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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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mutant protein, from which certain spatial relationships in the subunit and in dimer–dimer interactions may be inferred.

Experimental Procedures

Materials. Iodo[2-^{14}C]acetic acid was purchased from Amersham/Searle. Other materials were obtained as indicated earlier (Fowler & Zabin, 1978b).

Protein Isolation. β-Galactosidase was purified from *E. coli* strain A324-5 (Fowler, 1972). M15 protein was obtained from strain DZ291 (Langley et al., 1975b) by essentially the same procedure. However, because it was found to be less soluble in ammonium sulfate (see below), a 0–20% instead of a 0–33% ammonium sulfate precipitation step was used. Before use, the purified proteins were passed through a column of Bio-Gel A-1.5m to remove any aggregates higher than tetramers in the case of β-galactosidase and dimers in the case of M15 protein.

Analytical Procedures. β-Galactosidase activity was measured directly (Horiuchi et al., 1962). The activity of M15 protein was measured after complementation with an excess of the α-donor peptide CNBr2 (Langley et al., 1975a,b). Slab-gel electrophoresis on 5% polyacrylamide was performed in Tris–glycine buffer, pH 8.3 (Gabriel, 1971). Protein staining was done with 0.025% Coomassie Blue, and staining for β-galactosidase activity with 6-bromo-2-naphthyl-β-D-galactopyranoside/naphthanildiazo blue B (Erickson & Steers, 1974). The dried gels were submitted to autoradiography on Kodak Blue Brand X-ray film for 6 weeks.

Activation of M15 Protein with Anti-β-Galactosidase Antibodies. Serial dilutions of rabbit anti-β-galactosidase (Accolla & Celada, 1976) were made in 0.1 M sodium phosphate, 1 mM magnesium sulfate, 0.2 mM manganese sulfate, 0.1 M β-mercaptoethanol, pH 7.0, and 0.1 mL of each concentration was mixed with M15 protein or carboxymethyl M15 protein in 0.2 mL of the same buffer. After incubation at 28 °C for 3 h, enzyme activity was measured directly in the usual way.

Carboxymethylations. Reactions were performed in 0.1 M Tris-HCl buffer, pH 8.1, at 28 °C for 1 or 1.5 h at protein concentrations of 0.1–1 mg/mL under an atmosphere of nitrogen with exclusion of light. Sodium iodo[2-^{14}C]acetate at specific activities of 0.1–0.6 μCi/μmol was added in a molar excess of 10–10 000 compared with protein subunits, and the reactions were stopped by additions of large excesses (over 10-fold) of β-mercaptoethanol. For the small-scale incorporation studies, radioactivities were measured by removal of samples (0.1 mg), addition of bovine serum albumin (0.25 mg), and precipitation with equal volumes of 20% trichloroacetic acid at 4 °C. After centrifugation the precipitate was collected, washed twice with 10% trichloroacetic acid, and counted. At this scale, experimental variation in single samples was large but values determined were averages of three samples from each of two (M15 protein) or four (β-galactosidase) different carboxymethylation series.

For the large-scale studies, samples of 40 mg were treated for 1.5 h with [^{14}C]iodoacetate as indicated. After addition of β-mercaptoethanol to stop the reactions, and removal of small portions to test biological activities, proteins were dialyzed to remove the reagent. They were then treated with nonradioactive iodoacetate in 8 M urea to convert all cysteine residues to carboxymethylated derivatives.

Positions of Labeled Carboxymethyl Groups. β-Galactosidase and M15 protein which were fully carboxymethylated were treated with CNBr and the digest was chromatographed on CM-cellulose (Fowler, 1978). Pooled fractions were passed through Sephadex G-50.

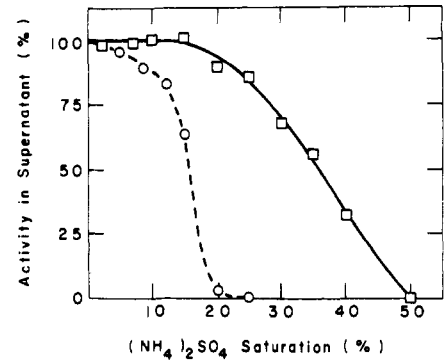

FIGURE 1: Precipitation curves with ammonium sulfate for β-galactosidase (—) and M15 protein (- - -). Proteins (0.1 mg/mL) were incubated at pH 7.0 for 15 h at 4 °C in various concentrations of ammonium sulfate, and the biological activity in the supernatant was determined after centrifugation.

Peptides were identified by their positions in the CM-cellulose and Sephadex elution patterns, by amino-terminal analysis, by amino acid analysis, and by automatic sequencer analysis as described earlier for β-galactosidase (Fowler & Zabin, 1978a) and M15 protein (Langley et al., 1975b). The amount of radioactivity in particular carboxymethylcysteine residues was determined by measurements on pooled fractions from columns, on pure peptides and fragments derived from them, and where applicable, on carboxymethylcysteine phenylthiohydantoin obtained from sequencer analysis. The experimental variation in values for a specific cysteine residue obtained from different samples was close to that of the background levels. Results are expressed in terms of the ^{14}C-label in carboxymethyl groups per residue in the subunit by comparison with the specific activity of the labeled reagent.

Results

Solubilities of β-Galactosidase and M15 Protein in Ammonium Sulfate. During the purification of β-galactosidase protein from the wild-type and mutant strains, it was noted that M15 protein was less soluble than β-galactosidase in ammonium sulfate. The salt precipitation curves of the pure materials were tested as shown in Figure 1. Under the conditions used, M15 protein is completely precipitated at ammonium sulfate concentrations of about 20% while the wild-type enzyme remains almost entirely in solution.

Carboxymethylation of β-Galactosidase and M15 Protein. The two proteins were treated with iodoacetate to determine the extent of incorporation of carboxymethyl groups and their effect on biological activity. When quantities of about 1 mg of native β-galactosidase were incubated for 1 h with [^{14}C]-iodoacetate at molar ratios of 10, 100, and 1000 (relative to the number of protein subunits) an increasing amount of radioactivity, but equivalent to less than 2 carboxymethyl groups per subunit, was incorporated (Table I, experiment 1). At a molar ratio of 10 000, the incorporation was considerably higher. Similar results were obtained with M15 protein, except that the incorporation of label at higher molar ratios of reagent was greater.

A marked difference was seen in the biological activities of the two proteins after reaction with iodoacetate. Even after treatment with the highest ratio of reagent, β-galactosidase was found to retain 90% of its initial enzyme activity. In contrast, the activity of M15, measured after complementation with CNBr2, dropped to 10% of the initial value.

These experiments were carried out in 0.1 M Tris buffer at

TABLE I: Incorporation of ^{14}C and Biological Activity after Carboxymethylation with [2-^{14}C]Iodoacetate.

expt	iodoacetate to subunit (molar excess)	incorp of label (CM[a] groups/subunit)		remaining biological act. (% of untreated)	
		β-galactosidase	M15 protein	β-galactosidase	M15 protein
1	10	0.1	0.1	100	90
	100	0.4	0.5	100	70
	1 000	1.4	2.0	95	30
	10 000	3.2	6.0	90	10
2	10	0.1		100	
	400		1.6		30
	1 000	1.8		90	
3	1 000	1.6	4.1	95	15

[a] CM, carboxymethyl.

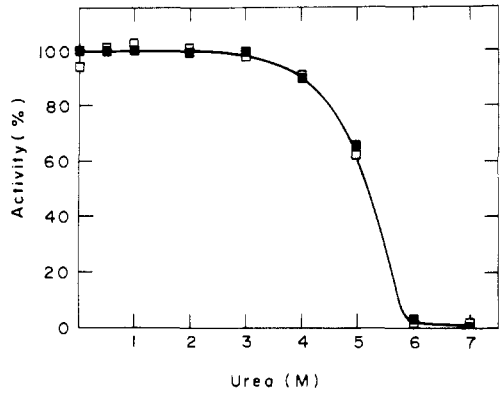

FIGURE 2: Urea inactivations of β-galactosidases. Inactivations of native (□) and carboxymethyl (■) β-galactosidase (1 mg/mL). Carboxymethylation was to 1.6 groups/subunit. Urea treatments were in 0.1 M phosphate buffer, pH 7.2, for 2 h at the indicated urea concentrations, followed by 100-fold dilutions and immediate measurements of enzymatic activities.

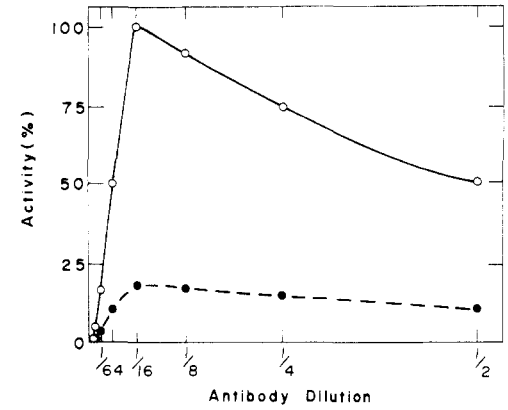

FIGURE 3: Activations of native (○) and carboxymethyl (●) M15 proteins by anti-β-galactosidase. Preincubations were for 3 h with the indicated dilutions of antiserum (100 μL) and enzyme (3 μg) in 0.1 M sodium phosphate, 1 mM magnesium sulfate, 0.2 mM manganese sulfate, 0.1 M β-mercaptoethanol, pH 7.0 (200 μL). Activities obtained are expressed as % of maximum antibody activation of unmodified M15 protein.

pH 8.1. The same results were obtained in 0.1 M phosphate buffer at the same pH in other experiments not shown here. When 40-mg quantities of β-galactosidase and M15 protein were treated with iodoacetate for 1.5 h, the extent of incorporation of carboxymethyl groups and the consequent effects on biological activities were similar (Table I, experiments 2 and 3).

Stabilities to Heat and Urea of Native and Carboxymethylated Proteins. Carboxymethylation of β-galactosidase (to 1.6 carboxymethyl groups per subunit, sample of Table I, experiment 3) does not change the stability of the enzyme to heat or urea. At 57 °C, native and modified protein had identical inactivation curves with a $t_{1/2}$ of about 20 min. Similarly, the two forms behaved identically with urea, starting to lose activity at concentrations of urea higher than 3 M (Figure 2).

M15 protein is extremely heat-labile, with a $t_{1/2} < 1$ min. Treatment with urea gave a complex pattern of inactivation because a partial reactivation occurs during the 2-h time period necessary to carry out the complementation reaction after removal of the sample from urea. However, complete loss of activity occurred in 4 M urea. Because M15 protein is so unstable, the modified protein was not tested for stability.

Antibody Activation of M15 Protein. Anti-β-galactosidase antibodies can activate M15 protein to about 10–15% of that obtained by complementation with CNBr2 (Accolla & Celada, 1976). To determine whether carboxymethyl M15 protein can also be activated, a sample containing 4.1 carboxymethyl groups and 15% of its initial biological activity (Table I, experiment 3) was treated with anti-β-galactosidase. This carboxymethyl M15 protein was activated to about 15% of the value obtained with unmodified M15 protein (Figure 3). Therefore modification with iodoacetate inhibits both activation processes to the same extent. It is also of interest that maximum activation with antibody occurs at identical antibody concentrations, indicating that antibody binding to the protein is not affected by carboxymethylation.

Biologically Active Form of M15 Protein. On addition of CNBr2, M15 protein, a dimer, is converted to an enzymatically active tetramer (Langley & Zabin, 1976). The effect of carboxymethylation on this process was investigated by electrophoresis in 5% polyacrylamide of modified and unmodified M15 protein before and after complementation with CNBr2. The results are shown in Figure 4. Samples in wells 1–5 were stained for protein. For comparison, β-galactosidase, which is a tetramer, was added to well 1. Wells 2 and 3 contained M15 protein and carboxymethyl M15 protein, respectively. Well 4 contained M15 protein and an excess of CNBr2. Well 5 was the same as well 4 except that M15 protein was carboxymethylated. It can be seen that addition of CNBr2 shifts the position of the M15 protein band close to that of β-galactosidase, and that this conversion is decreased by modification of M15 protein with iodoacetate. All enzyme activity in the complemented enzyme is associated with a migration corresponding to the position of the tetramer (well 6), while most, though not all, of the ^{14}C label remained associated with the dimer (well 7). These results show that carboxymethylation

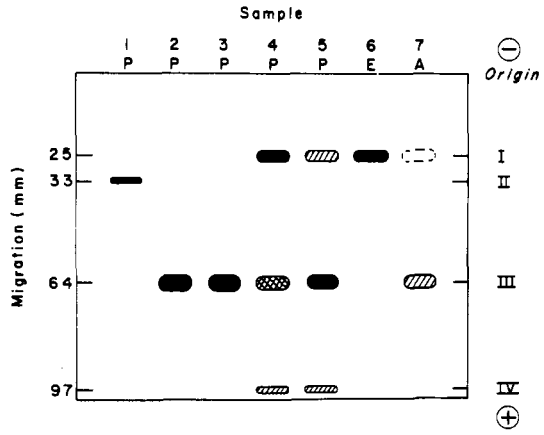

FIGURE 4: Different protein forms separated by electrophoresis in 5% polyacrylamide gel. (1) β-Galactosidase; (2) M15 protein; (3) carboxymethyl M15 protein (4.1 groups/subunit); (4) M15 protein complemented with CNBr2; (5–7) carboxymethyl M15 protein complemented with CNBr2. (P) Protein staining; (E) staining for enzymatic activity; (A) autoradiography. Roman numerals denote positions of complemented M15 protein (I), β-galactosidase (II), M15 protein (III), and CNBr2 (IV). Electrophoresis at 100 V for 3 h in Tris-glycine buffer, pH 8.3. Solid areas denote highest amounts of component. Hatched areas denote lower amounts.

inhibits conversion of M15 protein to the tetrameric form.

Position of Carboxymethyl Groups in β-Galactosidase and M15 Protein. Preparations of the two proteins which had been treated with 1000-fold excesses of [^{14}C]iodoacetate were denatured in urea, and carboxymethylation was carried out to completion with unlabeled iodoacetate. The samples were then cleaved with CNBr and the resulting peptide mixtures were chromatographed on CM-cellulose. The elution profile of radioactivity of a sample of β-galactosidase (containing 1.8 labeled carboxymethyl groups per subunit) is shown in the upper portion of Figure 5, and a profile for M15 protein (with 4.1 labeled groups per subunit) is shown in the lower part of Figure 5. It is evident that the two profiles are different. Data are not shown for another experiment with carboxymethyl β-galactosidase and carboxymethyl M15 protein (both containing 1.6 labeled groups per subunit), but similar patterns of radioactivity in the eluates were obtained.

All radioactive fractions from the CM-cellulose column were passed through Sephadex G-50 and peptides were identified by their positions in the elution profiles, and, as necessary, by amino acid analyses, amino-terminal analyses, and automatic sequencer analyses, as described earlier for the sequence determination of β-galactosidase (Fowler & Zabin, 1978a).

Radioactivity was found only in carboxymethylcysteine residues. Approximately half of the total label in β-galactosidase was found to be present at two residues (Table II), primarily at 1019 (peaks A and B, Figure 5) and to a lesser extent at 498 (peak A, Figure 5). The remaining label, equivalent to 0.9 carboxymethyl group, was found to be spread throughout the other 14 carboxymethylcysteine residues in the β-galactosidase polypeptide chain. No single one of these residues had a ^{14}C content of more than 0.1 carboxymethyl group.

In the M15 protein digest, 5 residues were labeled at levels above the background (Table II). These were residues 76 (from peak C, Figure 5), 387 and 498 (peaks F, G, and H), 600 (peak E), and 1019 (peaks C and D). The radioactivity in these residues accounted for 2.3 of the 4.1 carboxymethyl groups, or more than 50% of the total label in the protein. The background level in each of the remaining 11 carboxymethylcysteine residues varied from 0.1 to 0.2 carboxymethyl groups.

TABLE II: Position of Labeled Carboxymethylcysteine.[a]

position in subunit	^{14}C in CM[b] groups/residue	
	β-galactosidase	M15 protein
76	0.1	0.6
387	0.1	0.6
498	0.3	0.5
600	0.1	0.3
1019	0.6	0.3

[a] Background levels of radioactivity (0–0.1 for β-galactosidase and 0–0.2 for M15 protein) were found in the other carboxymethylcysteine residues at positions 122, 154, 247, 328, 400, 534, 746, 823, 884, 912, and 937. [b] CM, carboxymethyl.

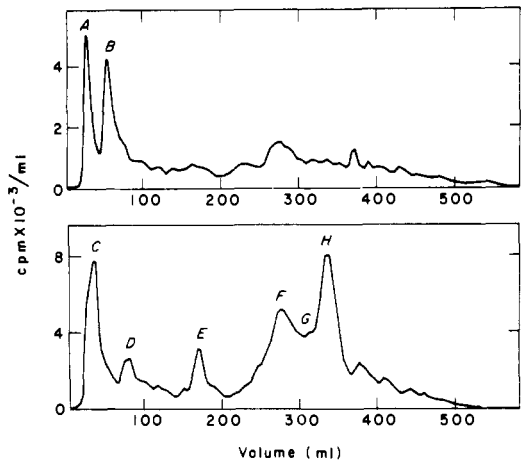

FIGURE 5: Chromatography on CM-cellulose of CNBr digests of [^{14}C]carboxymethyl β-galactosidase (top) and [^{14}C]carboxymethyl M15 protein (bottom). Column size: 1.5 × 15 cm. Elution was carried out with a salt gradient of 0–0.1 M NaCl (300 + 300 mL) in 0.02 M ammonium acetate buffer, pH 5.0, containing 8 M urea. Chromatographies were performed with peptide mixtures from 20 mg of protein. Letters refer to fractions discussed.

Discussion

Under the conditions used for the experiments reported here, reaction with iodoacetate was specific for cysteine residues. No other carboxymethyl products were detected upon amino acid analysis. Radioactivity measurements of denatured protein treated with [^{14}C]iodoacetate under the same conditions in other experiments showed values not exceeding 16 carboxymethyl groups per subunit, which is the number of cysteine residues in the β-galactosidase monomer. Finally, in all peptides from which the ^{14}C label was recovered on sequencer analysis, the position corresponded to a cysteine residue in the protein.

A differential reactivity of cysteine residues with iodoacetate was noted for both native β-galactosidase and M15 protein (which also contains 16 cysteine residues per subunit). This most likely reflects differential accessibilities to the reagent of cysteine-containing regions in both polypeptide chains. The differences in the degree of carboxymethylation of cysteine residues were, however, not absolute, but this is expected since iodoacetate is a small molecule which might penetrate into a folded protein. In the wild-type enzyme two residues were labeled with iodoacetate at levels significantly above background, cysteine-1019, and less strongly, cysteine-498. Little or no loss in enzyme activity was observed after this treatment, in agreement with earlier studies (Craven et al., 1965; Loontiens et al., 1970). Cysteine-1019, which is close to the carboxyl terminus, apparently occupies a position on or near the surface

of the tetrameric protein and is not involved in the catalytic mechanism or subunit interactions. The same conclusions must apply to cysteine-498. It is of interest that this residue is near the galactosyl binding site as indicated by covalent labeling of Met-500 by an active site specific reagent (Fowler et al., 1978).

In M15 protein, more residues are sensitive to modification and the biological activity is markedly affected, even at lower ratios of iodoacetate to protein. The increased sensitivity to chemical modification is probably due both to an intrinsic lability of the mutant structure and to exposure of new cysteine residues in the dimeric protein. A lower thermodynamic stability of M15 is also evident from heat and urea inactivation experiments, and the decreased solubility of M15 in ammonium sulfate may be related to the exposure of different surface areas. The conformation of the M15 subunit, however, must be basically similar to that of the wild-type structure, since the mutant form binds to the same affinity columns (Villarejo & Zabin, 1973), has a trace of activity by itself (Langley & Zabin, 1976), forms dimers (Langley et al., 1975b), and is activated by antibodies (Accolla & Celada, 1976). In addition, the two cysteine residues in the wild-type enzyme that are most accessible to iodoacetate are also among those significantly labeled in the M15 form. Consequently, labeling results with the M15 protein should be relevant to the wild-type structure.

Five cysteine residues in the M15 protein were found to be labeled above the background level. In addition to cysteine-1019 and -498 which react in β-galactosidase, residues at 76, 387, and 600 also were labeled. Since modification of residues 1019 and 498 had no effect on enzyme activity of β-galactosidase, carboxymethylation of one or more of the latter group of residues must be responsible for the loss of biological activity of M15 protein. The remaining 11 cysteine residues are insignificantly labeled and presumably less accessible in both protein forms.

The regain in activity by complementation involves conversion of dimeric M15 protein to a tetramer when the α-donor peptide CNBr2 is added (Langley & Zabin, 1976). Activation of M15 protein by antibodies also involves a dimer to tetramer change.[1] Since both activations are inhibited to the same extent by carboxymethylation, it seems likely that the cysteine residues affected are in areas involved in dimer–dimer interactions. This would also explain why thiols are necessary for complementation as previously found (Langley & Zabin, 1976), because the same cysteine residues are apparently very sensitive to oxidation or other modification. The effect of carboxymethylation of cysteine-76 in the protein is of particular interest, since carboxymethylation of this residue in the α-donor peptide does not inhibit α complementation (Langley et al., 1975a). This suggests that in complemented protein, which contains a duplicate of residues 42–92 in each subunit, only that portion of the polypeptide chain contributed by M15 protein, and not by CNBr2, is functional.

[1] F. Celada & I. Zabin, unpublished data.

The results of these experiments suggest that cysteine residues in β-galactosidase may be divided into three groups, those that are positioned superficially in native tetramers, those that are accessible in the dimeric state only, and those which are relatively unavailable to reagent. Evidence that supports these conclusions has also been obtained from experiments in which antibodies prepared from peptides of β-galactosidase were tested against the native protein (Celada et al., 1978). Thus, anti-CNBr14 and 24, prepared from CNBr14, which contains Cys-498, and CNBr24, which contains Cys-1019, react strongly with the native protein. Anti-CNBr2, on the other hand, does not react with β-galactosidase, in agreement with the lack of availability of Cys-76 to iodoacetate in the native structure (Celada et al., 1978), though it is exposed both to iodoacetate and anti-CNBr2 in M15 protein.[1]

Acknowledgments

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