Catalytic consequences of experimental evolution: catalysis by a ‘third-generation’ evolvant of the second β -galactosidase of *Escherichia coli*, ebg^{abcde} , and by ebg^{abcd} , a ‘second-generation’ evolvant containing two supposedly ‘kinetically silent’ mutations

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The kinetics of hydrolysis of a series of synthetic substrates by two experimentally evolved forms (‘evolvants’), ebg^{abcd} and ebg^{abcde} , of the second β -galactosidase of *Escherichia coli* have been measured. The ebg^{abcd} enzyme differs from the wild-type (ebg^o) enzyme by Asp⁹²→Asn (*a*) and Trp⁹⁷⁷→Cys (*b*) changes in the large subunit, as well as two changes hitherto considered to have no kinetic effect, Ser⁹⁷⁹→Gly in the large subunit (*c*) and Glu¹²²→Gly in the small subunit (*d*). The enzyme ebg^{abcde} contains in addition a Glu⁹³→Lys change in the large subunit (*e*). Comparison of ebg^{abcd} with ebg^{ab} [Elliott, K., Sinnott, Smith, Bommuswamy, Guo, Hall and Zhang (1992) *Biochem. J.* **282**, 155–164] indicates that the *c* and *d* changes in fact accelerate the hydrolysis of the glycosyl-enzyme intermediate by a factor of 2.5, and also decrease the charge on the aglycone oxygen atom at the first transition state; the charge on the glycone, however, is unaltered [see K., Konstantinidis, Sinnott and Hall (1993) *Biochem. J.* **291**, 15–17]. The *e* mutation causes a fall in the degalactosylation rate of about a factor of 3, and its occurrence only together with *c* and *d* mutations [Hall, Betts and Wootton (1989) *Genetics* **123**, 635–648] suggests that degalactosylation of

a hypothetical ebg^{abe} enzyme would be so slow that the enzyme would have no biological advantage over the ancestral ebg^{ab} . The transfer products from galactosyl- ebg^{abcd} and galactosyl- ebg^{abcde} to high concentrations to glucose have been measured; the predominant product is allolactose, but significant quantities of lactose are also formed; however, at apparent kinetic saturation of the galactosyl-enzyme, hydrolysis rather than transfer is the preponderant pathway. A knowledge of the rates of enzyme-catalysed exchange of ¹⁸O from [1-¹⁸O]galactose to water permits the construction of the free-energy profiles for hydrolysis of lactose by ebg^{abcd} and ebg^{abcde} . As with the other evolvants, changes in the profile away from the rate-determining transition state are essentially random, and there is no correlation between the changes in the free energies of intermediates and of their flanking transition states. We consider the aggregate of our kinetic data on the ebg system to be telling experimental support for the theoretical objections of Pettersson [Pettersson (1992) *Eur. J. Biochem.* **206**, 289–295 and previous papers] to the Alberly–Knowles theory of the evolution of enzyme kinetic activity.

INTRODUCTION

In addition to the well-known β -galactosidase of *Escherichia coli*, for which the X-ray crystal structure is at last available [1], the organism produces another β -galactosidase, ebg , which is 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 homology 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 Mg²⁺ [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 galactosylarabinose result in Class-IV mutations. A third round of selection of Class-IV-containing strains on lactobionate results in Class-V mutations [7].

These classifications were made on the basis of similarities of kinetic properties of isolated enzymes towards various β -galactosides [7]. The ebg system is one in which Cairnsian evolution takes place [8,9]: when the ebg operon is put under intense selection pressure for sensitivity to and efficient hydrolysis of galactoside sugars, multiple mutations take place with a frequency far exceeding the ‘resting’ mutation frequency. Thus it has been known for some time that mutations in regulatory and structural genes occur together in this system [10]. It was later established that ebg enzymes within the same class could contain different numbers of amino acid changes [4], with some changes being, to a first approximation, kinetically silent. The defining amino acid change for a Class-I enzyme is Asp⁹²→Asn in the large subunit; the resulting Asn⁹² is homologous with the active-site Asn¹⁰² 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 Trp⁹⁷⁷→Cys, also in the large subunit; Trp⁹⁷⁷ is homologous

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with the active-site Trp⁹⁹ of the *lacZ* enzyme, so in the case of Class-II enzymes 12 of the 15 active-site residues are conserved.

The Asp⁹²→Asn and Trp⁹⁷→Cys changes are denoted by the superscripts *a* and *b* respectively (the wild-type enzyme being *ebg*⁰). 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 Ser⁹⁷→Gly in the large subunit and Glu¹²²→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 Asp⁹²→Asn and Trp⁹⁷→Cys changes in the large subunit, is Glu⁹³→Lys, also in the large subunit (indicated by superscript *e*).

The chemical mechanism of both *ebg* and *lacZ* enzymes involves a double-displacement reaction in which a nucleophilic glutamate residue (537 in *lacZ* [11], 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 Mg²⁺ rather than acid catalysis by a proton donor to assist the departure of the leaving group, where this is possible and necessary [12–14]. 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 [3,14,15]. The (more efficient) *lacZ* enzyme is more complex kinetically; the species formed immediately after aglycone–glycone cleavage, namely the galactosyl enzyme with the leaving group still complexed to the enzyme as its magnesium salt, appears to accumulate with phenolic leaving groups [13,16].

This paper in the first instance addresses the question of whether the changes Ser⁹⁷→Gly in the large subunit and Glu¹²²→Gly in the small subunit have any kinetic effect at all or whether they are completely neutral mutations. The study of *ebg*^{abcd}, which is similar to that previously performed for *ebg*^{ab} [3,17], reveals that the *c* and *d* changes are not completely neutral.

In the second instance catalysis by a ‘third-generation’ evolvant is examined, largely for the purpose of further examining the changes in the free-energy profile of the catalysed reaction, as has already been done with *ebg*⁰, *ebg*^a, *ebg*^b [18], and *ebg*^{ab} [3], to test the conformity or otherwise of the observed changes to the theory of Albery and Knowles. In its original formulation, this theory proposed that evolutionary changes in the free-energy profile of the catalysed reaction occurred most readily by uniform changes in the energies of all bound states, next most readily by the adjustment of the relative energies of bound intermediates, and with the greatest difficulty by selective stabilization of a single transition state [19]. The original formulation of the theory (based on experimental work on modern triose phosphate isomerase) assumed that the enzyme maintained substrate and product close to equilibrium, that linear free-energy relationships existed between the energies of bound intermediates and their flanking transition states, and, most crucially, that the physiological concentrations of the substrates and products were invariant with respect to evolution.

Our demonstration [20] that, in the first generation of *ebg* evolvants, *ebg*^a and *ebg*^b, the first-order rate constant for the hydrolysis of the galactosyl-enzyme intermediate decreased by an order of magnitude compared with that of the wild-type enzyme *ebg*⁰ was clearly at variance with the Albery–Knowles theory in its original formulation; the easiest change was not an increase or decrease in the energies of all internal states, which would have left first-order rate constants unaltered. Chin [21] pointed out that if the enzyme did not catalyse a reaction

maintained at equilibrium, optimal catalysis was not observed with an equilibrium constant between bound species of unity, but rather a free-energy profile in which the energies of bound intermediates described a series of regular decreases (the ‘descending staircase’ model). The closeness of the physiological situation to chemical equilibrium was taken into account by the revised theory advanced by Burbaum et al. in 1989 [22]. This form of the theory now accommodated our earlier observations that the second, degalactosylation, step of the *ebg* enzymes got slower as biological selection improved k_{cat}/K_m for lactose hydrolysis. The predicted deceleration arose from the assumption of Brønsted-type relationships with coefficients of ~ 0.5 between bound intermediates and the transition states flanking them. Such a relationship would predict a lowering of the free energy of the galactosyl-enzyme intermediate by 0.5 kJ for a 1 kJ fall in the energy of the first chemical transition state, and a fall in the energy of the second chemical transition state of 0.25 kJ as a consequence of this. The barrier corresponding to hydrolysis of the galactosyl-enzyme intermediate would therefore be raised by 0.25 kJ. However, determination of the free-energy profile for *ebg*⁰, *ebg*^a, *ebg*^b [18] and *ebg*^{ab} [3] indicated that the changes were not so simply described.

MATERIALS AND METHODS

Materials

The enzymes *ebg*^{abcd} and *ebg*^{abcde} were purified to electrophoretic homogeneity from *E. coli* strains A27 and A272 respectively. Bacteria were grown up and enzymes isolated as described previously [3]. Substrates were those described previously [3,17] or were resynthesized by previously described routes.

Kinetic methods

All kinetic parameters refer to our standard buffer of 0.125 M potassium phosphate buffer containing 5 mM MgCl₂ and 25 mM 2,2′-dipyridyl adjusted to pH 7.5 with KOH. The enzyme used for each kinetic experiment was standardized with 5 mM 4-nitrophenyl galactoside in this buffer, using a value for $\Delta\epsilon$ of 14430 M⁻¹·cm⁻¹ at 400 nm at this pH.

Michaelis–Menten parameters for aryl galactoside substrates at 25 or 37 °C were estimated from initial rates at ten substrate concentrations between $K_m/3$ and $3K_m$ measured in the thermostatically controlled spectrometer Perkin–Elmer Lambda 6 [3]. Initial rates were estimated from the manufacturer’s PECS software, using absorption coefficient differences (M⁻¹·cm⁻¹) and wavelengths (nm) for the various leaving groups as follows: 3,4-dinitrophenol 14490 at 390; 3,5-dinitrophenol 1864 at 390; 4-nitrophenol 14430 at 400; 2-nitrophenol 3311 at 400; 4-cyanophenol 5222 at 281; 3-nitrophenol 296 at 400; 4-bromophenol 749 at 288; phenol 1170 at 277. Michaelis–Menten parameters for fluoride substrates were estimated from initial rates of liberation of fluoride ion, measured electrochemically [17,23]. Initial velocities were fitted directly to rectangular hyperbolae using Kaleidagraph (Synergy Software, Reading, PA, U.S.A.). The same program was used to calculate the lines shown in Figures 1–5.

k_{cat} values were estimated assuming that freshly isolated enzyme was 100% active and had an M_r per active site of 1.40×10^5 . Pre-steady-state measurements with other forms of the enzyme had indicated that this assumption was broadly correct [3,5]. Active-site titrations using the β -galactosidase inactivator 2′,4′-dinitrophenyl 2-deoxy-2-fluoro- β -D-galactopyranoside [11] produced a burst of 2,4-dinitrophenol of 83% of the theoretical size in the case of *ebg*^{abcd} and 94% in the case

of *ebg^{abcde}* (K. Srinivasan and M. L. Sinnott, unpublished work; [24]).

The enzyme-catalysed wash-out of the label from D-[1-¹⁸O]galactose was monitored by natural-abundance ¹³C-NMR spectroscopy of the anomeric carbon atom of both α and β anomers of the sugar. Measurements were made on solutions containing ~60% labelled galactose (approx. 100 mg), ³H₂O buffer (as lock) (0.1 ml) and enzyme solution to a total volume of 1 ml, as described previously [3]. The K_m value of galactose was estimated as a K_i by measurement of the K_m for 4-nitrophenyl galactoside as a function of five to six concentrations of fully mutarotated galactose.

Product analysis

In the presence of methanol

Portions (1.0 ml) of a solution of 4-nitrophenyl galactoside in standard buffer were placed in Eppendorf tubes and to each was added methanol, to give nine final concentrations from 0 to 4% (v/v). An aliquot of enzyme was added to each tube. After 24 h at 25 °C, the reaction was stopped by heating for 15 min at 60 °C. The precipitated enzyme was then filtered through 0.2 μ m syringe filters and the samples were analysed by HPLC on a Spectra Physics model SP8800 with an 8430 refractive-index detector. An Alltech 10 μ m-particle-size carbohydrate column (300 mm $\times$ 4.1 mm) was used, with 75% (v/v) acetonitrile (spectrophotometric grade) in water as isocratic eluent at a flow rate of 1.5 ml/min. Methyl galactoside was eluted with a retention time of 4.96 min and galactose with a retention time of 9.46 min. The relative molar response factor of the sugars was determined using two known concentrations, and was found to be 0.44:1, galactose/methyl galactoside. Linear least-squares treatment of plots of [GalOMe]/[Gal] against [methanol] gave the selectivity of the galactosyl-enzyme.

In the presence of glucose

Preliminary analyses to determine the proportion of total disaccharide that was lactose when glucose replaced methanol in experiments exactly analogous to the above were performed by HPLC under the same conditions. The disaccharides had retention times greater than 10 min, but the resolution was considered to be inadequate, especially as extrapolation to zero conversion was necessary because the saccharides were better substrates than methyl galactoside.

Gas-chromatographic analysis of total disaccharides was carried out on a Shimadzu model 17A capillary gas chromatograph fitted with a 30 m J & W Scientific fused DB-1 column, flame detection and CR601 Chromatopac integrator.

The samples of 4-nitrophenyl galactoside hydrolysed in the presence of glucose were analysed by the procedure of Huber and Hurlburt [25]. At various times after the addition of enzyme to a reaction mixture, 100 μ l aliquots were removed and placed in Eppendorf tubes with small holes drilled in the caps. The aliquots were immediately frozen in a solid CO₂/acetone bath. The contents of the tube were lyophilized overnight. Then dimethyl-formamide (450 μ l), hexamethyldisilazane (300 ml) and trimethylchlorosilane (150 μ l) were added to the lyophilized contents of the tubes and the tubes were capped with lids with no holes. The contents were thoroughly mixed and incubated at room temperature for 2 days until two layers had formed [26]. Then 200 μ l of the top layer (which contained the silylated sugars) was added to 1 ml of toluene and allowed to stand for 2 h. This was centrifuged in an Eppendorf Microfuge at full speed for 30 min, and 3 μ l aliquots were analysed by GLC. The

column temperature was held at 95 °C for 1 min and then increased at 50 °C/min to 250 °C and held for 37 min. The injector and detector ports were set at 280 °C and the helium carrier gas flow rate was 1 ml/min. The molar response factors for all disaccharide isomers were assumed to be the same. The fraction of galactosyl-enzyme that was channelled to disaccharide was measured by the use of an inositol internal standard; the relative response factors of inositol and lactose were 1:1.80.

RESULTS AND DISCUSSION

Hydrolysis of galactosyl-*ebg^{abcd}* and galactosyl-*ebg^{abcde}*

In Tables 1 and 2 are set out Michaelis–Menten parameters for hydrolysis of a series of aryl galactosides by *ebg^{abcd}* and *ebg^{abcde}*. It is seen that, at least for the more acidic leaving groups, k_{cat} is essentially invariant with respect to leaving group acidity. This

Table 1 Steady-state kinetic parameters for the hydrolysis of galactosyl derivatives by Mg²⁺-saturated *ebg^{ab}* and *ebg^{abcd}* at pH 7.5 and 25 °C

Data for *ebg^{ab}* are taken from [3], except for substrates with the fluoride leaving group, which are taken from [11].

Leaving group	<i>ebg^{ab}</i>		<i>ebg^{abcd}</i>	
	K_m (mM)	k_{cat} (s ⁻¹)	K_m (mM)	k_{cat} (s ⁻¹)
3,4-Dinitrophenol	0.047	3.3	0.052	9.04
3,5-Dinitrophenol	0.082	3.9	0.046	10.5
4-Nitrophenol	0.10	3.1	0.11	10.1
2-Nitrophenol	0.056	4.0	0.087	11.3
4-Cyanophenol	0.21	1.7	0.10	11.1
3-Nitrophenol	0.28	4.4	0.12	10.8
4-Bromophenol	0.21	2.7	0.16	9.34
Phenol	0.64	1.2	0.35	7.05
Fluoride	0.21	3.3	0.64	9.0
(Fluoride) ₂ *	0.11	0.13	0.085	0.114
3,4-Dinitrophenyl at 37 °C	0.049	10.2	0.095	21.9
Water at 37 °C	16†	15	16†	13

* Substrate is 1-fluorogalactosyl fluoride.

† Measured as a K_i against *p*-nitrophenyl galactoside.

Table 2 Steady-state kinetic parameters for the hydrolysis of galactosyl derivatives by Mg²⁺-saturated *ebg^{abcde}* at pH 7.5 and 25 °C

Leaving group	K_m (mM)	k_{cat} (s ⁻¹)
3,4-Dinitrophenol	0.028	3.9
3,5-Dinitrophenol	0.023	4.5
4-Nitrophenol	0.055	4.1
2-Nitrophenol	0.021	4.3
4-Cyanophenol	0.17	3.6
3-Nitrophenol	0.20	4.0
4-Bromophenol	0.082	4.4
Phenol	0.33	2.3
Fluoride [17]	0.18	2.7
(Fluoride) ₂ * [17]	0.34	0.26
3,4-Dinitrophenol†	0.033	8.6
Water†	23.5‡	13

* Substrate is 1-fluorogalactosyl fluoride.

† Measured at 37 °C.

‡ Measured as a K_i against *p*-nitrophenyl galactoside.

Figure 1 k_{cat} (a) and K_m (b) for 3,4-dinitrophenyl β -D-galactopyranoside as a function of added organic solvent for ebg^{abcd} enzyme

○, In the presence of methanol; □, in the presence of dioxan.

Figure 2 k_{cat} (a) and K_m (b) for 3,4-dinitrophenyl β -D-galactopyranoside as a function of added organic solvent for ebg^{abcde} enzyme

○, In the presence of methanol; □, in the presence of dioxan.

behaviour is commonly found for a two-step hydrolase proceeding through a substituted-enzyme intermediate, and is a preliminary indication that k_{cat} represents hydrolysis of that substituted-enzyme intermediate. It was observed for ebg^a , ebg^b [20] and ebg^{ab} [3], as well as the large-subunit-only species derived from ebg^o and ebg^a [5]; nucleophilic competition with methanol, as well as observation of a pre-steady-state 'burst' of leaving group in accessible cases, confirmed the expectation that k_{cat} did indeed represent degalactosylation.

Figures 1 and 2 show Michaelis-Menten parameters for hydrolysis of 3,4-dinitrophenyl galactoside by ebg^{abcd} and ebg^{abcde} respectively in the presence of methanol and (as a control) the non-nucleophilic solvent dioxan. In the presence of methanol, galactosyl- ebg enzymes preferentially react to yield methyl β -galactoside, so that the kinetic scheme becomes:

so that now

$$k_{cat} = k_{+2}(k_{+3} + k_{+4}[\text{MeOH}]) / (k_{+2} + k_{+3} + k_{+4}[\text{MeOH}])$$

and

$$K_m = (k_{+3} + k_{+4}[\text{MeOH}]) / (k_{-1} + k_{+2})$$

A linear increase in k_{cat} or K_m in the presence of methanol, but not dioxan, as observed, therefore indicates that hydrolysis of the

galactosyl-enzyme is rate-determining. The k_{+4}/k_{+3} values obtained kinetically for ebg^{abcd} (1.3 M⁻¹ from Figure 1a and 1.6 M⁻¹ from Fig 1b) are in fair agreement with that estimated by product analysis (1.1 M⁻¹); likewise the kinetic values for ebg^{abcde} (1.6 M⁻¹ and 1.8 M⁻¹) are in good agreement with the value estimated from product analysis (1.6 M⁻¹).

The preference of the galactosyl-enzyme for methanol over water (~ 85 for ebg^{abcde} and ~ 73 for ebg^{abcd} on a molar basis) is substantially unchanged from that of other evolants (~ 57 for ebg^{ab} [3], ~ 63 for ebg^b and 102 for ebg^a [20]) but very different from wild-type (~ 7 [14]).

Comparison of the absolute rate of hydrolysis of galactosyl- ebg^{ab} and galactosyl- ebg^{abcd} (Table 1) reveals that the latter process is around 2.5 times faster than the former. This is despite ebg^{abcd} and ebg^{ab} both being Class-IV enzymes [7]. Growth rates of $lacZ$ - ebg -containing *E. coli* strains on lactose as sole carbon source, i.e. biological fitness under the conditions of the experiments, correlate with k_{cat}/K_m for lactose hydrolysis. This parameter does not contain k_{+3} [6]. It is therefore reasonable that modified enzymes that confer the same selective advantage on the producing organisms should differ in the value of parameter that does not affect viability. Nonetheless, the existence of pronounced kinetic differences in non-critical processes between Cairnsian evolvant enzymes of the same biological fitness, but containing different numbers of amino acid changes, is suggestive. Cairnsian evolution of this type may be a means whereby systems can avoid kinetic bottlenecks in evolutionary space.

Examination of the third-generation evolvant, ebg^{abcde} , indicates that this may well be happening. The value of k_{+3} for

Figure 3 k_{cat} for 3,4-dinitrophenyl β -galactopyranoside hydrolysis by ebg^{ab} as a function of added glucose.

Figure 4 k_{cat} for 3,4-dinitrophenyl β -galactopyranoside hydrolysis by ebg^{abcd} as a function of added glucose

the first generation of *ebg* evolvants represented somewhat more than an order of magnitude fall from the value for wild-type [5,18]. The value of k_{+3} for ebg^{ab} represents a 5-fold further fall from ebg^a and a 3-fold fall from ebg^b , to the point where for lactose $k_{+2} = 2.4 k_{+3}$. A further fall in k_{+3} , even without any corresponding increase in k_{+2} , would have made degalactosylation rate-determining for lactose hydrolysis at the estimated intracellular concentration (and indeed far below it). It is therefore likely that the *e* (Glu⁹³→Lys) change by itself would not have affected biological fitness. The *c* and *d* changes, which speed up the degalactosylation rate, although biologically neutral a generation previously, are now required.

Attempts to locate the galactosyl-enzyme-glucose intermediate on the free-energy profile

In constructing the free-energy profile for the hydrolysis of lactose, the enzyme-lactose complex, the galactosyl-enzyme and the galactose-enzyme complexes are not the only forms of the enzyme that may be involved. It is well known that the *lacZ* enzyme at least possesses an aglycone-binding site [25]. Therefore it may be possible to locate the non-covalent complex of glucose with the galactosyl-enzyme. We attempted this kinetically by replacing methanol in the nucleophilic competition experiments with glucose, expecting to see a hyperbolic dependence of k_{cat} for 3,4-dinitrophenyl galactoside on glucose concentration. Although up to 1.0 M glucose had no effect on k_{cat} for galactosyl fluoride hydrolysed by ebg^o or 3,4-dinitrophenyl galactoside hydrolysed by ebg^b , and had only an uninterpretable 17% accelerating effect on the same substrate hydrolysed by ebg^a (results not shown), the accelerating effect on hydrolysis of this substrate by ebg^{ab} , ebg^{abcd} and ebg^{abcde} was quite marked (Figures 3–5). These data were fitted to

$$k_{\text{cat}} = k_0 + (k - k_0)[\text{glucose}] / ([\text{glucose}] + K)$$

where k_0 is the k_{cat} value at zero glucose concentration, and k the apparent rate constant for conversion of the galactosyl-enzyme-glucose complex into disaccharides. Values of K (the apparent binding constant of glucose to the galactosyl-enzyme) were 0.79 M for ebg^{ab} , 0.2 M for ebg^{abcd} and 0.46 M for ebg^{abcde} .

It is well known in the case of the *lacZ* enzyme that the β -D-galactopyranosyl residue is transferred from the galactosyl-enzyme to all possible hydroxy groups of glucose [25]. Therefore the fraction of initially formed disaccharide that was actually lactose was measured. The products of the enzyme reaction were analysed at various reaction times by the method of Huber and

Figure 5 k_{cat} for 3,4-dinitrophenyl β -galactopyranoside hydrolysis by ebg^{abcde} as a function of added glucose

Hurlburt [25] using capillary gas chromatography (for solubility reasons the galactosyl-enzyme had to be generated from the 4-nitrophenyl glycoside rather than the 3,4-dinitrophenyl glycoside for these experiments). The fraction of the total amount of disaccharide formed that was lactose was plotted against time and in each case the line was extrapolated to zero time. This fraction of total disaccharide that was lactose was 0.18 for ebg^{ab} , 0.30 for ebg^{abcd} and 0.45 for ebg^{abcde} . Chromatographic peaks corresponding to α - and β -lactose were identified by co-injection with a genuine sample. Although a range of other products was formed, the bulk of the disaccharides appeared to be allolactose (the 1→6 isomer). However, when by use of inositol as an internal standard we were able to determine the fraction of the total substrate (47.6 mM 4-nitrophenyl galactoside) being converted into disaccharides at 0.8 M glucose, it was only 32% for ebg^{abcd} and 29% for ebg^{abcde} . Clearly therefore the kinetic saturation observed in Figures 3–5 cannot be associated with complete conversion of the galactosyl-enzyme into disaccharides. At the very high glucose concentrations used, media effects [1 M glucose corresponds to an 18% (w/v) solution] or competition of glucose for the *galacto* site could have brought about the kinetic effects set out in Figures 3–5. It is clear, however, that the curves of Figures 3–5 do not represent real saturation of the aglycone-binding site in the galactosyl-enzyme with glucose.

Figure 6 Free-energy profile for the hydrolysis of lactose by *ebg* enzymes (drawn to scale for *ebg*^o)

Transition-state structure changes

Two probes of transition-state structure have been applied: the measurement of the β_{lg} (V/K) value for aryl galactosides and the ratio of k_{cat}/K_m values of β -D-galactopyranosyl fluoride and 1-fluorogalactopyranosyl fluoride.

The β_{lg} value has to be calculated on k_{cat}/K_m , since degalactosylation is largely rate-determining for aryl galactosides. The parameter therefore contains polar contributions to binding as well as measures of the degree of charge development at the leaving group oxygen atom. In the absence of changes in these polar interactions, the more negative the β_{lg} value, the more charge there is on the leaving oxygen atom at the first chemical transition state. There is evidence that this decreases as the enzymes become more highly evolved. Values of -0.46 ± 0.08 for *ebg*^o [5], -0.37 ± 0.04 for *ebg*^a [15], -0.28 ± 0.04 for *ebg*^b [15] and -0.30 ± 0.04 for *ebg*^{ab} [3] can be compared with the values derived from the data in Tables 1 and 2 (-0.19 ± 0.05 for *ebg*^{abcd} and -0.26 ± 0.09 for *ebg*^{abcde}). Although the comparatively high errors in these values make the changes in β_{lg} (V/K) as a consequence of any one amino acid change somewhat ambiguous, the trend over three generations towards less negative values is unmistakable. Two alterations in the details of the catalytic machinery of the enzyme could contribute to the change: a more effective application of electrophilic catalysis by the active-site Mg^{2+} and less C–O bond cleavage at the transition state.

The second probe of transition-state structure we applied was the ratio of the rates of hydrolysis of β -D-galactopyranosyl

fluoride and 1-fluoro-D-galactopyranosyl fluoride. We have advanced arguments elsewhere [23] that, because of the roughly comparable sizes of hydrogen and fluorine, and the substantial motions executed by the anomeric hydrogen during nucleophilic displacements at the anomeric centres of glycosides, the kinetic effect of the second fluorine is exclusively electronic. The ratio of rates of mono- to di-fluoride, converted into a free-energy difference, is then a measure of the charge development on the glycone at the transition state. Since there is no simple way of knowing whether formation or hydrolysis of the fluorogalactosyl enzyme limits the rate of hydrolysis of 1-fluorogalactosyl fluoride, the comparison has to be made on k_{cat}/K_m , rather than k_{cat} . We have previously shown that the parameter $\Delta\Delta G^\ddagger$ $\{= RT \ln [k_{cat}/K_m]_{F_1} / (k_{cat}/K_m)_{F_2}\}$ where F_1 and F_2 refer to galactosyl fluoride and 1-fluorogalactosyl fluoride respectively} showed marked changes in the course of evolution [17]. Values (kJ/mol^{-1}) of 10.4 for *ebg*^o, 6.8 for *ebg*^a, 7.2 for *ebg*^b, 6.3 for *ebg*^{ab} and 7.3 for *ebg*^{abcde} indicate a decrease in charge development at the first chemical step of *ebg* enzymes for the first two generations and then a slight increase for the third evolutionary change. The movement in the dimension of evolutionary space defined by this parameter for the first two generations was away from the *lacZ* enzyme, for which $\Delta\Delta G^\ddagger = 15.2$. The *c*, *d* and *e* mutations together produce an apparent shift towards the *lacZ* enzyme. From the data in Table 1 can be calculated a value of $\Delta\Delta G^\ddagger = 6.0$ for *ebg*^{abcd}. This is close to the value for *ebg*^{ab}, indicating that the *c* and *d* mutations do not affect charge development on the glycone. A corollary of this is that the increase in $\Delta\Delta G^\ddagger$, seen previously in the third-generation evolvant, *ebg*^{abcde}, is entirely attributable to the *e* (Glu⁹³→Lys) change increasing the charge development on the glycone. The β_{lg} (V/K) values for *ebg*^{abcd} and *ebg*^{ab} are significantly different, although, in the sense of the *c* and *d* mutations, reducing the charge on the aglycone somewhat. It thus appears that even changes that are to a first approximation ‘kinetically silent’ can produce detectable changes in transition-state structure.

Free-energy profiles for *ebg*^{abcd} and *ebg*^{abcde}

The free-energy profile for a simple double-displacement glycosidase, such as the *ebg* enzymes, is given schematically in Figure 6. We constructed the profile on the assumptions used previously [3,18], namely an assumed diffusional constant of enzyme–ligand combination of $10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ and an estimated intracellular concentration of 53 mM for cells growing on lactose as sole carbon source. The free energies of the intermediates and transition states are given in Table 3.

Table 3 Free energies (kJ/mol) of intermediates and transition states for hydrolysis of lactose during experimental evolution of *ebg* enzymes

All data refer to a standard state of 53 mM concentration. Data for *ebg*^o, *ebg*^a, and *ebg*^b are from [3].

Species (see Figure 6)	<i>ebg</i> ^o	<i>ebg</i> ^a	<i>ebg</i> ^b	<i>ebg</i> ^{ab}	<i>ebg</i> ^{ab} (predicted*)	<i>ebg</i> ^{abcd}	<i>ebg</i> ^{abcde}
E + lactose	0.0	0.0	0.0	0.0	—	0.0	0.0
‡1	42.0	42.0	42.0	42.0	—	42.0	42.0
E · lactose	2.7	−1.5	1.2	−10.2	−3.0	−10.0	−10.7
‡2	77.2	67.7	71.8	61.8	62.3	61.7	63.7
E · αGal	−19.4	−21.3	−23.7	−24.4	−29.3	−22.0	−23.8
‡3	40.7	45.1	42.6	45.4	47.0	45.7	46.3
E · GalOH	−22.2	−25.4	> −23.7	> −23.6	—	−23.4	> −22.8
‡4	21.6	21.6	21.6	21.6	—	21.6	21.6
E + GalOH	−20.4	−20.4	−20.4	−20.4	—	−20.4	−20.4

* The values were predicted by adding the differences (*ebg*^a − *ebg*^o) and (*ebg*^b − *ebg*^o) to the *ebg*^o value.

Because of the similarity of k_{cat} for galactose and the degalactosylation rate, it is not possible to locate the E·galactose complex for *ebg^{abcde}*. However, it is clear that the higher degalactosylation rate of *ebg^{abcd}* than *ebg^{ab}* arises from the higher free energy of the galactosyl-enzyme in the evolvant containing four mutations, rather than a lowering of ‡3. Since Class IV enzymes can be obtained from either *ebg^a* or *ebg^b*, and *ebg^{abcd}* or *ebg^{ab}* can result, it is instructive to consider the direction of the consequent changes in the free energy of the galactosyl-enzyme. The *ebg^a→ebg^{ab}*, *ebg^b→ebg^{ab}* and *ebg^a→ebg^{abcd}* changes all result in a decrease in the free energy of the galactosyl-enzyme, but the *ebg^b→ebg^{abcd}* change results in an increase, i.e. two evolutionary changes that have the same effect on function have, on closer examination, opposite effects on the free energy of a non-critical species.

We consider this is to be the most telling single piece of evidence yet uncovered against the very idea that changes in the free-energy profiles of the catalysed reaction can be analysed in terms of tidy changes in the biologically irrelevant thermodynamics of non-crucial parts of the free-energy profile. In 1983 we wrote 'There is no compelling reason why (the) limited repertoire of amino-acid changes should oblige the physical scientist by causing a rate-enhancement attributable to a single clear-cut physico-chemical phenomenon' [18]. It is now known that the repertoire of amino acid changes that will result in a better β -galactosidase is very limited indeed and confined to those changes already observed (i.e. the small number of possibilities is dictated by the limited possibilities presented by the existing protein structure, not by the replicative machinery of *E. coli*) [27]. The examination of the thermodynamics of more advanced evolvants, the completion of which is described in this paper, only further supports the correctness of our 1983 assessment. Although *ebg^{abcde}*, having arisen by selection on lactobionate, in fact shows a slight increase in the free energy of ‡2 for hydrolysis of lactose, changes after the loss of the leaving group are still germane. The effects of the *e* amino acid change are compatible with no version of the Albery–Knowles theory; the free energy of the galactosyl-enzyme decreases significantly, but this is associated with a slight increase in the free energy of one of the flanking transition states.

In summary, changes in the free-energy profile of the catalysed reaction, except for that of the rate-determining transition state, are essentially random in the *ebg* system. This provides telling experimental support for the trenchant criticism by Pettersson [28,29] of the basis of the Albery–Knowles treatment. He pointed out that the assumption of Brønsted-type relationships between the free energies of intermediates and of their flanking transitions states was unfounded. In the *ebg* system we find indeed that they do not exist. One of many possible causes is suggested by the treatment of Ellington and Benner [30], who showed that it was an implicit assumption of the Albery–Knowles treatment that the slope and intercept of the proposed linear free-energy relationship remained invariant with respect to evolution. Such constancy implies a constant transition-state structure [29]. We find, however, quite large alterations in transition-state structure

in the *ebg* system [17]. Therefore, even with an infinite range of structural possibilities available to an enzyme, there are no good reasons for expecting the Albery–Knowles treatment to be valid.

The structural possibilities available to the *ebg* enzymes that permit it to become a better β -galactosidase are, however, very limited indeed [27]. At least in this system therefore, and quite possibly generally, it appears that detailed consideration of the full free-energy profiles of the catalysed reaction is an irrelevance in understanding the evolution of enzyme catalytic activity. The pathway of evolution of this, and probably other, enzymes is dictated by protein structure and the energy demands of the growing organism [31], and not reaction thermodynamics.

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