ANTIBODY-MEDIATED ACTIVATION OF A DELETION-MUTANT β-GALACTOSIDASE DEFECTIVE IN THE α REGION

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1. Introduction

A number of effects attributable to changes in conformation have been described to occur upon the interaction of specific antibody with wild type and mutant β-galactosidases. These effects range from stabilization under normally denaturating conditions [1], to disruption of the quaternary structure [2], facilitation of ω-complementation [3], and activation of missense mutant gene products [4]. The latter can be regarded as the repair of the damage induced by a minor genetic defect, the substitution of a single amino acid in a polypeptide chain of 135,000 daltons. The repair of a more severe genetic defect is described in the present paper: it deals with the antibody-mediated activation of the deletion mutant product M15. This β-galactosidase lacks 30 residues (AA 11–42) in the N-terminal region, is a dimer instead of tetramer, and has been well characterized as an acceptor in α-complementation [5].

2. Materials and methods

2.1. *Diluents and chemicals*

Buffer B was used for the dilution of the enzyme in all experiments of activation; it contained 10 mM (hydroxymethyl)aminomethane (tris), 10 mM $MgCl_2$, 0.1 M NaCl and 0.05 M 2-mercaptoethanol; its pH was adjusted to 7.2 (23°C) with acetic acid; the complete buffer was prepared daily by adding mercaptoethanol to the stock salt solution. Buffer Hepes 0.1 M was used for all experiments of complementation; its pH was adjusted to 7.2 with concentrated NaOH. Thiophenyl-ethyl-β-D-galactopyranoside (TPEG) was a gift from Dr. Agnès Ullmann, Institut Pasteur. *o*-Nitrophenyl-β-D-galactopyranoside (ONPG) was purchased from Mann Research Labs., Inc.

2.2. *Extraction of β-galactosidase (β-gal) and M15 protein*

The *E.coli* constitutive mutant 3300, grown in tryptone-lactate broth, was used for the production of normal β-D-galactosidase. The deletion mutant 3000-M15 [6], was the source of α acceptor.

Procedures followed for extraction of the enzymes were similar to those described by Celada et al. [7]. The purification of β-gal yielded a preparation with a specific activity of ~500,000 EU/mg, which showed only minor contaminants in SDS acrylamide electrophoresis, while M15 was used as a crude bacterial extract.

2.3. *Assay of β-galactosidase*

The enzymatic assay was done at 37°C in a 2 ml volume of buffer B containing 3 mM ONPG; the reaction was stopped by adding 0.5 ml of 1.2 M Na_2CO_3, and its optical density at 420 nm was measured and converted to *o*-nitrophenol concentration using a molar extinction coefficient (1 cm pathway) of 4700. An enzyme unit (EU) is defined as the amount of β-galactosidase which hydrolyzes 10^{-9} moles of substrate/min under the above conditions.

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2.4. *Animals and immunization procedures*

Male and female rabbits weighing 2–3 kg each were used. Immunization was done by injecting into the foot pad 1 ml of buffer containing 3–5 mg β-gal protein, emulsified with 1 ml of Complete Freund's Adjuvant. Some procedures were repeated for secondary stimulation and one week after challenge bleedings were done by puncturing the central artery of the ear. Collected serum was kept at -30°C in small samples after sterilization by filtration through millipore.

2.5. *Preparation of peptides for complementation*

Seven mg of β-gal were dissolved in 2 ml 8 M urea (previously deionized), 0.25 ml 3 M Tris–acetic acid buffer (pH 8.6), 0.1 ml EDTA 5% and 0.025 ml 2-mercaptoethanol. The solution was kept in the dark for 4 h under nitrogen atmosphere, then aminoethylation was performed at room temperature according to Raftery and Cole [8] with the following schedule: three aliquots of 0.2 ml ethylenimine were added at time 0, 10 min and 20 min. At 30 min the reaction was stopped by slowly adding 1 ml glacial acetic acid, while lowering the temperature to 0°C. The mixture was dialyzed for 72 h against 20% acetic acid and finally lyophilized. On this material, CNBr cleavage was performed according to Steers et al. [9]: the sample was resuspended in 3 ml 70% formic acid, and a 50-fold excess (with respect of the methionine residues present) of crystalline CNBr (Eastman Organic Chemicals) was added. The reaction was allowed to proceed at room temperature for 24 h in the dark; then the reaction mixture was diluted ten-fold with distilled water and lyophilized.

The peptide mixture was used in complementation experiments without further purification and will be hereafter designated as 'α peptides'. The dry material was dissolved in 70% formic acid, and 25 μl aliquots thereof or of a further dilution in the same solvent were placed in the tubes where the complementation reaction was to take place; the liquid was evaporated under vacuum at room temperature. The dry peptides were resuspended in 0.1 ml of Hepes buffer.

2.6. *Activation of M15 by antibody*

A fixed concentration of M15 crude extract in 0.1 ml of buffer B was mixed with 0.1 ml of buffer B and 0.1 ml of scalar antibody concentrations. The mixture was incubated at 28°C for 3 h, and then assayed for enzyme activity.

2.7. *α-complementation procedure*

0.1 ml of M15 crude extract (or a dilution thereof in buffer B was mixed with 0.1 ml of buffer B and 0.1 ml of buffer Hepes containing the chosen concentration of the peptides preparation, dissolved from the lyophilized stock solution (see: preparation of peptides). The mixture was incubated at 28°C and, where not indicated otherwise, was assayed for enzymatic activity after 2 h. When the effect of antibody on complementation was investigated the 0.1 ml of buffer in the mixture was substituted with the chosen concentration of antiserum.

3. Results

Figure 1a shows the effect (in terms of specific enzyme activity), of 1 h incubation of scalar amounts of anti β-gal antiserum with a constant amount of M15 extract. The catalytic activity increases with

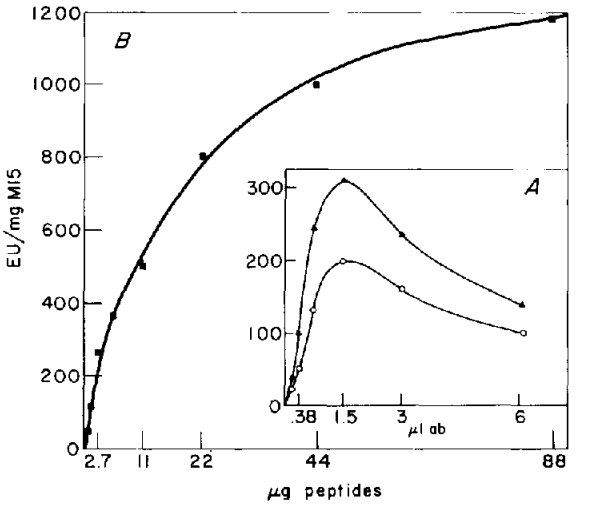

Fig.1A. Activation of M15 by increasing concentrations of antibody. Ordinate: enzymatic activity expressed as EU/mg M15 crude extract. Abscissa: μl antibody added. (○—○) Whole anti β-gal antiserum. (▲—▲) Monovalent Fab fragments obtained from the same antiserum. Fig.1B. Complementation of M15 by increasing concentrations of α-peptides. Ordinate: same as Fig.1A in a different scale. Abscissa: μg peptides added to M15.

antibody concentration to reach a maximum; then it decreases in antibody excess. The two curves shown indicate that this pattern is similar for native antibody and for monovalent Fab fragments obtained from the same antiserum.

For comparison, the enzyme activity resulting from α complementation of the M15 extract in the presence of increasing amounts of peptides obtained by CNBr cleavage of wild type β-galactosidase is plotted in fig.1b Since the same M15 extract was used for antibody activation and α-complementation the data are directly comparable and show that about 15% of the maximum activity attainable by complementation is obtained by antibody-M15 interaction.

Several three-component experiments were done to determine the effect of anti β-gal antibody on the α-complementation reaction. Table 1 shows the enzyme activity at equilibrium observed when three concentrations of peptides and three concentrations of antibodies were mixed in all combinations with the M15 extract. The results van be described as follows: (a) At low ('1') and optimal ('16') activating antibody concentration there is simple additivity of antibody and α peptides effects, when α peptides concentration is low ('1') or medium ('8'), (b) At high antibody concentration there is depression of activity in all combinations. The extent of this depression reaches ~65% of the maximum activity for all peptide concentrations as well as in the absence of peptides. (c) When α is high ('128'), which in our conditions is saturating, there is a significant depression even at 'optimal' ('16') antibody concentration.

The effect of anti β-gal antibodies on the kinetics of α-complementation was studied by measuring the

Table 1
Effect of different combinations of anti β-gal antibodies and α-peptides on the activity (EU/10^{-1} mg) of a fixed quantity of M15

Relative peptide concentration	Relative antibody concentration			
	0	1	16	128
0	<1	5	120	25
1	18	28	140	23
8	220	250	340	170
128	2000	2000	1600	700

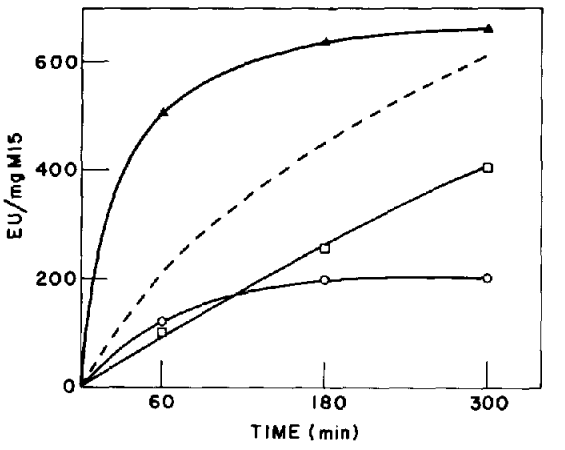

Fig.2. Effect of antibody on the kinetics of α-complementation. Ordinate: enzymatic activity. Abscissa: reaction time. (○—○) M15 + anti β-gal antibody. (□—□) M15 + α peptides. (▲—▲) M15 + β-gal + α peptides. The concentration of antiserum corresponds to optimal activation. The concentration of α peptides is 100-fold lower than at required for saturation.

enzyme activity at given times after mixing M15 protein with (i) α peptides, (ii) antibody and, (iii) α peptides and antibodies. Figure 2 shows the results. There is a significant increase of activity during the early stages of complementation in experiment (iii). This increases about 100% the sum of activity of (i) and (ii) at 60 min, and about 30% the same sum at 180 min. At 300 min the activity in (iii) is only 7% higher than the sum of (i) and (ii).

4. Discussion

Among several effects of antibodies on the conformation and the function of β-galactosidases, the present one is the first involving reactivation of a molecule that lacks a considerable part (30 AA) of the primary structure. Even on the basis of the present data obtained with a crude preparation of M15 it is possible to reach a number of provisional conclusions.

First, the activity brought about by antibody is a relatively small proportion of that obtainable by α-complementation (10–15%). Since the latter is known to activate M15 almost quantitatively, this means that either all M15 reach at best 10% of the wild type activity or, possibly, only one out of ten

M15 molecules are fully active after interaction with antibody.

Second, the activation of M15 by antibody is a complex phenomenon, as suggested by the distinct peak in the antibody dose–response curve, contrasting with α-complementation [10] and the antibody-mediated activation of point mutant β-galactosidases [11], both of which are described by parabolic saturation curves. The peak of activity and the following depression in antibody excess cannot be interpreted as phenomena related to lattice formation, since the same patterns are observed with monovalent Fab fragments. Instead, it is tempting to attribute activation and depression to the presence in the anti β-gal serum of at least two different antibodies capable of binding M15 and endowed with competing properties, and reaching its effective concentration at a different dilution of the antiserum. This hypothesis is strengthened by the finding of antibodies causing inhibition of the wild type enzyme activity [2]. An alternative possibility is that the same antibody may cause opposite effects in the enzyme function depending on the number bound per enzyme polymer. In order to decide between these alternatives it will probably be necessary to purify 'clonal' antibodies and assess their activating–inhibiting activity. However, the fact that also α-complementation can be partially inhibited by antibody, and that this can take place even at the antiserum concentration that corresponds to the antibody mediated activation peak, seems to favor the first hypothesis.

To evaluate the effect of antibody on the complementation process one must recall the recent finding [3] that anti β-gal enhances 5–20 times the yield of ω-complementation without visibly changing the kinetics of the reaction. The present data differ from these in more than one way. In fact they show a significant increase in the speed of enzyme activation, not accounted for by summation of complementation and activation, but lack of any increase in final yield of enzyme activity. It should be noted that another difference between α- and ω-complementations is the susceptibility of the latter but not of the former to be facilitated by substrate analogs [12].

The kinetics of α-complementation has been recently analyzed using pure reagents. After binding of the pure α peptide by pure M15 dimers, a tetrameric enzyme is formed (Langley and Zabin, personal communication). Collaborative experiments are now in progress te determine the effect of the antibody on the quaternary structure of M15.

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