Larger increases in sensitivity to paracatalytic inactivation than in catalytic competence during experimental evolution of the second β-galactosidase of *Escherichia coli*

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Second-order rate constants ($M^{-1} \cdot s^{-1}$) at 25 °C and pH 7.5 for inactivation of first-generation (*ebg*^{a} and *ebg*^{b}), second-generation (*ebg*^{ab} and *ebg*^{abcd}) and third-generation (*ebg*^{abcde}) experimental evolvants of the title enzyme by 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside are 0.042, 0.30, 10, 24 and 57 respectively. Only partial inactivation is observed, except for *ebg*^{abcde}. At a single high inactivator concentration, inactivation of the wild-type *ebg*^{o} is also seen. The changes in sensitivity to the paracatalytic inactivator (over a range of $10^{3.3}$) are larger than changes in k_{cat}/K_m for lactose (over a range of $10^{2.7}$) or nitrophenyl galactosides (over a range of only $10^{1.3}$), or changes in degalactosylation rate (over a range of $10^{1.7}$). These data raise the possibility that evolution in the reverse sense, towards insensitivity to a paracatalytic inactivator with a proportionally lower effect on transformation of substrate, may become a mechanism for the development of bacterial resistance to antibiotics that act by paracatalytic enzyme inactivation.

INTRODUCTION

In addition to the well-characterized *lacZ* β-galactosidase of *Escherichia coli* [1], the organism produces another β-galactosidase, *ebg*, also under negative control [2]. Unlike the *lacZ* enzyme, which is a tetramer of identical subunits, the *ebg* enzyme has an $\alpha_4\beta_4$ quaternary structure in which the holoenzyme contains four *ebgA* gene products and four *ebgC* gene products [3]. There is a 50 % nucleotide identity between the *lacZ* and *ebgA* genes, which results in a 33.7 % amino acid identity in the gene products [4]. Of the 15 amino acid residues that make up the active site of the *lacZ* enzyme [1], 13 are conserved in the wild-type *ebgA* gene product. The presence of the *ebgC* gene product is essential for full catalytic function, despite the active-site residues all belonging to the *ebgA* gene product; it appears that the small subunit is essential for the optimal operation of electrophilic catalysis by the active-site Mg^{2+} [5].

The *ebg* system is of interest as a model for acquisitive evolution [6]. The wild-type enzyme, *ebg*^{o}, is too catalytically feeble to permit growth on lactose or other β-galactoside disaccharides, but selection on lactose as sole carbon source results, 90 % of the time, in Class-I mutations, and 10 % of the time in Class-II mutations, both of which permit growth on lactose. Selection on lactulose results exclusively in Class-II mutations. Further selections of Class-I-containing strains on lactulose, or Class-II-containing strains on galactosyl-arabinose, result in Class-IV mutations. A third round of selection of Class-IV-containing strains on lactobionate results in Class-V mutations [7].

The defining amino acid change for a Class-I enzyme is Asp-92 → Asn in the large subunit: the resulting Asn-92 is homologous with the active-site Asn-102 of the *lacZ* enzyme, so that in Class-I enzymes 14 of the 15 *lacZ* active-site residues are conserved. The defining amino acid change for a Class-II enzyme is Trp-977 → Cys, also in the large subunit: Trp-977 is homologous with the active-site Trp-999 of the *lacZ* enzyme, so in the case of Class-II enzymes 12 of the 15 active-site residues are conserved.

The Asp-92 → Asn and Trp-977 → Cys changes are denoted by the superscripts *a* and *b* respectively (the wild-type enzyme being *ebg*^{o}). However, some Class-IV enzymes (such as ebg^{ab}) have only these two amino acid changes, whereas others have additional mutations. In ebg^{abcd} these additional amino acid changes are Ser-979 → Gly in the large subunit and Glu-122 → Gly in the small subunit (indicated by superscripts *c* and *d* respectively). The defining amino acid change in a Class-V enzyme, in addition to the ancestral Asp-92 → Asn and Trp-977 → Cys changes in the large subunit, is Glu-93 → Lys, also in the large subunit (indicated by superscript *e*).

Studies with chemical, UV and mutator-gene mutagenesis have established that the only way to get a more efficient β-galactosidase from *ebg*^{o} is to change Asp-92 to Asn or Trp-977 to Cys; the pathway taken during spontaneous mutation is dictated by the catalytic possibilities inherent in the protein, not the replicative machinery of *E. coli* [8].

The chemical mechanism of both *ebg* and *lacZ* enzymes involves a double-displacement reaction in which a nucleophilic glutamate residue (537 in *lacZ* [9], homologous with 512 in *ebg*) forms a covalent galactosyl-enzyme intermediate with the substrate, with expulsion of the leaving group. Both enzymes appear to use electrophilic catalysis by the active-site Mg^{2+}, rather than acid catalysis by a proton donor, to assist the departure of the leaving group, where this is possible and necessary [10–12]. The *ebg* enzymes are kinetically simple, with either glycone–aglycone cleavage or hydrolysis of the glycosyl-enzyme intermediate governing steady-state rates for the hydrolyses of aryl galactosides or galactosyl pyridinium salts [3,12,13]. At the chemical transition states leading to and from the galactosyl-enzyme there is a build up of positive charge on the sugar ring, although this is much more pronounced for the *lacZ* enzyme than *ebg* enzymes. Evolution, indeed, reduces the charge on the glycone so that in this sense it is away from *lacZ* in mechanism even though it is towards *lacZ* in function [14].

Withers' group has introduced a very useful class of paracatalytic inactivators and active-site titrants for retaining

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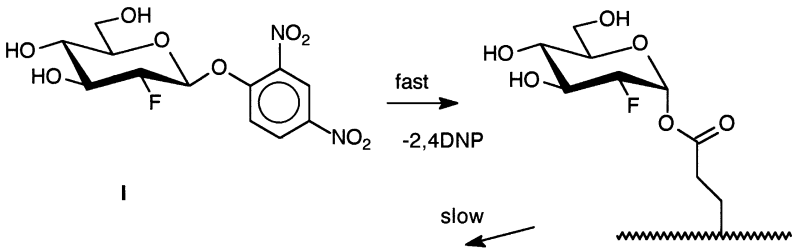

Scheme 1. Molecular events leading to complete and transient inactivation of *ebg* β-galactosidase by 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside (I)

The reagent reacts with loss of 2,4-dinitrophenolate (2,4-DNP) to give a glycosyl-enzyme intermediate (an α-D-galactopyranosylated glutamate residue, as shown). This intermediate does not react further, or reacts further (presumably to give 2-deoxy-2-fluoro galactose) only slowly.

glycosidases [15]. These agents are based on the principle of destabilizing the oxocarbonium-like transition state by replacement of the 2-OH by an electron-withdrawing fluorine, while at the same time making the glycosyl-enzyme intermediate accessible by incorporating a good leaving group, such as fluoride or 2,4-dinitrophenolate. 2-Fluoro-2-deoxy-glycosyl enzyme intermediates are formed which do not turn over, or turn over very slowly. The formation of these intermediates is associated with the liberation of a concentration of leaving group equivalent to the concentration of enzyme active sites: in the case of the intensely chromophoric 2,4-dinitrophenolate leaving group therefore, the reagents constitute attractive active-site titrants. 2′,4′-Dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside [compound (1), Scheme 1] has been shown to label the nucleophile Glu-537 of *lacZ* β-galactosidase [9].

We now report kinetic studies of the interaction of paracatalytic inactivator (I) with *ebg* enzymes.

MATERIALS AND METHODS

Materials

Some samples of compound (I) were kindly given by Professor Stephen G. Withers, University of British Columbia; later samples were synthesized via electrophilic fluorination of triacetoxy D-galactal [16] and had the properties reported [9].

The various *ebg* enzymes were purified from the sources and by the procedures already described for ebg^{o}, ebg^{a}, ebg^{b}, ebg^{ab} [3,7], ebg^{abcd} and ebg^{abcde} [17], and for the large (α) subunits of ebg^{a} and ebg^{o} [5]. ψebg^{a} and ψebg^{b} are versions of the enzyme with the PCR-induced Leu-9 → His mutation, as described previously [5].

Methods

Inactivation of the enzymes was monitored by incubating them in 50 mM potassium phosphate buffer, pH 7.5, containing 5 mM $MgCl_2$, at 25 °C in the presence of various concentrations of compound (I). Residual enzyme activity was measured at appropriate time intervals by removal of an aliquot of the inactivation mixture (30–50 μl) and addition to a solution of 4-nitrophenyl α-L-arabinopyranoside (5 mM, 1.0 ml) in 0.125 M potassium phosphate buffer, pH 7.5, with 5 mM $MgCl_2$ and 25 μM 2,2′-bipyridyl. The release of 4-nitrophenolate was monitored by the change in A_{400} in a Perkin–Elmer Lambda 6 or Lambda 3 spectrophotometer fitted with a thermostatically controlled cell compartment.

Release of 2,4-dinitrophenol from the inactivator by high concentrations of enzyme was monitored at 400 nm. Protein was assayed by the biuret method and an absorption coefficient of 11300 $M^{-1}\cdot cm^{-1}$ was used to calculate burst size [9].

The arabinoside (rather than galactoside) was used to monitor inactivation since the re-activation of 2-deoxy-2-fluoro-glycosyl enzymes can be much accelerated by transglycosylation to suitable sugar acceptors, and the absence of a primary hydroxyl on the assay substrate would minimize this process during the assay.

Pseudo-first-order rate constants (k_{obs}) were determined by fitting each inactivation curve (fraction of remaining active enzyme against time) to the first-order rate equation with a residual with the use of the program Kaleidagraph (Synergy, Philadelphia, PA, U.S.A.). Since saturation of k_{obs} with the inactivator concentration was not achieved, the individual values for k_i (the inactivation rate constant) and K_i (the dissociation constant) could not be determined by fitting the observed rate constants to eqn (1):

$$k_{obs} = k_i[I]/(K_i + [I]) \quad (1)$$

Values of k_i/K_i were obtained from the slopes of plots of $1/k_{obs}$ versus 1/[I] for ebg^{abcd} and ebg^{abcde}, but for other enzymes, where there was less likelihood of saturation, from the slopes of plots of k_{obs} versus [I].

RESULTS AND DISCUSSION

The inactivation of *lacZ* β-galactosidase by compound (I) is quite efficient: it obeys saturation kinetics [eqn (1)], with $k_i = 1.8 \times 10^{-3}\ s^{-1}$ and $K_i = 0.16$ mM ($k_i/K_i = 11\ M^{-1}\cdot s^{-1}$). The 2-

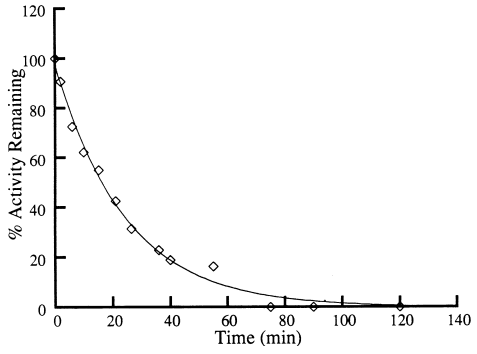

Figure 1 Time-dependent inactivation of ebg^{abcde} at pH 7.5 and 25 °C with 16.8 μM

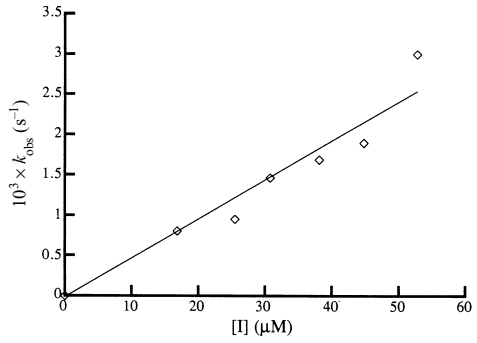

Figure 2 Plot of first-order rate constant for inactivation of *ebg*^{abcde} against concentration of inactivator, at pH 7.5 and 25 °C

deoxy-2-fluorogalactosyl-enzyme intermediate does turn over, but only slowly ($\tau_{1/2} = 11$ h) [9]. We therefore initially only intended to use the reagent as an active-site titrant for *ebg* enzymes, but were surprised to discover that the wild-type enzyme and the first-generation evolvants were inactivated only very slowly indeed.

Faster inactivation was observed for the second- and third-generation evolvants, but only the third-generation evolvant *ebg*^{abcde} was completely inactivated.

The complete inactivation of *ebg*^{abcde} is illustrated in Figure 1. Even when complete inactivation was observed, however, enzyme saturation could not be seen (Figure 2).

Partial inactivation of less evolved enzymes is illustrated with the data for *ebg*^{ab} in Figure 3. Partial inactivation with the Withers-type reagents is commonly associated with a slow steady-state turnover: if the enzyme is saturated with inactivator, after the transient phase the fraction of enzyme present as the 2-deoxy-2-fluoroglycosyl-enzyme is $k_{+2}/(k_{+2}+k_{+3})$, where k_{+2} is the first-order rate constant for conversion of the Michaelis complex into the 2-fluoro-2-deoxy-glycosyl-enzyme and k_{+3} is the rate of hydrolysis of the fluoroglycosyl-enzyme. Slow steady-state turnover can just be discerned when the inactivator is incubated with a high concentration of *ebg*^{abcd}. In the experiments displayed in Figures 4 and 5, a high concentration of enzyme was incubated with the inactivator, and the liberation of 2,4-dinitrophenol was monitored. It is seen that in the case of *ebg*^{abcd} there is a steady-state rate which is not apparent in the experiment with *ebg*^{abcde}, confirming that partial inactivation does arise from slow steady-state turnover.

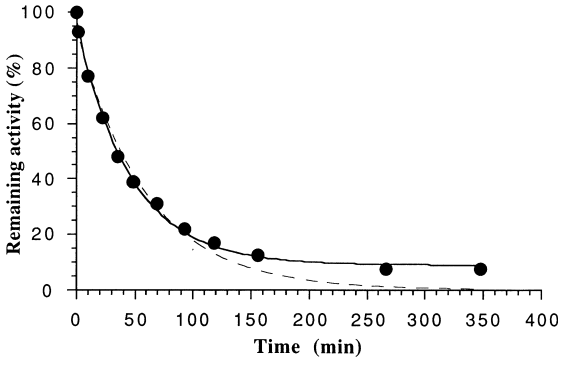

Figure 3 Time-dependent inactivation of *ebg*^{ab} at pH 7.5 and 25 °C with 29.9 μM inactivator

The solid line is the best fit to eqn (3) and the dotted line is the best fit to an exponential decay to zero.

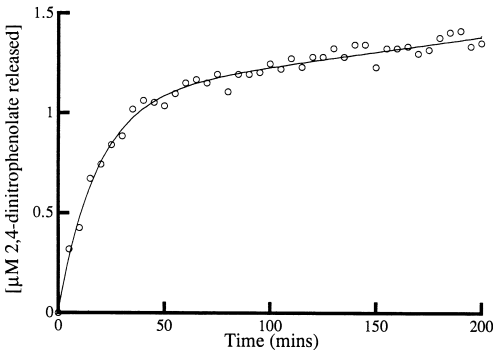

Figure 4 Release of 2,4-dinitrophenolate on hydrolysis of inactivator (60 μM) at pH 7.5 and 25 °C by *ebg*^{abcd} enzyme (1.2 μM)

The data are fitted (using Kaleidagraph; see the Materials and methods section) to an exponential approach to a steady-state, i.e.

$[P] = At + B[1 - \exp(-kt)]$

If the data are fitted to a simple exponential the correlation coefficient falls from 0.993 to 0.98 and the data are visibly misfitted.

If the enzyme is assumed to be 100 % pure and 100 % active protein of molecular mass per active site of 140 kDa, then the size of the burst displayed in Figures 4 and 5 corresponds to 83 and 94 % of the concentration of enzyme respectively. These data provide assurance that the k_{cat} values quoted in previous papers are close to absolute. In the case of active-site titrants such as the present one, where the modified enzyme does in fact slowly turn over, the active-site titration becomes a conventional pre-steady-state experiment, if an experiment is on the laboratory rather than rapid-reaction timescale. In such experiments the burst amplitude (Π) is given by eqn (2):

$$\Pi = [E]_o[k_{+2}/(k_{+2}+k_{+3})]^2\{[S]/([S]+K_m)\}^2 \quad (2)$$

Burst size is therefore reduced if $k_{+2} \sim k_{+3}$. For evolvants where there is turnover therefore, even fully active enzyme will give a substoichiometric burst. It is therefore likely that the *ebg* enzymes as we isolated them were, for practical purposes, 100 % active and 100 % pure. [The K_i parameter of eqn (1) corresponds to K_s, not the K_m of eqn (2).]

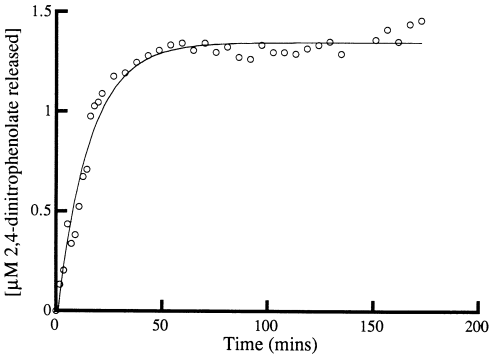

Figure 5 Release of 2,4-dinitrophenolate on hydrolysis of inactivator (60 μM) at pH 7.5 and 25 °C by *ebg*^{abcde} enzyme (2.3 mg/ml)

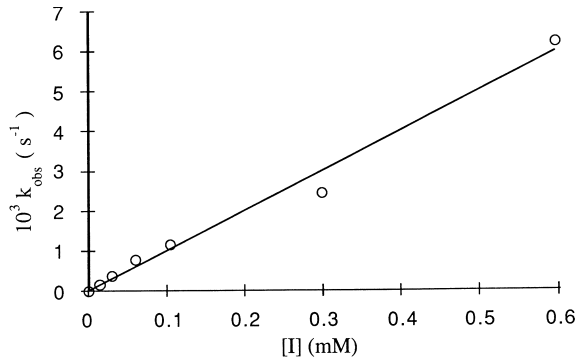

Figure 6 Plot of first-order rate constant for inactivation versus inactivator concentration for *ebg*^{ab}

Table 1 Second-order rate constants for inactivation of *ebg* mutants by 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside

Data were obtained from at least five concentrations of the inactivator.

	ebg^{a}	*ebg*^{b}	*ebg*^{ab}	*ebg*^{abcd}	*ebg*^{abcde}
k_i/K_i($M^{-1}\cdot s^{-1}$)	0.042	0.33	10	24	57

Table 2 First-order rate constants for inactivation of *ebg* mutants by 2′,4′-dinitrophenyl 2-deoxy-2-fluoro-β-D-galactopyranoside at a single inactivator concentration (pH 7.5, 25 °C, 5 mM $MgCl_2$)

	ebg^{o}	*ebg*^{o} α-subunit	*ebg*^{a}*	*ebg*^{a}*	*ebg*^{a} α-subunit	ψ *ebg*^{a}	*ebg*^{b}*	ψ *ebg*^{b}
Inactivator concn. (mM)	3.7	3.7	3.7	1.8	3.7	1.8	1.8	1.8
$10^5 \times k_{obs}$ (s^{-1})	9.8	2.6	20	6.8	4.3	6.4	62	82

* Data at several concentrations obtained (see Table 1).

First-order rate constants for partial inactivation were obtained by fitting the measured activities to eqn. (3), rather than a simple exponential decay to zero.

$$\text{Activity} = A + B\exp(-k_{obs}\, t) \quad (3)$$

The values of k_{obs} so obtained in no case showed saturation-dependence, so we were able to obtain only second-order rate constants for inactivation. A typical plot of k_{obs} versus [I] is given in Figure 6, and k_i/K_i values are given in Table 1.

For a number of enzymes and enzyme species, slow inactivation was observed only at one high inactivator concentration (Table 2); the inactivator is not readily accessible and extensive measurements at high concentrations were not possible. However, since no sign of the inactivation rate constant reaching a plateau value was observed for any *ebg* inactivation, and K_m values for substrates are generally much higher for *ebg*^{o} than for evolvants, it is unlikely that *ebg*^{o} is saturated at 3.7 mM inactivator. An approximate second-order rate constant for wild-type enzyme can be estimated.

The data in Table 2 indicate that the PCR-induced amino acid change has no effect on inactivation rate, despite these changes in some cases reducing k_{cat} values by nearly an order of magnitude. The inactivation rates in the absence of the small subunit in the two cases examined (*ebg*^{o} and *ebg*^{a}) show modest decreases over the inactivation rates of the corresponding intact enzyme. This is expected in the light of the evidence that we presented [5] that indicated that the main function of the small subunit was to alter the active-site conformation such that application of electrophilic catalysis was more effective. (Given the extensive homology between *lacZ* and *ebg* enzymes, it is reasonable to assume that a single active site of the *ebg* enzyme, like that of the *lacZ* enzyme [1], is composed of residues from two subunits. Mechanisms for subtly altering the disposition of catalytic groups in such an active site by binding of a different protein can readily be envisaged.) However, 2,4-dinitrophenolate does not require any electrophilic or acidic catalysis to promote its departure, and so the effects of any electrophilic optimization are minimal.

The main finding of this work is that the evolutionary changes in the kinetics of this paracatalytic inactivation are bigger than those in transformation of substrate. If the second-order rate constant for inactivation of *ebg*^{o} can be identified with the quotient of the measured first-order constant and the inactivator concentration, then the data in Tables 1 and 2 indicate that evolutionary changes alter the second-order rate constant for inactivation over a range of 3.3 orders of magnitude, from 0.026 $M^{-1}\cdot s^{-1}$ for *ebg*^{o} to 57 $M^{-1}\cdot s^{-1}$ for *ebg*^{abcde}. Changes in k_{cat}/K_m ($M^{-1}\cdot s^{-1}$ at 37 °C) for lactose hydrolysis (9 for *ebg*^{o}, 360 for *ebg*^{a}, 36 for *ebg*^{b}, 4100 for *ebg*^{ab}, 4100 for *ebg*^{abcd} and 1900 for *ebg*^{abcde} [7]) range over 2.7 orders of magnitude. The bigger changes for the inactivator than for the substrate are not a consequence of an 'unnatural' leaving group in the inactivator: k_{cat}/K_m values for *o*- and *p*-nitrophenyl galactosides for these evolvants vary over a range of only 1.3 orders of magnitude [7].

The changes in k_{cat}/K_m for lactose and nitrophenyl galactosides are in the same sense as changes in k_i/K_i: the more evolved the enzyme, the bigger the second-order rate constant. However, changes in the first-order rate constant for hydrolysis of the galactosyl-enzyme are in the opposite sense, changes over a range of 1.7 orders of magnitude being seen as the hydrolysis of the glycosyl-enzyme gets steadily slower as the enzyme evolves.

The observation of incomplete inactivation and turnover for the *ebg* enzymes in general is entirely in accord with our previous conclusion that the sugar ring of the substrate carried only one-half to two-thirds of the positive charge at the first chemical transition state for its transformation by *ebg* enzymes than it did at the comparable transition state for transformation by *lacZ* enzymes [14]. The lower the degree of charge development at the sugar, the more readily the fluoroglycosyl-enzyme will turn over, because of the proportionately lower effect of the 2-fluoro-group. We detected a slight increase in transition-state charge development on going from *ebg*^{ab} to *ebg*^{abcde} [14], and the absence of detectable turnover with *ebg*^{abcde}, unlike *ebg*^{ab} is in accord with this.

It is, though, not easy to rationalize other changes within the *ebg* series. Transition-state positive-charge build up is greatest for *ebg*^{o}, and decreases by about a third on the first round of evolution. Thereafter it remains approximately constant until it increases slightly as a consequence of the *ebg*^{ab} to *ebg*^{abcde} change. It is now clear that the effect of the replacement of 2-OH groups by fluorine is not entirely inductive: paracatalytic inactivators of α-glycosidases by the Withers principle requires two fluorines at position 2 (and hence a picrate leaving group) [16,18]. Hydrogen-bonding interactions between groups on the protein and the 2-OH group, which strengthen at the transition state, may be important in promoting catalysis against natural substrates, and

replacement of 2-OH by 2-F may result in new patterns of hydrogen-bonding which are subtly different from enzyme to enzyme and which have different kinetic effects; in the case of the *lacZ* β-galactosidase, increased hydrogen-bonding by the 2-OH group at the transition state is estimated to be responsible for around 34 kJ/mol of the overall rate acceleration [19].

The changes in the second-order rate constant for inactivation do not parallel the second-order rate constant for transformation of the substrate on which the selection was made, and changes in this parameter for 'unnatural' substrates such as nitrophenyl galactosides are modest. It seems that, in addition to the effect of change of aglycone charge, the inactivation is particularly sensitive to other evolutionary changes, which affect transformation of substrate less.

Many antibiotics (most notably β-lactams) appear to act as paracatalytic enzyme inactivators. Changes in the target protein (rather than the emergence of transport-based defences) in response to selection pressure by therapeutic agents are well-recognized if not particularly common events. This work delineates an evolutionary route for producing bigger effects on paracatalytic inactivation than transformation of substrate. As it happens, the changes have occurred in the sense of increased sensitivity to a paracatalytic reagent as a consequence of selection for increased catalytic competence. However, since the evolutionary possibilities in the *ebg* system are dictated by the structure of the protein rather than the replicative machinery of *E. coli* [8], the reverse of this, evolution towards insensitivity to a paracatalytic reagent with only modest changes in catalytic competence, could clearly happen.

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REFERENCES

1 Jacobson, R. H., Zhang, X.-J., DuBose, R. F. and Matthews, B. W. (1994) Nature (London) **369**, 761–766
2 Hall, B. G. and Hartl, D. L. (1975) Genetics **81**, 427–435
3 Elliott, A. C., K, S., Sinnott, M. L., Smith, P. J., Bommuswamy, J., Guo, Z., Hall, B. G. and Zhang, Y. (1992) Biochem. J. **282**, 155–164
4 Hall, B. G., Betts, P. W. and Wootton, J. C. (1989) Genetics **123**, 635–648
5 Calugaru, S. V., Hall, B. G. and Sinnott, M. L. (1995) Biochem. J. **312**, 281–286
6 Hall, B. G. (1976) J. Mol. Biol. **107**, 71–84
7 Hall, B. G. (1981) Biochemistry **20**, 4042–4049
8 Hall, B. G. (1995) Mol. Biol. Evol. **12**, 514–517
9 Gebler, J. C., Aebershold, R. and Withers, S. G. (1992) J. Biol. Chem. **267**, 11126–11130
10 Sinnott, M. L., Withers, S. G. and Viratelle, O. M. (1978) Biochem. J. **175**, 539–546
11 Sinnott, M. L. and Selwood, T. (1990) Biochem. J. **268**, 317–323
12 Burton, J. and Sinnott, M. L. (1983) J. Chem. Soc. Perkin Trans. **2**, 359–364
13 Li, B. F. L., Holdup, D., Morton, C. A. J. and Sinnott, M. L. (1989) Biochem. J. **260**, 109–114
14 K, S., Konstantinidis, A. K., Sinnott, M. L. and Hall, B. G. (1993) Biochem. J. **291**, 15–17
15 Withers, S. G. and Aebershold, R. (1995) Protein Sci. **4**, 361–372
16 McCarter, J. D., Adam, M. J., Braun, C., Namchuk, M., Tull, D. and Withers, S. G. (1993) Carbohydr. Res. **249**, 77–90
17 Krishnan, S., Hall, B. G. and Sinnott, M. L. (1995) Biochem. J. **312**, 971–977
18 Braun, C., Brayer, G. D. and Withers, S. G. (1995) J. Biol. Chem. **270**, 26778–26781
19 McCarter, J. D., Adam, M. J. and Withers, S. G. (1992) Biochem. J. **286**, 721–727