# Probes of β-Galactosidase Structure with Antibodies. Reaction of Anti-Peptide Antibodies against Native Enzyme†

Franco Celada,‡ Audrée V. Fowler, and Irving Zabin*

ABSTRACT: Antibodies were prepared against 18 tryptic and cyanogen bromide peptides from β-galactosidase ranging in size from 15 to 96 amino acid residues representing more than 80% of the polypeptide chain. They were tested for binding capacity and affinity toward their homologous antigens and toward the whole native protein. Nine antisera bound to β-galactosidase; these had been raised against certain peptides from the central and carboxyl-terminal regions of the polypeptide chain. Based on these results a preliminary model of the three-dimensional structure of the folded protein is suggested.

The enzyme β-galactosidase (β-D-galactoside galactohydrolase, EC 3.2.1.23) of *Escherichia coli* is an excellent immunogen. It is a tetramer of four identical subunits, each containing 1021 amino acids (Fowler & Zabin, 1978). Antisera of high titer can be prepared; these precipitate but do not inactivate the enzyme (Cohn & Torriani, 1952). Such antisera, as expected considering the size of the immunogen, contain antibodies specific for a large number of different determinants, and cross-react with β-galactosidase proteins produced by missense, deletion, and termination mutant strains (Perrin, 1963; Fowler & Zabin, 1966, 1968). Some of these defective proteins, which may retain low levels of enzyme activity, can be activated by antibody (Rotman & Celada, 1968; Messer & Melchers, 1970; Melchers & Messer, 1970). Fragments comprising as little as one-third of the β-galactosidase polypeptide chain also react with anti-β-galactosidase (Fowler & Zabin, 1968; Celada et al., 1974).

Antigenic structures of many proteins including lysozyme, flagellin, bovine serum albumin, and myoglobin have been analyzed (Crumpton, 1973; Atassi & Habeeb, 1977); in most cases antibodies were prepared against the whole, native proteins, and their reaction with fragments was studied. There are a number of examples where fragments were used to prepare antibodies which were then tested against the complete protein. Antibodies raised against the "loop" peptide cross-reacted with native lysozyme (Maron et al., 1971), and antibodies made against a carboxyl-terminal peptide of the β chain of human hemoglobin S cross-reacted with the native human hemoglobin S (Curd et al., 1976). Fragments of β-galactosidase comprising the amino-terminal two-thirds of the molecule (Berg et al., 1970), and the ω fragment, the carboxyl-terminal third (Celada et al., 1974), have also been used as immunogens.

In the present study we have prepared antibodies against many tryptic and cyanogen bromide peptides of β-galactosidase. We have asked whether these antibodies, raised against defined portions of the polypeptide chain, can be used to probe the structure of the native protein on the assumption that antigenic determinants recognized are exposed on the native structure. We present data here showing that certain of these antibodies do bind β-galactosidase while others do not and we suggest that these results afford a preliminary picture of the topography of the molecule.

## Experimental Procedures

*Materials.* β-Galactosidase was isolated as previously described (Fowler, 1972). Carrier-free sodium [$^{125}$I]iodide was obtained from New England Nuclear. Goat anti-rabbit immunoglobulin antiserum (catalogue no. 539844, 1 unit precipitates 40 μg of rabbit γ-globulin), lactoperoxidase (B grade), and *o*-nitrophenyl β-D-galactoside were purchased from Calbiochem, and complete Freund's adjuvant was from Difco. Bio-Gel P-2 was a product of Bio-Rad Laboratories. Spectrapor 3 dialysis tubing was obtained from Spectrum Medical Industries, Inc., Los Angeles.

*Preparation and Radioactive Labeling of Peptides.* All peptides were prepared from carboxymethyl β-galactosidase. Cyanogen bromide (CNBr) and tryptic (T) peptides were purified as described elsewhere (Fowler, 1978; Fowler et al., 1978a).

Most of the peptides were labeled with $^{125}$I by the lactoperoxidase method using conditions described previously (Brake et al., 1977). For peptides devoid of or containing few tyrosyl residues, the Chloramine-T method (Greenwood et al., 1963) was used. The iodination of CNBr2, which contains no tyrosine, was carried out as follows: to 12.5 μg of peptide in 25 μL of 0.05 M potassium phosphate, pH 7.5, were added 50 μL of sodium[$^{125}$I] iodide solution and 50 μg of Chloramine-T in 25 μL 0.5 M potassium phosphate buffer, pH 7.5. After 25 s the reaction was stopped with 100 μL of sodium metabisulfite (2.4 mg per mL) and 200 μL of potassium iodide (10 mg per mL) each in the 0.05 M phosphate buffer. The mixture was applied to a column of Bio-Gel P-2 (0.9 × 50 cm) equilibrated with 0.1 M sodium phosphate, pH 7.0, and eluted with the same buffer. Fractions corresponding to the first peak of radioactivity were pooled, bovine serum albumin was added to a concentration of 1 mg per mL, and 0.2-mL portions were frozen and kept at −40 °C until needed.

*Immunization Procedures.* Peptides were normally stored in 30% acetic acid in the frozen state. Solutions of 0.1–0.2 mL containing 0.5 mg of peptide were added to 1 mL of 10 M freshly deionized urea, 0.2 mL of 0.1 M sodium phosphate, pH 7.2, was added to each sample, and the mixtures were then dialyzed for several hours against 0.02 M sodium phosphate, pH 7.2, containing 8 M urea using Spectrapor 3 dialysis tubing.

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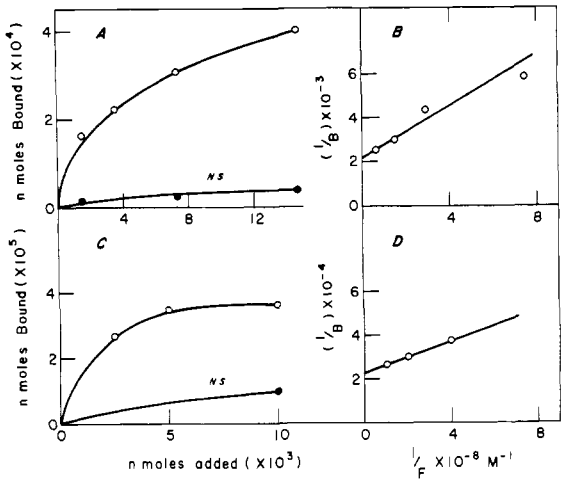


FIGURE 1: Binding curves of CNBr14 and of β-galactosidase with anti-CNBr14. (A) One microliter of anti-CNBr14 tested against [$^{125}$I]-CNBr14. NS, normal serum. (B) Double-reciprocal plot of the data shown in A. B, bound; F, free. (C) One microliter of anti-CNBr14 tested against β-galactosidase. (D) Double-reciprocal plot of the data shown in C. Each point is the mean of two experiments. Lines were fitted graphically. Titer and binding avidity of the antisera to the homologous peptide and to the native enzyme were calculated from graphs B and D, as follows. The titer is defined as the number of nanomoles of antigen bound by 1 mL of undiluted antiserum under conditions of extreme antigen excess. It was derived from the intercept of the regression line on the ordinate after correcting for volume and serum concentration. The avidity is expressed as $1/K_D$ (in L/mol) and corresponds to the concentration of free antigen at which the binding reaches 50% of the maximum value.

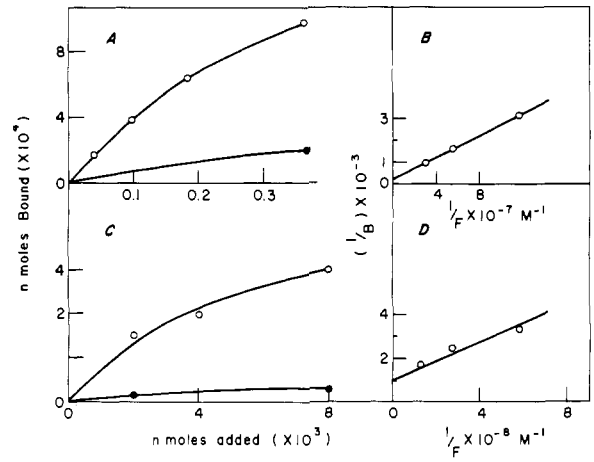


FIGURE 2: Binding curves of CNBr24 and of β-galactosidase with anti-CNBr24. Legend as in Figure 1.

They were then dialyzed against 0.05 M sodium phosphate, pH 7.2, with several changes to remove the urea. All peptides with the exception of CNBr20 remained soluble. Each antigen was injected into two white female New Zealand rabbits. Quantities of 200 μg of peptide were emulsified with an equal volume of complete Freund's adjuvant and were injected into the footpads. An identical injection was made 30 days later. A final injection of 10 μg of antigen in 0.2 mL of buffer was given intradermally, distributed in five or six sites on the back of the animals. Suspensions of CNBr20 were emulsified and injected in the same way. Bleedings from the central artery of the ear were done 10 days after the second and 7 days after the last injection. Sera were stored at −40 °C.

*Radioimmunoassay of Antisera.* The antigen-binding capacity and the binding avidity of each antibody were measured by incubation of a fixed amount of antisera with increasing amounts of the corresponding labeled antigen. Bound and free antibody were separated by precipitation of the complex with anti-antibody. In a typical determination, 50 μL of 1:50 diluted antiserum was mixed with 50 μL of 0.1 M sodium phosphate buffer, pH 7.2, containing 0.01 M magnesium sulfate, and 50 μL of 1% Triton X-100 in a series of tubes. Quantities of 10, 25, 50, and 100 μg of labeled peptide (approximately $10^5$ cpm per μg) in 50 μL of the same buffer were added and the mixtures were incubated for 30 min. To each tube was added 25 μL of goat anti-rabbit γ-globulin antiserum containing 0.5 precipitating unit, sufficient to precipitate 20 μg of rabbit γ-globulin. After a further incubation for 2 h, the mixtures were centrifuged and the supernatant solutions were discarded. The precipitate was washed twice with 0.5 mL of 0.25% Triton X-100 in the phosphate buffer and counted in a Beckman Biogamma counter. All manipulations were performed at room temperature and each assay was done in duplicate. Normal serum was used as a control. Determinations of antibody titer and binding avidity are described in the legend to Figure 1.

*Antisera Binding to β-Galactosidase.* Quantities of 50 μL of 1:50 diluted antiserum and 100 μL of 0.1 M sodium phosphate buffer, pH 7.2, containing 0.01 M magnesium sulfate were mixed with 1, 2.5, 5, and 10 pmol of β-galactosidase (1 pmol is 116 ng) in 100 μL of the same buffer. Incubations, precipitation with anti-antibody, and washings were identical with those used for the radioimmunoassay described above except that 2.5 mL of 1% Triton X-100 was used for enzyme activity (Celada et al., 1976).

## Results

*Preparation of Anti-Peptide Antibodies.* Eighteen peptides isolated from β-galactosidase, which ranged in size from 15 to 96 amino acid residues, were mixed with complete Freund's adjuvant and injected into rabbits *without* protein carrier. Sera thus obtained were tested by incubation with homologous $^{125}$I-labeled peptides and precipitation with goat anti-rabbit antiserum as described in Experimental Procedures. The size of the peptide and its position within β-galactosidase are shown in Table I.

It may be seen that every peptide was an effective immunogen (Table I). Binding capacities of the antisera produced varied within a 30-fold range, from 0.4 to 13.3 nmol of antigen bound per mL of serum. Some representative binding curves are shown in Figures 1 and 2. There was no apparent correlation between titer of antibody and size of the immunogen (Table I). Avidities were within the range to be expected for antibody and antigen, from $3.3 \times 10^5$ to $2 \times 10^8$ L per mol.

It is of interest that an active antibody was elicited by the injection of a suspension of CNBr20. The anti-CNBr20 was tested with CNBr20B. This peptide was produced by cleavage of CNBr20 at low pH in guanidine (Fowler et al., 1978b) and is soluble.

*Binding of Anti-Peptide Antibodies to β-Galactosidase.* Each antibody was next tested for its ability to bind to the whole, native β-galactosidase. Antibody and enzyme were incubated and precipitated with anti-antibody, and the amount of β-galactosidase in the precipitate was measured by enzyme assay. Control experiments showed that antisera had no effect on enzyme activity, as had been observed earlier with anti-β-galactosidase itself (Cohn & Torriani, 1952).

Nine of the anti-peptide antibodies bound to β-galactosidase to a significant extent (Table II). These were anti-CNBr10, 14, 15, 16, 18, 20, 20B, 23, and 24. Their binding capacities for β-galactosidase were lower than for the homologous peptide antigen, but the avidities were high, from $2 \times 10^7$ to $1.6 \times 10^8$. None of the antibodies corresponding to peptides derived from

TABLE I: Anti-Peptide Antibodies.

| designation | peptide: size (amino acid residues) | peptide: position in β-galactosidase (residue no.) | antibody: binding capacity (nmol of antigen per mL of serum) | antibody: avidity (L/mol) |
|---|---|---|---|---|
| CNBr2 | 90 | 3-92 | 1.3 | $5 \times 10^7$ |
| T8 | 81 | 60-140 | 1.0 | $3 \times 10^7$ |
| CNBr3 | 95 | 93-187 | 2.3 | $1 \times 10^6$ |
| CNBr4 | 15 | 188-202 | 0.4 | $2.4 \times 10^6$ |
| T16 | 20 | 211-230 | 4.0 | $5 \times 10^7$ |
| T28-30 | 36 | 351-386 | 8.0 | $1 \times 10^7$ |
| CNBr10 | 41 | 378-418 | 1.0 | $2 \times 10^8$ |
| CNBr14 | 59 | 442-500 | 0.45 | $8 \times 10^7$ |
| CNBr15 | 40 | 501-540 | 2.2 | $7 \times 10^7$ |
| CNBr16 | 61 | 541-601 | 1.7 | $4 \times 10^6$ |
| CNBr18 | 90 | 654-743 | 5.9 | $3.6 \times 10^6$ |
| CNBr19 | 23 | 744-766 | 1.4 | $4 \times 10^5$ |
| CNBr20 | 96 | 767-862 | 11.9[a] | $4.6 \times 10^6$ |
| CNBr20B | 62 | 801-862 | 13.3 | $4 \times 10^6$ |
| CNBr21 | 61 | 863-923 | 3.5 | $4.4 \times 10^7$ |
| CNBr22 | 43 | 924-966 | 6.7 | $3.3 \times 10^5$ |
| CNBr23 | 23 | 967-989 | 0.6 | $2.5 \times 10^7$ |
| CNBr24 | 32 | 990-1021 | 5.0 | $2 \times 10^6$ |

[a] Binding capacity and avidity were tested against CNBr20B.

TABLE II: Binding of Anti-Peptide Antibodies to β-Galactosidase.

| antibody to peptide | binding capacity (nmol of antigen per mL of serum) | avidity (L/mol) | rel binding capacity[a] (%) |
|---|---|---|---|
| CNBr2 | none | | |
| T8 | none | | |
| CNBr3 | none | | |
| CNBr4 | none | | |
| T16 | none | | |
| T28-30 | trace | | |
| CNBr10 | 0.11 | $1 \times 10^8$ | 11 |
| CNBr14 | 0.043 | $1.4 \times 10^8$ | 9.5 |
| CNBr15 | 0.01 | $1.6 \times 10^8$ | 0.5 |
| CNBr16 | 0.2 | $4 \times 10^7$ | 12 |
| CNBr18 | 0.4 | $1.4 \times 10^8$ | 7 |
| CNBr19 | none | | |
| CNBr20 | 1.6 | $2 \times 10^7$ | 13 |
| CNBr20B | 0.14 | $1.3 \times 10^8$ | 1 |
| CNBr21 | none | | |
| CNBr22 | none | | |
| CNBr23 | 0.022[b] | $2 \times 10^7$ | 4 |
| CNBr24 | 1.0 | $4.5 \times 10^7$ | 20 |

[a] Binding capacity for β-galactosidase compared to homologous antigen. [b] The binding to control serum was three-fourths of the experimental value.

the amino-terminal part of the β-galactosidase polypeptide chain bound to the protein, nor did anti-CNBr19, 21, and 22. No anti-peptide antibody precipitated β-galactosidase whether or not it bound to the protein.

*Relative Binding Capacity.* The binding capacity for β-galactosidase, as compared with the homologous antigen for each of the nine antibodies which bind to the enzyme is shown in the last column of Table II. Anti-CNBr10, 14, 16, 20, and 24 reacted about 10-20% as well with β-galactosidase as with the corresponding peptides. Anti-CNBr18 reacted to about 7% and lower relative binding was seen with the three other antisera. The numerical values for binding capacity and for relative binding capacity are calculated on the assumption that (a) all peptides are monomers in solution and antibodies react with the monomers and (b) when titers are determined under conditions of antigen excess, only one monomer of the four in β-galactosidase will form a complex with the antibody, yet each contributes enzyme activity when the precipitate is assayed for catalytic activity. For these reasons the calculations have been based on the tetrameric weight of the molecule, 465 000.

## Discussion

We have shown that a number of tryptic and cyanogen bromide peptides from β-galactosidase ranging in size from 15 to 96 amino acid residues were immunogenic to the rabbit when injected in complete Freund's adjuvant without the use of any carrier protein. Titers ranged from 0.4 to 13.3 nmol of antigen bound per mL of homologous antiserum. These are relatively active antibodies. By way of comparison, the binding capacity of 5.0 nmol of CNBr24 per mL of anti-CNBr24 is equivalent to an antibody content capable of binding 0.25 mg of a protein of 50 000 daltons. The best β-galactosidase antibodies prepared in this laboratory precipitate 4-5 mg per mL. It is also worth noting that a peptide insoluble at neutral pH in aqueous solution (CNBr20) also was able to elicit the production of antibodies.

One surprising feature of these experiments was the success in obtaining antibodies against all injected peptides, especially considering that some of them were at the very limit of the size considered critical for expressing immunogenicity. One reason for obtaining positive reactions was the sensitivity of the radioimmunoassay, and the fact that each serum was tested against a range of antigen concentrations, allowing the detection of low-affinity antibodies. Another possibility that would explain the immunogenicity of small peptides might be self aggregation to give larger complexes. In any case this would not affect interpretation of the results.

The peptides used, which include several overlapping fragments, comprise more than four-fifths of the β-galactosidase molecule. They are identical in primary structure to the corresponding segment of the whole protein except that all 16 cysteine residues are carboxymethylated and the CNBr peptides contain homoserine and homoserine lactone in place of methionine. Antibodies raised against peptide immunogens,

therefore, are directed against discrete portions of the polypeptide chain of 1021 amino acid residues.

Antigenic sites on proteins fall into two major categories, sequential or conformation dependent (Sela et al., 1967). The former are made up of sequences of 3–5 amino acids, and the latter consist of a cluster of amino acid residues which may be situated far apart in the linear sequence but close together in the folded molecule. It seems likely that antibodies raised against peptides are directed only against sequential determinants. Such antibodies should therefore be ideal probes for determining which portions of the linear polypeptide sequence are available on the "surface" of the native enzyme. If certain of the peptides have actually retained the shape that region has in β-galactosidase, their antibodies also would be useful probes.

Nine of the 18 different antisera were found to bind to β-galactosidase. Six of these, anti-CNBr10, 14, 16, 18, 20, and 24, reacted relatively strongly. These are antisera raised against regions comprising residue numbers 378–418, 442–500, 541–601, 654–743, 767–862, and 990–1021. Therefore, these portions of the sequence, or a part of each, evidently are exposed in the folded molecule. There was no relationship between the size of the peptide and the degree of binding of the corresponding antibody to β-galactosidase. Most peptides probably contain more than one antigenic determinant. This may account for the lower binding to β-galactosidase and suggests that only part of the sequences are exposed on the surface of the native protein.

Several other antisera, anti-CNBr23 and 20B, also bound to the whole protein but less strongly. The reaction of anti-CNBr20B is of some interest. CNBr20B is a peptide (residues 801–862 of β-galactosidase) which contains only part of the sequence of CNBr20 (residues 767–862). Anti-CNBr20B has a binding capacity for β-galactosidase only about 10% that of anti-CNBr20. This suggests that the antigenic determinant(s) exposed on β-galactosidase which is recognized by anti-CNBr20 is in the region 767–800 of the folded chain. Anti-CNBr15 had a very low relative binding capacity, and a trace was found with anti-T28-30. No binding could be detected with anti-CNBr2, CNBr3, T8, CNBr4, T16, CNBr19, CNBr21, and CNBr22.

These results are represented graphically in Figure 3. It seems reasonable to conclude that those segments of β-galactosidase available to antisera, parts of the central and the carboxyl-terminal regions of the polypeptide, are indeed on the outside of the protein. Some supporting evidence for this conclusion has been obtained from probes with chemical reagents. When β-galactosidase was treated with iodoacetate, the 2 of the 16 cysteine residues in the protein which were most available to the reagent were Cys-498 and -1019 (Jörnvall et al., 1978). These are present in CNBr14 and 24, respectively. Antibodies prepared against both these peptides bind to β-galactosidase. Also, the active site reagent, β-D-galactopyranosylmethyl *p*-nitrophenyltriazene, specifically binds to Met-500, in CNBr14 (Fowler et al., 1978c).

The lack of binding of certain antibodies to β-galactosidase suggests but does not prove that the corresponding segments are not on the surface of the folded protein. The antigenic determinant of a peptide may in fact be buried but the remainder of that sequence might not. It is also possible that an isolated peptide might have a different shape in the free state than that sequence has in the protein. In such a case the antibody raised against the peptide would similarly not react with the protein. However, the consistent negative results obtained with peptides from the amino-terminal part of the polypeptide chain suggest that they may indeed be hidden. Evidence to support the conclusion that the region 3–92 (CNBr2) is a dimer-dimer binding area has been obtained from an examination of the effect of anti-CNBr2 on α complementation.[1]

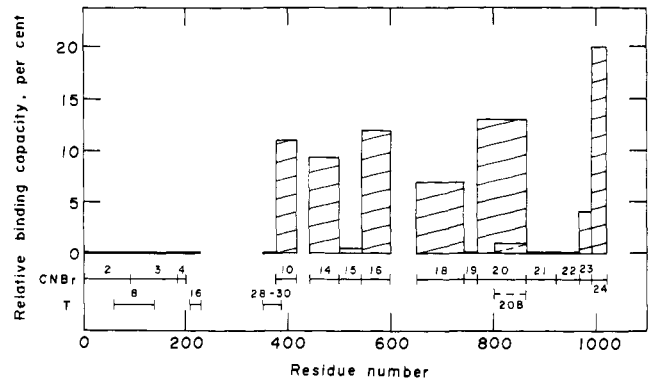


FIGURE 3: Relative binding of β-galactosidase by anti-peptide antibodies. The figure shows a linear map of the polypeptide chain of β-galactosidase and the cyanogen bromide (CNBr) and tryptic (T) peptides which were used to prepare antibodies. Solid lines and hatched segments represent the binding of antibodies to β-galactosidase as compared with the homologous peptide.

A possible limitation to the significance of the binding of a given anti-peptide antibody to the native protein may originate from cross-reaction, i.e., the binding of the same antibody to other peptides besides the immunogen. In preliminary experiments this phenomenon has been observed to happen for some of the anti-peptide antisera. However, in all cases the binding avidity for the cross-reacting peptide was lower than for the immunogen (F. Celada, unpublished). Since the avidity of all anti-peptide antibodies binding to β-galactosidase is very high (from $2 \times 10^7$ to $2.5 \times 10^8$ L/mol), it is unlikely that any of these reactions may be attributed to cross-reactivity.

Subject to these limitations, a very preliminary model of the three-dimensional structure of β-galactosidase has been outlined as indicated in Figure 3. The experimental data obtained so far indicate that antibody probes prepared from peptide fragments can be useful for investigating topological features of proteins. Further refinements are possible by localizing antibody binding sites in the peptide antigen.

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# Probe of β-Galactosidase Structure with Iodoacetate. Differential Reactivity of Thiol Groups in Wild-Type and Mutant Forms of β-Galactosidase†

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ABSTRACT: Carboxymethylation with $^{14}C$-labeled iodoacetate of cysteine residues in wild-type β-galactosidase from *Escherichia coli* and in a defective β-galactosidase from deletion mutant strain M15 was investigated in order to determine accessible positions in the tetrameric wild-type form and the dimeric mutant M15 protein. The extent of carboxymethylation, the effects on biological activity, antibody activation, physical stability, and the labeling of particular residues were studied. The results distinguish three groups of spatial relationships for cysteine residues in the protein, define possible regions for subunit interactions, and confirm that no cysteine residue is specifically involved in catalysis. Residue 1019 and to a lesser extent 498 are accessible in the tetrameric protein and probably represent exposed areas. In the M15 protein, these two, and three additional residues, at 76, 387, and 600, were found to react significantly with reagent. One or more of the latter are suggested to be in the dimer-dimer interface. Complementation and activation by antibody are inhibited by carboxymethylation of M15 protein.

The β-galactosidase of *Escherichia coli* (β-D-galactoside galactohydrolase, EC 3.2.1.23) is a tetrameric protein composed of identical subunits, each containing 1021 amino acids. The primary structure is known (Fowler & Zabin, 1978a), but little information is available on structure-function relationships, on residues at the active site, or on conformation of the native molecule. On the basis of complementation studies with enzymatically inactive mutant forms, it has been suggested that the carboxyl-terminal third of the β-galactosidase polypeptide chain forms a distinct globular structure (Goldberg, 1970). Amino acid sequence similarities within the amino-terminal three-fourths of the chain (Hood et al., 1978) also suggest the possibility of separate domains within this portion of the molecule.

In the present study, modification of native β-galactosidase with iodoacetate was carried out in order to probe the topography of the protein. The reaction was chosen because it can be made highly specific for cysteine residues and because cysteine, at 16 residues per subunit, is the least common amino acid in β-galactosidase. The reaction of iodoacetate with a defective β-galactosidase from deletion mutant strain M15 was also studied. This protein, designated here as M15 protein, contains all 16 cysteine residues but lacks amino acid residues 11-41 of the wild-type polypeptide (Langley et al., 1975b). The difference not only results in the loss of enzyme activity but also alters the quaternary structure of M15 protein, making it a dimer instead of a tetramer (Langley & Zabin, 1976). Enzyme activity may be fully restored by intracistronic complementation with fragments of β-galactosidase (α donors) supplying the missing sequence (Lin et al., 1970). A less complete activation may also be obtained by interaction with anti-β-galactosidase antibodies (Accolla & Celada, 1976). The stoichiometry and kinetics of the completion of M15 protein with the α-donor peptide CNBr2, derived from residues 3-92 of β-galactosidase, have been studied in some detail (Langley & Zabin, 1976).

The extent of the cysteine modification, the effects on biological activity and on other properties, and the positions of carboxymethyl groups on both proteins were determined. The results reveal a differential reactivity of cysteine residues and show some interesting differences between wild-type and

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