Activation of β -galactosidase by monoclonal antibodies

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Four monoclonal cell lines secreting antibodies that activate the β -galactosidase protein from *lac*⁻_{aba} strains of *Escherichia coli* have been isolated. One of the antibodies, BG 79, inhibits the normal β -galactosidase from *E. coli* in addition to its activation of the protein from mutants. Moreover, when in combination with any of the other activating antibodies, BG 79 exhibits synergistic activation of the β -galactosidase protein, and the synergistically activated enzyme is stimulated by methanol, although most of the proteins activated by single antibodies are inhibited by methanol. The equilibrium of binding of BG 79 to the β -galactosidase protein is not affected by the presence of a second antibody, and the half-time for activation by BG 79 is only slightly, though significantly, increased by preincubation of the protein with the second antibody. Our results imply that activation of β -galactosidase proteins is not a simple correction of a conformational defect, and that many distinct active conformations are available to the enzyme.

The β -galactosidase (EC 3.2.1.23) proteins synthesized by certain mutant forms of *Escherichia coli* have little or no ability to catalyse the hydrolyses of galactosides unless activated by treatment with antibodies directed against the enzyme from *E. coli* not mutated at the same locus (Celada *et al.*, 1971; Messer & Melchers, 1970). The interaction between antibodies and a protein may be studied with relative simplicity in this system because the appearance of enzymic activity after the interaction can be used to assay the extent or time course of combination. More insight into the mechanism of the interaction may be obtained by the use of monoclonal antibodies, directed against single antigenic sites of the protein, than by the use of a whole antiserum, which may react simultaneously with several different antigenic sites. Indeed, studies of the reaction between single monoclonal antibodies and β -galactosidase proteins have been initiated in several laboratories (Frackelton & Rotman, 1980; Accolla *et al.*, 1980). An investigation of the activation of β -galactosidase proteins by two monoclonal antibodies known to bind at distinct antigenic sites has not been previously reported, although such experiments, exploiting the unique epitopic binding characteristic of monoclonal antibodies, could be expected

to yield a wealth of information not available if only a single antibody were used.

Some of the properties of our monoclonal antibodies presented here have appeared in abstract form (Hewitt *et al.*, 1981) and in summary (Duncan, 1982).

Methods

Enzymes and antibodies

Strains of *E. coli* producing a constitutive normal β -galactosidase (strain A-324-5) or constitutive mutant forms of the enzyme (strains *lac*⁻_{aba} 13, 71, 627, 645) were grown aerobically at 37°C in medium 63 (Herzenberg, 1959) supplemented with 1 mg of thiamine/litre. β -Galactosidase and its various mutant forms were purified by methods based on those described by Craven *et al.* (1965) and were virtually homogeneous in their protein content as judged by density-gradient electrophoresis and by chromatography. Purified enzymes were stored as precipitates in 50%-satd. (NH₄)₂SO₄ and were stable for many months.

Monoclonal antibodies directed against normal β -galactosidase were raised in Balb/c mice and in HO rats. Animals were immunized with purified unmutated β -galactosidase and the splenic lymphocytes from the mice were fused with P3/NSI/1-Ag4-1 (NS1) myeloma cells (Köhler *et al.*, 1976); those from the rats were fused with either NS1- or 210 RCY 3-Ag 1.2.3 (Y123)-myeloma cells (Galfre

Abbreviations used: 2-Nph-Gal, 2-nitrophenyl β -D-galactoside; 4-Nph-Gal, 4-nitrophenyl β -D-galactoside.

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et al., 1979) as described previously (Hewitt & Liew, 1979). Colonies of cells which produced antibodies capable of activating the mutant forms of β -galactosidase were cloned by limiting dilutions, and the isolated stable monoclonal lines were cultured either *in vitro* or as ascites tumours in syngeneic animals. Hybridoma BG18 is a rat $\times$ mouse hybrid and was grown only *in vitro*. Antibodies were purified from tissue-culture medium either by chromatography on protein A–Sephadex (Pharmacia, Uppsala, Sweden) by the method of Ey *et al.* (1978) or by precipitation with $(\text{NH}_4)_2\text{SO}_4$ or Na_2SO_4 and chromatography on DEAE-cellulose. Ascitic fluid was purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and chromatography on DEAE-cellulose. The immunoglobulin isotype of each hybridoma line was determined with isotype-specific antisera from Miles Laboratories Ltd., Slough, Berks., U.K., by double immunodiffusion in agar.

A sample of purified activating antibody was radioiodinated by the iodogen technique (Fraker & Speck, 1978).

Electrophoresis of the enzymes and antibodies was performed as described by Shapiro *et al.* (1967) or on gels with a gradient of polyacrylamide concentration (PAA 4/30 from Pharmacia).

Assay conditions

Assays and incubations were conducted at 37°C, except as noted. β -Galactosidase was assayed with kinetically saturating concentrations of 2-Nph-Gal or 4-Nph-Gal in 50 mM-Tris/HCl buffer, pH 7.5, containing 10 mM-MgCl₂ supplemented with 25 mM-NaCl or -KCl as appropriate (Roth & Rotman, 1975) and with 2.5 molal methanol on occasions. In order to assay the mutant forms of the enzyme it was necessary to activate them by incubation with antibodies raised against the normal enzyme. A variety of enzyme and antibody concentrations were required for the present study, but the usual procedure was to incubate for 20 min the enzyme solution and antibody, both dissolved in 50 mM-Tris buffer, pH 7.5, containing 0.5 mg of normal rabbit globulin and 10 μ mol of dithiothreitol/ml, and then to initiate the enzymic reaction by the addition of sufficient substrate solution to dilute the incubation mixture at least 10-fold. Except where noted, the β -galactosidase protein used in the present study was isolated from strain *lac*⁻_{aba} 13.

The equilibrium constants and stoichiometry of the overall reaction between an activating antibody and enzyme were estimated kinetically and by a comparative-binding technique. For the kinetic method, 124 fmol of enzyme in 1 ml of the NaCl/Tris/MgCl₂ assay buffer was incubated for 3 h with a series of concentrations of the activating antibody (from 54.6 to 1090 pM) and the reaction was initiated by the addition of 100 μ l of 50 mM-2-Nph-Gal in the

buffer. Under these conditions the reaction rate was constant for at least 90 min. In some cases a fixed concentration (10 nM) of a second activating antibody was added to the incubation at the same time as the enzyme. In the binding method a solid-phase radioimmunoassay was performed in poly(vinyl chloride) microtitre plates. β -Galactosidase was immobilized in the wells of the plates by passive adsorption, any unfilled protein-binding sites being blocked subsequently with bovine albumin. A series of concentrations of a radioiodinated activating antibody at a known specific radioactivity, diluted with 3% albumin in 10 mM-sodium phosphate/150 mM-NaCl, pH 7.6, was incubated overnight at 0°C in the plate and after thorough washing the amount of antibody bound to the plate was calculated with corrections for non-specifically bound radioactivity. On occasions an unlabelled monoclonal antibody was added along with the labelled species.

Rate constants for the activation of the mutant enzymes by antibody were investigated by a kinetic study of the approach to a steady state after mixing of the enzyme and antibody (at concentrations described in the Results section) into 8 mM-2-Nph-Gal. In some experiments the enzyme was pre-incubated for 2 h with an excess of a second activating antibody before dilution into the substrate and antibody under study.

The kinetic effect of interaction between monoclonal antibodies and the *normal* β -galactosidase was investigated by incubating 0.1 pM of the enzyme with 25 μ l of tissue-culture medium containing the antibody concerned and after 20 min adding 250 μ l of substrate.

Experimental values obtained from equilibrium procedures were fitted to theoretical equations by methods similar to those of Duggleby (1981); enzymic Michaelis constants and associated maximum velocities were obtained as described by Cornish-Bowden & Eisenthal (1978). Median values were compared by the Mann–Whitney U-test and mean values, presented with their standard errors, by Student's *t*-test. Kinetic constants were obtained from pre-steady-state experiments by fitting to theoretical equations using a non-linear least-squares regression routine (from the Wellcome Statistical Services section). Binding equations and pre-steady-state kinetic equations were derived by standard techniques (Dixon & Webb, 1979).

Results and discussion

Antibodies activating β -galactosidase

Four stable hybridoma lines that secrete antibodies able to activate the β -galactosidases from mutant strains of *E. coli* have been established. Their

Table 1. *Monoclonal hybridoma lines*

Designation	Origin	Immunoglobulin (Ig) isotype
BG 18	HO rat $\times$ NSI	Rat IgG ₁
BG 19	HO rat $\times$ Y123	Rat IgG ₁
BG 79	Balb/c mouse $\times$ NSI	Mouse IgG _{2a}
BG 81	Balb/c mouse $\times$ NSI	Mouse IgG ₁

Table 2. *Activation of strain 13 lac⁻ β -galactosidase protein*

Antibodies used	2-Nitrophenol produced (μ mol/min per nmol of enzyme; mean $\pm$ S.E.M., $n = 4$ or 5)	
	No methanol	2.5 molal methanol
BG 18	12 $\pm$ 0.3	17 $\pm$ 0.3
BG 18 + BG 79	42 $\pm$ 1	93 $\pm$ 4
BG 19	1 $\pm$ 0.1	0.9 $\pm$ 0.01
BG 19 + BG 79	11 $\pm$ 0.2	17 $\pm$ 0.3
BG 81	0.86 $\pm$ 0.04	0.2 $\pm$ 0.004
BG 81 + BG 79	28 $\pm$ 1	46 $\pm$ 2
BG 79	7.6 $\pm$ 0.4	4.6 $\pm$ 0.2

hybrid nature and immunoglobulin isotypes are presented in Table 1. One of the antibodies, BG 79, in combination with another antibody, has a synergistic action on the maximum activation of each of the mutant enzymes, and this occurs with all the substrates tested (2-Nph-Gal, 4-Nph-Gal and 4-methylumbelliferyl β -D-galactoside). The synergism is shown in Table 2. Only those combinations of antibodies including BG 79 exhibit synergistic enzyme activation, which appears to be due to action of both types of antibody on the same molecule of β -galactosidase because: (i) the strains of *E. coli* used in the present study have not been reported to produce more than one type of β -galactosidase; (ii) the enzymes activated by BG 79 plus another activating antibody are stimulated by methanol when 2-Nph-Gal (but not 4-Nph-Gal) is used as substrate, whereas the enzymes when activated by a single antibody (with the exception of BG 18; Table 2) are, typically, inhibited by methanol (Table 2); and (iii) the combined activation is greater than the sum of the activations produced by the individual antibodies (Table 2).

Binding equilibria

The dependence on the concentration of BG 79 on the extent of activation of β -galactosidase in the presence and absence of BG 81 is shown in Fig. 1. Application of the method of Duggleby (1981) to an equation expressing the relevant equilibrium shows

Fig. 1. *Binding equilibria in β -galactosidase activation* Enzyme protein (124 fmol) was allowed to react with various concentrations of BG 79 in the absence (■) or presence (●) of 10 pmol of BG 81. After 3 h the activity of the enzyme was measured. The points are mean experimental values; the lines are calculated from the best-fit constants as described in the text.

that the binding constant between BG 79 and the enzyme is not altered statistically by the presence of BG 81 [405(263–704)pM with BG 79 alone; 263(166–400)pM with both BG 79 and 81] (median estimates; 97% confidence range), although the maximum enzymic velocity is increased synergistically as in Table 2. Examination of appropriate extensions of the binding equation shows that the equilibrium constants obtained by these techniques may contain terms for the equilibrium between antibody and enzyme and any other (e.g. conformational) equilibria that occur either before or after the enzyme–antibody interaction or during measurement of the activity of the enzyme. However, the enzymic reaction rate does not vary over the period used for the measurements for the estimations of the binding constants, so disturbances of the equilibrium because of changes among the kinetic intermediates after reaction of the enzyme with substrate must be small. The method of Dixon (1972) allows determination of the number of binding sites on an

enzyme for a ligand to which it binds tightly. This method, along with a knowledge of the concentrations of both enzyme and antibody, applied to the values from experiments similar to that of Fig. 1, gives a mean value of 3.4 binding sites for BG 79/molecule of β -galactosidase from three determinations in the absence of BG 81, and 3.43 in the presence of BG 81 (four determinations).

The results obtained when radioiodinated BG 79 is allowed to react with β -galactosidase (strain 13) immobilized in plastic wells yield an apparent binding constant of 53 (33–79; 95% confidence) pmol/well with 3 (1.7–4.6) pmol as the maximum binding. In the presence of an excess of BG 81 the values are 64 (31–93) and 3.6 (2.5–5.4) respectively and are not statistically different from those in the absence of BG 81. Similarly, neither BG 18 nor BG 19 affects the binding of BG 79, but non-iodinated BG 79 does compete with labelled BG 79 for binding, as expected. Interpretation of binding experiments in heterogeneous systems is not straightforward, but the present results are in agreement with the above kinetic binding studies and imply that the constants for the equilibria of binding of BG 79 are not affected by the presence of a second, different, antibody. As in kinetic studies, direct-binding techniques yield values that may contain terms other than those for the single equilibrium constant of the enzyme–antibody reaction.

Rate constants of activation

Melchers & Messer (1973) have suggested that the activation of mutant-strain-645 β -galactosidase by an activating antiserum raised in rabbits involves a rate-limiting unimolecular reaction, possibly a conformational change in the protein. We have been able to confirm this observation by a study of the appearance of 2-nitrophenol after mixing of the strain-13 β -galactosidase protein and BG 79 into 2-Nph-Gal. With the knowledge of the stoichiometry and equilibrium of binding between BG 79 and the β -galactosidase protein it was possible to select concentrations of enzyme and BG 79 so that essentially all the enzyme would be converted into the activated form, with only a relatively small change in the concentration of unbound BG 79. Under these conditions curves of the general form shown in Fig. 2 might be obtained, either because of the time taken for the actual combination between enzyme and antibody or because of secondary slow changes in the system. The former possibility may be excluded, because at a fixed enzyme concentration (2.4 nM) the computed rate constant of the activation reaction as defined in Fig. 2 is independent of the concentration of BG 79 over the range 20–52 nM (r , coefficient of linear correlation = 0.55; $P > 0.1$ for $r = 0$). This rate constant is also independent of the

Fig. 2. *Pre-steady state of activation by monoclonal antibodies*

β -Galactosidase protein (1.5 pmol) was preincubated for 2 h with tissue-culture medium (B, D) or BG 81 in this medium (A, C). At zero time a solution of 2-Nph-Gal containing the medium (D, C) or BG 79 in the medium (A, B) was added and the absorbance changes were monitored. The numerical values obtained from A and B were fitted to the following equation:

$$P = aE_0t + bE_0(e^{-t(j+k)} - 1)$$

to obtain rate constants for activation by BG 79. P is the product formed at time t by a total enzyme concentration E_0 equilibrating between forms F and G, with associated rate constants j and k . a and b are complex constants including contributions from rate constants for the enzymic reactions of G and F as well as j and k .

enzyme concentration ($P > 0.90$). The intercept of the asymptote to the product–time curve with the product–concentration axis (Fig. 2) has a negative value directly proportional to the enzyme concentration in the range 0.6–2.4 nM ($r = 0.96$), but independent of the antibody concentration ($P > 0.90$). These results are all consistent with the rate-limiting reaction of activation being a unimolecular reaction and other than the interaction of the antibody with the enzyme. The rate constant of the overall reaction of activation calculated from the 19 values obtained in the present study is $0.17 \pm 0.01 \text{ min}^{-1}$, giving a mean half-time of acti-

vation of 4.2 min. The rate constant for activation by BG 79 determined in the presence of enzyme pre-activated with an excess of BG 81 (10 pmol of antibody/pmol of enzyme) is 0.12 ± 0.01 (10 min) $^{-1}$, a value that is significantly less ($P < 0.01$) than that for activation by BG 79 alone. These values for the half-time of activation are in good agreement with that of Melchers & Messer (1973) (about 12 min at 35°C), especially as different methods for evaluating the constants were used. As pointed out before, the *equilibrium* constant measured kinetically includes terms not only for the actual binding reaction but also for any other changes that occur. Activation being a unimolecular reaction under some conditions is strong evidence of the existence of such changes and may provide a basis for understanding the equilibrium constant's unusually low value.

Inhibition of β -galactosidase

A further property of BG 79 is that of inhibition of *normal* β -galactosidase. Such inhibition, like the capacity for synergistic activation, is restricted to BG 79 among our monoclonal antibodies. With 2-Nph-Gal as substrate, BG 79 causes a significant ($P > 0.001$) inhibition of β -galactosidase, from 514 ± 3 μ mol/min per mg to 347 ± 3 μ mol/min per mg, and the *extent* of this inhibition is affected neither by substituting K^+ for Na^+ in the assay medium, nor by the presence or absence of methanol, nor by the replacement of 2-Nph-Gal by 4-Nph-Gal in any of the above situations (cf. Roth & Rotman, 1975).

A variety of active states

Activation of mutant forms of β -galactosidase is usually regarded as being mediated via conformational changes in the protein (Melchers & Messer, 1973; Celada *et al.*, 1971). The observation reported here that each enzyme form may be activated to different extents by different monoclonal antibodies implies that interaction of several different activating antibodies with the protein does not induce a single activated state. This inference is supported by the finding of synergistic activation by one of the monoclonal antibodies; an already active state may be activated yet further. Table 2 shows that the synergistically activated enzyme is stimulated by methanol when 2-Nph-Gal is used as substrate. Stimulation of β -galactosidase by methanol indicates that, for the substrate concerned, degalactosylation of the enzyme is partly rate-limiting; with the unmutated enzyme this is the case with 2-Nph-Gal but not with 4-Nph-Gal (Viratelle *et al.*, 1969). It may be concluded from these observations that the synergistically activated enzyme has a rate-limiting step in its kinetic reaction pathway that is different from that of the protein activated by a

single activating antibody, but is similar to that of the unmutated protein. This is supported by the observation that hydrolysis of 4-Nph-Gal by the synergistically activated enzyme is not stimulated by methanol. The K_m for 2-Nph-Gal of the synergistically activated enzyme (510 μ M, 98% confidence limits at 390 and 665 μ M) is significantly ($P < 0.001$) different from that of the enzyme activated by BG 79 alone (921 μ M, limits at 840 and 1000 μ M). The K_m for 2-Nph-Gal of normal β -galactosidase is about 100 μ M (Viratelle *et al.*, 1969). Overall, then, these results show that not only are a variety of levels of activity available to the β -galactosidase proteins, but that the catalysis may occur with various rate-limiting steps dependent on the nature of the activation.

Lack of interaction between activating antibodies

Both the solid-phase-binding experiments and the kinetic measurements show that the equilibrium of binding of BG 79 is not significantly affected by the presence of BG 81, even though this combination of antibodies causes synergistic activation of the β -galactosidase protein. This suggests that the site at which BG 79 binds is not affected by the presence of BG 81, so any conformational changes involved in activation by BG 81 probably do not extend over the whole of the protein. A unimolecular reaction, presumably a conformational change, is rate-limiting in the activation by BG 79, and the rate constant for this reaction is only slightly (although significantly) affected by the presence of BG 81, as would be expected if the activation by BG 79 were by a change essentially independent of that brought about by BG 81. Analysis of the kinetic binding data shows that combination of approximately four molecules of BG 79 is required to activate each molecule of mutant enzyme maximally, whether or not BG 81 is present. It is likely that our mutant forms of β -galactosidase are tetrameric (Melchers & Messer, 1970), so it appears that each subunit requires activation separately. The divergence of the experimental values from precisely one molecule of BG 79 per subunit may be due to inactive protein in the enzyme preparation. Inasmuch as each subunit binds a molecule of BG 79, it is unlikely that the lack of interaction between BG 79 and BG 81, as measured from equilibria and rate constants, merely represents binding of the two types of activating antibody to different subunits of the same polymeric enzyme.

Our studies show that a range of monoclonal antibodies activate the various β -galactosidase proteins via changes that are, at least in some cases, independent, but which together have major effects on the kinetic reaction pathway of the enzyme. Further applications of these antibodies, particularly BG 79 with its unique properties, should lead to

a deeper understanding of the changes inducible in proteins, and of the mechanics of β -galactosidase.

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