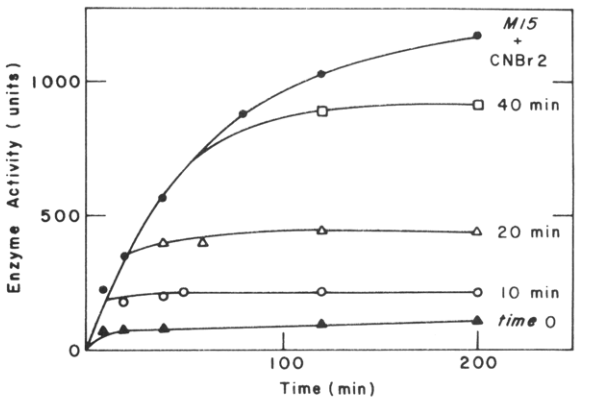

FIGURE 2: Effect of anti-CNBr2 on α-complementation. Equal volumes of 10^{-7} M CNBr2 and 10^{-7} M M15 protein were mixed and anti-CNBr2 diluted 1:2 with normal serum was added to separate portions of the mixture at times 0, 10, 20, and 40 min after the initial mixing. The control tube received normal serum at the time of mixing.

Anti-M15 protein cross-reacted with all antigens, though most weakly with T8.

Effect of Anti-CNBr2 on α-Complementation. The effect of anti-CNBr2 on the formation of α-complemented enzyme was determined by adding the antibody to a mixture of CNBr2 and M15 protein at various times (Figure 2). It can be seen that when anti-CNBr2 was added at the time of mixing (time 0), complementation was inhibited almost completely. If complementation had already occurred, however, anti-CNBr2 had little or no effect.

Specificity of Inhibition of α-Complementation by Antibodies. To test for specificity of inhibition of the formation of complemented enzyme, anti-CNBr3, anti-T8, and anti-M15 protein were also added to CNBr2–M15 protein mixtures (Table III). Under conditions of limiting CNBr2, only anti-CNBr2 prevented the formation of complemented enzyme to any significant extent. When M15 was limiting, both

anti-CNBr2 and anti-M15 protein were inhibitory.

Discussion

In the experiments reported here, we have used the α-complementation reaction to help define the sequences involved in dimer–dimer interaction in β-galactosidase. The formation of α-complemented enzyme involves the noncovalent association of CNBr2 from residues 3–92 of β-galactosidase with the dimeric protein lacking residues 11–41 from deletion mutant strain M15. We have asked first whether the segment 3–92 is indeed buried within the native enzyme, as suggested by probes with antibody prepared from CNBr2 (Celada et al., 1978). Experiments with trypsin described here support this conclusion. Treatment of native enzyme with trypsin had no effect on the subsequent release of active α-donor peptide by CNBr, while under the same conditions, α-donor activity was completely lost from urea-denatured protein. It might be argued that this portion of the polypeptide chain is not buried but that every one of the trypsin-sensitive bonds is in the wrong configuration for proteolysis. This seems very unlikely because, although CNBr2 has no lysine residues, it contains arginine residues subject to hydrolysis at positions 13, 14, 26, 37, 43, 52, and 59. The first four are thus within the region missing in M15 protein, and it seems safe to conclude that CNBr2 is in fact folded within the native protein.

Experiments with antibodies, which were carried out next, indicate that a dimer–dimer binding area might be localized with more precision. Binding studies with anti-CNBr2 show that the CNBr2 segment of the chain is available in the M15 protein dimer but not in β-galactosidase. Anti-T8 reacts less strongly and anti-CNBr3 reacts not at all with M15 protein. Anti-M15 protein binds CNBr2 and, less strongly, CNBr3 and T8. The finding that CNBr3 cross-reacts with anti-CNBr2 was unexpected. This appears to be a true cross-reaction, because [^{121}I]CNBr3 binding was inhibited far more efficiently by unlabeled CNBr2 than by unlabeled CNBr3.[1]

Anti-CNBr1 was found to almost completely inhibit the formation of α-complemented enzyme when it was added at the time of mixing of CNBr2 and M15 protein. Anti-M15 protein also inhibited the reaction but only under conditions of limiting M15 protein. Neither anti-T8 nor anti-CNBr3 inhibited complementation to any significant extent. This suggests that the sequence 60–187 does not contain dimer–dimer contact residues and that these are instead in the segment amino terminal to residue 60.

Structural studies on a *lac* repressor–β-galactosidase fusion protein have shown that the first 23 residues of β-galactosidase can be substituted by another sequence without preventing the formation of β-galactosidase tetramer (Brake et al., 1978). Therefore it is unlikely that this segment is involved in dimer–dimer binding. Since the deletion in M15 protein extends only to residue 41, it seems probable that the segment 42–59 is also excluded. Recently, the amino acids missing from the defective β-galactosidase made by the deletion mutant strain M112 have been identified as residues 23–31.[2] M112 protein, like M15 protein, is a dimer. Therefore the specific region important for dimer–dimer binding may be further limited to the segment 24–31.

These results by no means exclude involvement of other contact residues elsewhere in the polypeptide chain. Indeed, probes with iodoacetate have shown that cysteine residues at positions 76, 387, and 600 are available to the reagent in M15 protein but not in β-galactosidase (Jörnvall et al., 1978). Experiments with other antipeptide antibodies might also help point to other possible binding regions.

Finally, it is interesting that the sequences involved in monomer–monomer interaction are different from those of the dimer–dimer binding region. Termination mutant strains which produce β-galactosidase lacking the carboxyl terminus of the chain form polypeptides which are monomeric (Fowler & Zabin, 1966). Therefore, a part of the sequence near the carboxyl terminus is important in the initial interaction of subunits to form dimers.

Acknowledgments

We are indebted to Dr. A. V. Fowler for stimulating discussions and to Dora Centis for excellent technical assistance.

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