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The catalytic consequences of experimental evolution

Studies on the subunit structure of the second (*ebg*) β-galactosidase of *Escherichia coli*, and on catalysis by *ebg*^{ab}, an experimental evolvant containing two amino acid substitutions

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1. The ratio of *ebgA*-gene product to *ebgC*-gene product in the functional aggregate of *ebg* β-galactosidases was determined to be 1:1 by isolation of the enzyme from bacteria grown on uniformly radiolabelled amino acids and separation of the subunits by gel-permeation chromatography under denaturing conditions. 2. This datum, taken together with a recalculation of the previous ultracentrifuge data [Hall (1976) J. Mol. Biol. **107**, 71–84], analytical gel-permeation chromatography and electron microscopy, strongly suggests an $\alpha_4\beta_4$ quaternary structure for the enzyme. 3. The second chemical step in the enzyme turnover sequence, hydrolysis of the galactosyl-enzyme intermediate, is markedly slower for *ebg*^{ab}, having both Asp-97→Asn and Trp-977→Cys changes in the large subunit, than for *ebg*^{a} (having only the first change) and *ebg*^{b} (having only the second), and is so slow as to be rate-determining even for an *S*-glycoside, β-D-galactopyranosyl thiopicrate, as is shown by nucleophilic competition with methanol. 4. The selectivity of galactosyl-*ebg*^{ab} between water and methanol on a molar basis is 57, similar to the value for galactosyl-*ebg*^{b}. 5. The equilibrium constant for the hydrolysis of lactose at 37 °C is 152 ± 19 M, that for hydrolysis of allolactose is approx. 44 M and that for hydrolysis of lactulose is approx. 40 M. 6. A comparison of the free-energy profiles for the hydrolyses of lactose catalysed by the double mutant with those for the wild-type and the single mutants reveals that free-energy changes from the two mutations are not in general independently additive, but that the changes generally are in the direction predicted by the theory of Burbaum, Raines, Albery & Knowles [(1989) Biochemistry **28**, 9283–9305] for an enzyme catalysing a thermodynamically irreversible reaction. 7. Michaelis–Menten parameters for the hydrolysis of six β-D-galactopyranosylpyridinium ions and ten aryl β-galactosides by *ebg*^{ab} were measured. 8. The derived β_{lg} values are the same as those for *ebg*^{b} (which has only the Trp-977→Cys change) and significantly different from those for *ebg*^{o} (the wild-type enzyme) and *ebg*^{a}. 9. The α- and β-deuterium secondary isotope effects on the hydrolysis of the galactosyl-enzyme of 1.08 and 1.00 are difficult to reconcile with the pyranose ring in this intermediate being in the $^4C_1$ conformation.

INTRODUCTION

Escherichia coli contains two β-galactosidases, encoded by genes in the *lac* and *ebg* operons. The *lac* operon has been the subject of many classic studies: its β-galactosidase is encoded by a single structural gene (*lacZ*; Ullmann *et al.*, 1968), and is produced as a 1021-residue polypeptide (Fowler & Zabin, 1977). The active form of the *lacZ* β-galactosidase is the tetramer (Sund & Weber, 1963; Melchers & Messer, 1973), although higher aggregates are known (Karlsson *et al.*, 1964). The *ebg* β-galactosidase was discovered in 1973 (Campbell *et al.*, 1973). Strains with the *lacZ* gene deleted, but still containing the gene for galactoside permease (*lacY*), were plated on medium containing lactose (or other β-galactosides) as sole carbon source. After a time, certain colonies began to grow. The gene giving the ability to hydrolyse lactose, and hence to grow, was located on the *E. coli* chromosome almost directly opposite the *lac* operon (at 66 min compared with 8 min for the *lac* operon; Hall & Hartl, 1974). It turns out that there must be spontaneous mutations in the genes coding for *ebg* β-galactosidase before growth can be sustained, since the wild-type *ebg* β-galactosidase, *ebg*^{o}, is too catalytically feeble to allow growth on lactose. When *ebg*^{o}-containing strains are placed under the intense selection pressure of having only lactose (or another β-galactoside) as a carbon source, strains producing mutant forms of the enzyme, with enhanced catalytic competence, are selected. The system is under negative control, and spontaneous mutations in the repressor gene (*ebgR*), to increase the sensitivity of the repressor protein to lactose, are also required. Mutations in the repressor gene and in the structural genes coding for the enzyme occur together at a frequency some 10^8 times that of the product of the frequencies with which they can be estimated to occur separately, and it is now clear that the system is one in which directed evolution (see, e.g., Hall, 1990) occurs.

Various genes of the *ebg* operon have been discovered and mapped: *ebgR* (Hall & Hartl, 1975), a structural gene for the β-galactosidase (*ebgA*) and a structural gene *ebgB* of presently unknown function (Hall & Zuzel, 1980). The sequence of the *ebgA* gene shows 50% nucleotide identity with that of the *lacZ* gene (Stokes *et al.*, 1985), and the sequence of the *ebgR* gene shows 44% nucleotide identity with the *lacI* gene, which encodes the *lac* repressor (Stokes & Hall, 1985).

The *ebg* enzymes were originally reported as homohexamers on the basis of sedimentation-equilibrium studies, which gave a molecular mass of 720 kDa, and the electrophoretic migration of the enzyme in polyacrylamide gels under denaturing conditions, which apparently gave one band of molecular mass 120 kDa (Hall, 1976). *ebg* enzymes isolated at Bristol, however, always showed an additional band of molecular mass approx. 15 kDa, even when the enzyme was isolated in the presence of phenyl-

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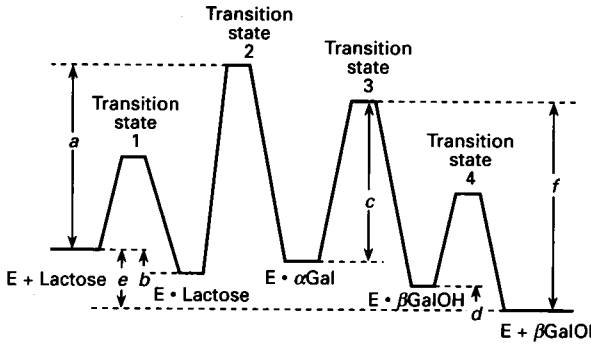

Fig. 1. Free-energy profile for the hydrolysis of lactose by *ebg* enzymes, illustrating how it may become kinetically accessible by the following measurements; (*a*) $k_{cat.}/K_m$ for lactose, (*b*) K_s for lactose, (*c*) k_{+3}, (*d*) K_s for galactose, (*e*) standard free energy of hydrolysis for lactose and (*f*) $k_{cat.}/K_m$ for hydrolysis of [1-^{18}O]galactose

Barriers corresponding to transition states 1 and 4 are for diffusion and for enzyme–substrate combinations that commonly correspond to rate constants of 10^7 $M^{-1} \cdot s^{-1}$.

methanesulphonyl fluoride (L. Hosie & M. L. Sinnott, unpublished work). These observations were confirmed by Hall *et al.* (1989), who subsequently discovered a second structural gene for the *ebg* β-galactosidase, *ebgC*, which coded for a polypeptide of molecular mass 20 kDa. The start of the *ebgC* gene (**ATG/AGG/ATC**..., corresponding to Met-Arg-Ile...) overlaps by four nucleotide residues the terminus of the *ebgA* gene (...**AAG/CAA/TGA**, corresponding to Lys-Gln-End). Expression of the *ebgC* gene, as well as the *ebgA* gene, is necessary for a lactose-positive phenotype in *lacZ*$^-$ mutants. The first part of the present paper sets forth our evidence that the catalytically active *ebg* enzyme is an aggregate of *ebgA*-gene and *ebgC*-gene products in a 1:1 ratio. Recalculation of the previous ultracentrifugation data (Hall, 1976) with a partial specific volume calculated from the sequences of *ebgA* and *ebgC* genes and the 1:1 association of their products in the active enzyme, together with new analytical gel-permeation data, strongly suggest that the active form of *ebg* enzymes is an $\alpha_4\beta_4$ hetero-octamer.

The *ebg* system is of interest as a model for the biology of acquisitive evolution (reviewed by Hall, 1982*a*) and for the chemistry of the evolution of catalytic function (Hall *et al.*, 1983; Li *et al.*, 1989). In these connections the repertoire of evolutionary changes brought about in catalysis by the enzyme as a result of spontaneous mutations appears limited. Selection experiments starting from the wild-type enzyme *ebg*^{o} appear to produce just two types of mutation: selection on lactose produces a Class I enzyme 90% of the time and a Class II enzyme 10% of the time; Class II enzymes are also produced exclusively by selection on lactulose (Hall, 1981). A typical representative of a Class I enzyme is *ebg*^{a}, which differs from *ebg*^{o} in an Asp-92→Asn substitution in the large (*ebgA*-gene product) subunit. A typical Class II enzyme is *ebg*^{b}, which differs from *ebg*^{o} by a Trp-977→Cys change, also in the large subunit (Hall *et al.*, 1989).

If Class I-containing strains are selected on lactulose as sole carbon source, or Class II-containing strains are selected on galactosylarabinose, then strains producing Class IV enzymes are produced. Both the Class IV alleles that have been sequenced produce *ebg* enzymes with both the Asp-92→Asn and Trp-977→Cys changes in the large subunit, but one of them also contains an additional Ser-979→Gly change in the large subunit and a Glu-122→Gly change in the small subunit (Hall *et al.*, 1989). The kinetic properties of the Class IV enzymes are broadly similar, so we have focused on the enzyme, *ebg*^{ab}, with just the two critical amino acid changes.

In the context of evolution of enzyme catalytic function we have investigated the mechanism of the *ebg*^{o} enzyme (Burton & Sinnott, 1983) and of the single mutants *ebg*^{a} and *ebg*^{b} (Li *et al.*, 1983, 1989; Hall *et al.*, 1983). The *ebg*^{o} enzyme gives β-galactopyranose as first product, and in the presence of methanol significant quantities of methyl β-D-galactopyranoside (Burton & Sinnott, 1983). The enzyme is therefore a standard retaining glycosidase, in which a glycosyl-enzyme intermediate is an obligatory intermediate. As with all such enzymes so far examined, this intermediate is the covalent glycosyl ester of an aspartic acid or glutamic acid side chain: affinity labelling experiments have identified the nucleophilic group in *ebg* enzymes as Glu-382 of the large subunit (Fowler & Smith, 1983; Stokes *et al.*, 1985).

Since there is no evidence with this enzyme of protein conformational changes or product-release steps being kinetically important, the kinetic mechanism is simple:

$$E + \beta GalX \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E \cdot \beta GalX \xrightarrow{k_{+2}} E \cdot \alpha Gal \xrightarrow{k_{+3}} E + \beta GalOH$$

For such a kinetic mechanism, of course;

$$k_{cat.} = k_{+2}k_{+3}/(k_{+2}+k_{+3})$$

$$K_m = k_{+3}(k_{-1}+k_{+2})/k_{+1}(k_{+2}+k_{+3})$$

$$k_{cat.}/K_m = k_{+2}k_{+1}/(k_{-1}+k_{+2})$$

i.e. $k_{cat.}/K_m$ does not contain k_{+3}. Hall (1976) showed that there was a good correlation between growth rates of *lacZ*$^-$ *ebg*$^+$ strains of *E. coli* on lactose and $k_{cat.}/K_m$ of the appropriate purified *ebg* enzyme for hydrolysis of lactose, i.e. k_{+3} did not affect fitness. It was therefore not wholly unexpected that the improvement of $k_{cat.}/K_m$ for lactose hydrolysis on evolution of *ebg* enzymes from *ebg*^{o} to *ebg*^{a} and ebg^b is accompanied by a dramatic fall in k_{+3}: at 25 °C it falls from in excess of 186 s^{-1} for *ebg*^{o} to 14.8 s^{-1} for *ebg*^{a} and 11.6 s^{-1} for *ebg*^{b}. It is therefore of interest to discover whether the double mutant, *ebg*^{ab}, is characterized by a further fall in k_{+3}.

The simple double-displacement mechanism followed by the *ebg* enzymes corresponds to a free-energy profile for the catalysed reaction of the type shown in Fig. 1. Most of such a profile can be made experimentally accessible by the appropriate measurements, and thus the *ebg* system can be an informative vehicle for the testing of theories of the evolution of enzyme catalysis in terms of such free-energy profiles. Such theories have their origin in the work of Albery & Knowles (1976) and Knowles & Albery (1977) on triose phosphate isomerase. These authors found that the modern enzyme had reached 'evolutionary perfection', in that diffusion of enzyme and substrate together limited the rate of the reaction in the thermodynamically favoured direction with physiological concentrations of substrate. This 'evolutionary perfection' was associated with a balanced thermodynamics of all internal states (all ES and EP complexes had the same free energy with respect to free enzyme and substrate). These authors further suggested that evolutionary changes in the free-energy profile of a catalysed reaction could be classified in terms of their increasing difficulty. The easiest change to bring about was a lowering of the free energy of all internal states (by, for example, an increase in the strength of a binding interaction at a site remote from the site of catalysis). The next most difficult change was considered to be an adjustment of the relative energies of internal states, and the most difficult change to accomplish was specific acceleration of an individual step.

Our discovery that a single quantum of evolutionary change in two instances dramatically decreased the rate of an individual step meant that in our system the easiest evolutionary change

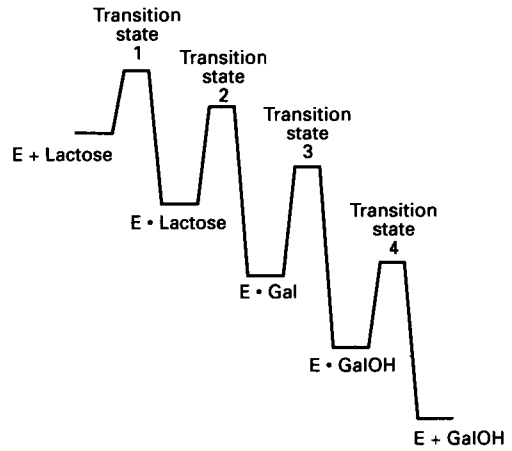

Fig. 2. Hypothetical optimized free-energy profile for *ebg* catalysing the hydrolysis under thermodynamically irreversible conditions

Note the 'descending staircase' of bound intermediates.

was not a lowering of the free energy of all internal states. Moreover, changes in the profile other than at the rate-determining transition state (state 2) were essentially random (Hall *et al.*, 1983). We therefore suggested that the Albery–Knowles picture of evolutionary change in the free-energy profile of the catalysed reaction, although probably a good guide to the whole evolutionary history of the enzyme, was a poor guide to the changes brought about by a single evolutionary event, which were dominated by the essentially random nature of such changes.

Chin (1983), however, pointed out that triose phosphate isomerase under physiological conditions maintained its substrate and product at near thermodynamic equilibrium, whereas many enzymes catalysed essentially irreversible reactions. In these cases one would expect the free energies of internal states to describe a 'descending staircase' on the free-energy profile, not matched internal thermodynamics. This is illustrated for *ebg* enzymes in Fig. 2. Burbaum *et al.* (1989) presented a refinement of the ideas of Albery & Knowles (1976), in which the departure of the physiological concentrations of reactants and products from their equilibrium values was explicitly taken into consideration. An important prediction from this work was that for an enzyme catalysing an essentially irreversible reaction catalytic improvement of the first, rate-determining, step should result in the slowing down of subsequent steps. This (at first sight counter-intuitive) prediction arises from the linear free-energy relationship assumed between the free energies of transition states and the free energies of the bound states flanking them. From Fig. 2 it is clear that, if the free energy of transition state 2 is lowered as a consequence of evolutionary change, then, if the assumption of a linear relationship between the free energy of transition state 2 and the free energy of E·αGal is correct, there will be a lowering of the free energy of E·αGal. If the free energy of transition state 3 remains unaltered, then k_{+3} will decrease. The changes observed as a consequence of single mutations in the *ebg* enzymes could thus well be consistent with the ideas of Burbaum *et al.* (1989). Examination of the changes in the profiles on going from the single mutants to the double mutant ebg^{ab} should provide more information on this point.

A further reason for studying the free-energy profile of ebg^{ab}-catalysed hydrolysis of lactose is to examine the question of additivity of the changes in a free-energy profile as a consequence of sequential amino acid changes in the protein. If these changes are additive then the free-energy profile for ebg^{ab} should be predictable from a knowledge of the $ebg^{o} \rightarrow ebg^{a}$ and $ebg^{o} \rightarrow ebg^{b}$ changes. The question has been addressed with respect to the consequences of site-directed mutagenesis: it is generally found that, unless the two sites interact, there is additivity of the effects of site-directed mutations in enzymes (Wells, 1990). However, recent work on dihydrofolate reductase (Howell *et al.*, 1990) indicates that the effect of a site-directed and countervailing spontaneous mutation are not additive.

We also present structure–reactivity and kinetic isotope effect data relating to the structure of the transition states 2 and 3 for ebg^{ab}, similar to data already reported for ebg^{a} and ebg^{b}. The structure–reactivity data are discussed, as is conventional, in terms of the gradient, β_{lg}, of the plot of the logarithm of a rate parameter against the pK_a of the leaving group. The more negative β_{lg}, the greater the change in charge at the leaving group atom (N or O) between the ground state and the transition state. Two chemical classes of substrate have been employed, β-D-galactopyranosylpyridinium salts and aryl galactosides. Since acidic or electrophilic catalysis to the departure of a pyridine is structurally impossible, β_{lg} values for this class of substrates correlate directly with the degree of C–N cleavage at the transition state. For aryl galactosides, partial proton donation to the leaving group atom can offset charge development caused by rupture of the glycosyl–O bond. β_{lg} values can be calculated from either $k_{cat.}$ or $k_{cat.}/K_m$ and we suggest, by a simple adaptation of the Northrop nomenclature system for enzyme isotope effects, that these be designated β_{lg} (V) and β_{lg} (V/K) respectively. (In this system, the type of isotopic substitution is specified by a preceding superscript, the kinetic parameter on which the effect is measured is in parentheses, and effects are reported conventionally, i.e. k_{light}/k_{heavy}.)

This paper therefore addresses three questions: the subunit composition of *ebg* enzymes, the changes in the free-energy profiles between ebg^{a} and ebg^{ab} and between ebg^{b} and ebg^{ab}, and the nature of any detectable changes in transition-state structure between the double mutant and the single mutants.

MATERIALS AND METHODS

Materials

ebg^{o}, ebg^{a}, ebg^{b} and ebg^{ab} enzymes were isolated from the constitutive strains described by Hall (1981), which were grown as described by him. Their purification (at 4 °C) was based on his procedure, with the modification that cells were disrupted by sonication rather than grinding with alumina paste, and that the DEAE-Sepharose ion-exchange step preceded the gel-filtration step, which was carried out on a 90 cm × 9 cm column of Sephacryl S-300.

Later work on ebg^{ab} was performed on enzyme that had been isolated by ion-exchange chromatography at room temperature on a Waters 650 system fitted with a PROTEIN PAK Glass DEAE-5PW (serial no. G9E83H089) column. Proteins were eluted by a linear salt gradient obtained from a 50 mM-potassium phosphate buffer (pH 7.5, containing 0.15 M-KCl and 3.5 mM-dithiothreitol) and the same buffer solution with the KCl concentration increased to 0.7 M.

ebg^{o} uniformly labelled with ^{14}C at a specific radioactivity of 253 Bq/mg, and ebg^{a} and ebg^{b} uniformly labelled with ^{3}H at specific radioactivities of 175 Bq/mg and 332 Bq/mg respectively were obtained by growing the appropriate strains in medium containing 9260 Bq of uniformly ^{14}C-labelled amino acids/ml or 26450 Bq of uniformly ^{3}H-labelled amino acids/ml (Amersham International, Amersham, Bucks., U.K.). *O*- and *N*-Glycoside substrates have been described elsewhere (Burton & Sinnott, 1983; Li *et al.*, 1983, 1989).

2,4,6-Trinitrophenyl 1-thio-β-D-galactopyranoside has been described previously (Sinnott *et al.*, 1978), but for the present

work we used a modified route that gave a cleaner product [m.p. 152–158 °C (decomp.)]. Picrylation of 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranose was achieved by refluxing a solution of the thiol and picryl chloride (1 equivalent) in dry acetone, in which anhydrous K_2CO_3 was suspended, for 2 h. *O*-Acetyl groups were removed by refluxing with a methanolic solution of 0.5% KCN (Herzig *et al.*, 1986). 2,4-Dinitrophenyl 1-thio-β-D-galactopyranoside was made analogously, with 1-fluoro-2,4-dinitrobenzene replacing the picryl chloride in the reaction with 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-galactopyranose [m.p. 146–148°; $[\alpha]_D^{25}$ −41° (*c* 0.1 in water)].

Allolactose was made by the method of Bredereck *et al.* (1962).

Methods

Kinetic measurements. All kinetic data refer to measurements in 0.125 M-potassium phosphate buffer containing 25 μM-2,2-bipyridyl and 5 mM-$MgCl_2$.

Michaelis–Menten parameters for *O*-glycosides and *N*-glycosides were determined spectrophotometrically as described previously (Burton & Sinnott, 1983; Li *et al.*, 1983), as were $^{\alpha^2}H(V)$ and $^{\beta^2}H(V)$ for 3,4-dinitrophenyl galactoside (Li *et al.*, 1989). Michaelis–Menten parameters for *S*-glycosides and for 3,4-dinitrophenyl galactoside at 37 °C, and the K_i value for galactose at 37 °C, were determined in a Perkin–Elmer Lambda 6 spectrophotometer fitted with a Peltier-effect thermostatically controlled cell block. Liberation of thiopicrate was monitored at 412 nm ($\Delta\epsilon$ 1.12×10^{-4} $M^{-1} \cdot cm^{-1}$) and of 2,4-dinitrothiophenolate at 402 nm ($\Delta\epsilon$ 9.6×10^{-3} $M^{-1} \cdot cm^{-1}$).

ebg^{ab}-catalysed wash-out of label from D-[1-^{18}O]galactose was monitored by natural-abundance ^{13}C-n.m.r. spectroscopy of the anomeric carbon atom of both α and β anomers of the sugar in a broadly similar manner to that described previously (Hall *et al.*, 1983), except that the spectrometer was a Bruker model AM-400, so the higher field strength eliminated the necessity for the zero-filling and resolution-enhancement procedures used earlier. A spectral width of 600 Hz, with 2k data points and 100 scans per spectrum, was used. The resolution was good enough for rate constants to be estimated from plots of $\ln[h_{18}/(h_{16}+h_{18})]$ against time, where h_{16} and h_{18} are the heights of the two peaks due to the two isotopomers. The blank rate was estimated as 2.8×10^{-5} s^{-1} in the present experiments, compared with 2.2×10^{-5} s^{-1} measured earlier. At 37 °C *ebg* enzymes lose activity over the time of this experiment, so the average of the activities at the beginning and end of the experiment was taken.

The pre-steady-state burst of *p*-nitrophenol aglycone during hydrolysis of *p*-nitrophenyl galactoside by *ebg*^{ab} was measured by using a SFA-12 rapid kinetics accessory (Hi-Tech Scientific, Salisbury, Wilts., U.K.) linked to a Perkin–Elmer Lambda-3 spectrophotometer and R100A recorder. Samples [150 μl each of the enzyme and the substrate (1 mM)] thermostatically controlled at 25 °C in the umbilicus were mixed, and liberation of aglycone was monitored at 400 nm.

Analytical measurements. The hydrolytic equilibrium between glucose, galactose, lactose and allolactose was measured at 37 °C in 30 mM-Tes/NaOH buffer, pH 7, containing 1.45 M-NaCl and 1 mM-$MgCl_2$. The buffer system was the minimum necessary to ensure continued activity of the *lacZ* β-galactosidase (Cambrian Chemicals, Croydon, Surrey, U.K.) used to establish the equilibrium. After a time established by trial and error to represent attainment of equilibrium, the solution was heated briefly to 100 °C, the precipitated protein was filtered off and the sugars were analysed by h.p.l.c. using a Spectrophysics 8800 system with an 8430 refractive-index detector. An Alltech 10 μm-particle-size carbohydrate column (300 mm × 4.1 mm) (Alltech Associates, Deerfield, IL, U.S.A.) was used, with 75% (v/v) acetonitrile in water as isocratic eluent (1.5 ml/min). This column has groups that catalyse the mutarotation of reducing sugars, so that only one peak per sugar is obtained. Glucose and galactose had a retention time of 5.5 min, lactose 9.0 min and allolactose 10 min. The relative molar response factors of glucose, galactose and lactose were 1:1:1.92; the relative molar response factor of allolactose was assumed to be the same as that of lactose.

The same analytical system was used to determine the equilibrium constant for lactulose hydrolysis: lactulose had a retention time of 8.0 min and fructose one of 4.8 min. The relative molar response factors of lactulose, fructose and galactose were assumed to be in the ratio of their molecular masses.

For determination of the selectivity of the galactosyl-enzyme between water and methanol, 16.8 mM-*p*-nitrophenyl galactoside in standard buffer (3.0 ml) and eight concentrations of methanol were incubated with 20 μl of *ebg*^{ab} solution (0.85 mg/ml) for 24 h at 25 °C. The mixture was heated at 55 °C for 20 min, the denatured enzyme being precipitated. The supernatant was analysed by h.p.l.c., with refractive-index detection, in the Spectrophysics system and with the column described above. Isocratic elution with water at a flow rate of 1.2 ml/min was employed. The relative molar response factor of the detector, galactose/methyl galactoside, was 1.00. The value of k_{+4}/k_{+3} of 1.04 M^{-1} was obtained by linear least-squares treatment of a plot of [GalOMe]/[GalOH] versus [MeOH].

For the determination of the mass ratio of small subunits to large subunits of *ebg* enzymes, the radiolabelled enzymes (approx. 5 mg/ml) were dissolved in a solution containing dithiothreitol (30 mM), guanidinium chloride (6 M) and K_2HPO_4 (0.2 M), adjusted to pH 8.4 with conc. KOH. The solution was stirred for 1 h under N_2, and then the solution was made 0.15 M in potassium iodoacetate and stirred in the dark for a further 15 min. The reaction was stopped by the addition of 2.5% (v/v) 2-mercaptoethanol, and dialysed against 80 vol. of 8.0 M-urea in 0.2 M-potassium phosphate buffer, pH 8.4. Small subunits and large subunits were separated by gel-permeation chromatography on a pre-equilibrated 90 cm × 3.2 cm column of Sephacryl-S300 with upward elution by 8.0 M-urea in 0.2 M-potassium phosphate buffer, pH 8.4, at a flow rate of 33 ml/h; 120-drop fractions were collected. The chromatography, as monitored by absorbance at 280 nm, produced near-baseline separation of the two subunits (the absorbance at the minimum between the two peaks corresponded to 2% of the peak height for the large subunit or 10% of that for the small subunit). For radiocounting 0.5 ml portions of each sample were diluted with 0.5 ml of water, 10 ml of scintillant was added, and the solutions were shaken and left overnight to equilibrate before their radioactivities were counted at room temperature. The scintillant consisted of 1% (v/v) 5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole (Koch–Light Laboratories, Colnbrook, Bucks. U.K.) in Triton X-100 (BDH Chemicals, Poole, Dorset, U.K.)/toluene (1:2, v/v). For gravimetric determination of subunit composition the pooled fractions were dialysed against double-distilled water (3 × 4.5 litres), freeze-dried and weighed.

An estimate of the molecular mass of the functional aggregate of *ebg*^{ab} was obtained by analytical gel-permeation chromatography on a Pharmacia f.p.l.c. system. A 30 cm × 0.8 cm column of Superose 6 was equilibrated with 0.125 M-potassium phosphate buffer, pH 7.5, containing 5 mM-$MgCl_2$ and 25 μM-bipyridyl. Injections (100 ml) of proteins at a concentration of 0.84 mg/ml were eluted at a flow rate of 0.2 ml/min. Protein was detected in the eluate by absorbance at 280 nm. Protein molecular-mass standards were purchased from Sigma Chemical Co. (Milwaukee, WI, U.S.A.).

Electron-micrograph pictures of *ebg*^{o} were obtained as follows: *ebg*^{o} (6.5 mg/ml) was diluted 500-fold into aq. 3.7% (v/v) formaldehyde. After 5 min, an equal volume of 1% uranyl acetate

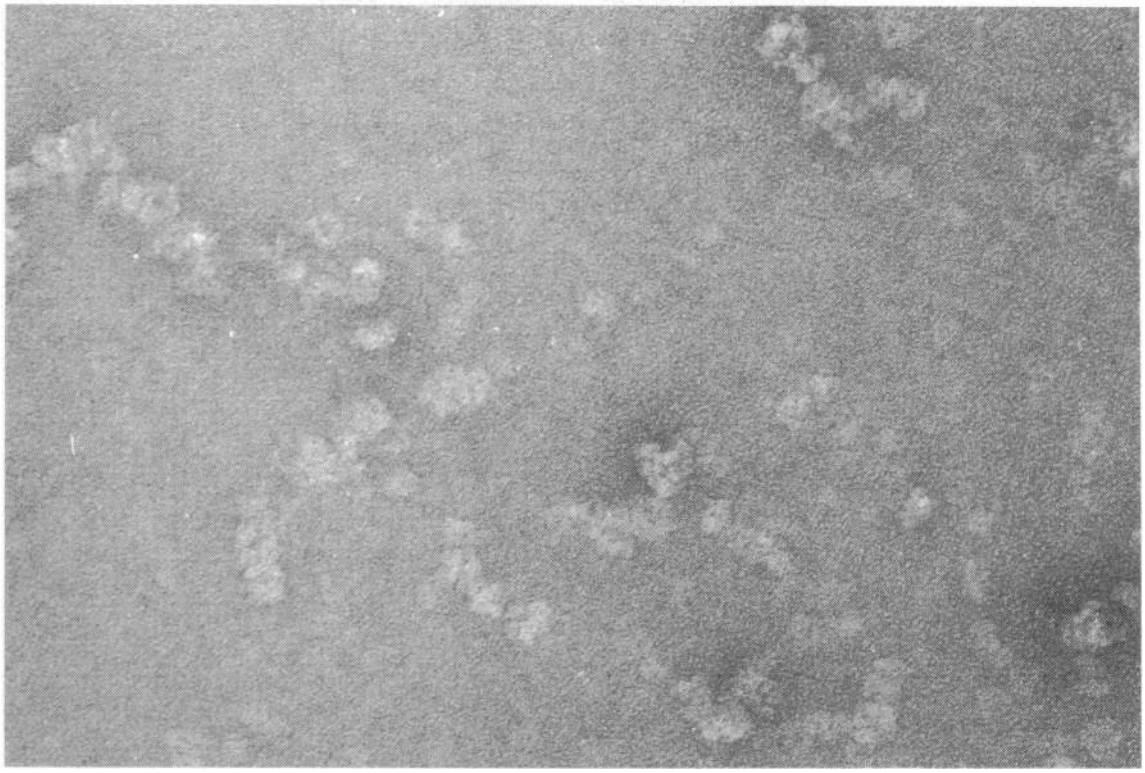

Fig. 3. Electron micrograph of uranyl acetate-stained *ebg*^{o} at 373300 × magnification

For details see the text.

solution was added, and a drop of the mixture was left to evaporate on the carbon grid at room temperature. Control experiments were performed in the absence of protein. The samples were examined in a JEOL Jem 100 Cx instrument at 66000 × and 100000 × magnification.

RESULTS AND DISCUSSION

Subunit composition of *ebg* enzymes

Isolated enzymes were reduced and carboxymethylated, and the two types of subunit were separated by gel-permeation chromatography. Enzymes were isolated from bacteria grown up in the presence of uniformly tritiated or uniformly ^{14}C-labelled amino acids. It was therefore possible to estimate the relative masses of the two subunits by radiocounting. The experiment was, however, done on the 30–80 mg scale (with a view to determining the nature and site of the amino acid changes at the protein level), so that it was possible to check the result by dialysis and weighing.

Two independent radiochemical measurements of the ratio with ^{3}H-labelled *ebg*^{a} gave values of 0.136 and 0.158; a similar measurement with ^{3}H-labelled *ebg*^{b} gave 0.144, and a radiochemical measurement with *ebg*^{o} gave 0.209. The gravimetric results for *ebg*^{o}, *ebg*^{a} and *ebg*^{b} were respectively 0.209, 0.156 and 0.163. The ratio of the molecular masses of the *ebgA* and *ebgC* peptides is 0.169 (Hall *et al.*, 1989), acceptably close to the average of the seven measured values (0.168). We therefore conclude that the large and small subunits are in a 1:1 ratio.

This ratio, and the sequence published by Hall *et al.* (1989) for *ebgA* and *ebgC* genes, enable us to comment on the previous ultracentrifugation studies (Hall, 1976), performed with *ebg*^{o} by the Canadian national ultracentrifugation service. These used the meniscus-depletion technique (Yphantis, 1964). Hall (1976) displayed a plot of logarithm of the fringe displacement against the square of the distance from the centre of the rotor. This plot was linear, but conversion of its gradient ($\sigma/2$) into a value of molecular mass involved an assumption that is probably incorrect. The molecular mass (M) is given by:

$$M = \sigma RT/(1-\gamma\rho)\omega^2$$

where γ is the partial specific volume, ρ is the solvent density and ω is the angular velocity. The value of γ was guessed as 0.77. From the amino acid compositions of the *ebgA* and *ebgC* peptides, the knowledge that these peptides are in a 1:1 ratio in the enzyme and the residue specific volumes given by McMeekin *et al.* (1949) it is possible to calculate a partial specific volume for the protein of 0.728. From the original data this now gives a molecular mass of 609 kDa, close to the value expected for the $\alpha_4\beta_4$ structure (551 kDa) expected by analogy with the analogous *lacZ* enzyme. Ultracentrifugation gives a weight-average molecular mass, and *ebg* enzymes (especially *ebg*^{o}) are known to aggregate.

The molecular mass of the form was checked by analytical gel-permeation chromatography. On a Superose 6 column *ebg*^{ab} was eluted with a retention time of 1.0, relative to thyroglobulin (669 kDa) 0.815, *lacZ* β-galactosidase (465 kDa) 1.038, apoferritin (433 kDa) 1.061 and β-amylase (200 kDa) 1.146. The protein is somewhat bigger than the *lacZ* enzyme, and considerably smaller than thyroglobulin. This experiment therefore allows us to rule out the $\alpha_5\beta_5$ structure, which would give a molecular mass close to that of thyroglobulin (689 kDa).

We also examined the *ebg* enzymes using electron microscopy. With uranyl acetate staining (but not with phosphomolybdate staining) protein molecules are just discernible. Although not without ambiguity if they are taken in isolation, in some of the highest-magnification photographs (372900 ×), one of which is shown in Fig. 3, some of the molecules appear to show fourfold symmetry. Such structures are approx. 13.0 nm on a side. If they

are assumed to arise by square packing of four spherical subunits, then the molecular mass of the subunit ($4\pi r^3/3\gamma$, where $r = 3.25$ nm and $\gamma = 0.728$) is calculated to be about 110 kDa, clearly in the correct region, particularly given the crudeness of our assumptions.

These data taken together therefore strongly suggest an $\alpha_4\beta_4$ subunit association for *ebg* enzymes.

Free-energy profiles for disaccharide hydrolysis

The free-energy profile of a uni-bi enzyme such as β-galactosidase strictly should be determined in three dimensions, since the fates of the two fragments from the hydrolysis of lactose (glucose and galactose) are, in principle at least, independent of each other. Since, however, loss of the two monosaccharide products from the enzyme is strictly ordered, a profile in two dimensions is adequate. The free-energy profile compares unimolecular and bimolecular processes, and therefore free-energy differences obtained are dependent on the thermodynamic standard state chosen. Burbaum *et al.* (1989) address this problem by converting bimolecular into unimolecular rate constants by taking as standard state the physiological concentration of the substrate. The intracellular concentrations of galactose and of glucose, fructose or arabinose in cells growing on the appropriate disaccharide are not known, but as hydrolysis is rate-limiting for growth (Dean, 1989) it is likely that the monosaccharides do not accumulate and that the *ebg* enzymes catalyse a thermodynamically irreversible reaction. We therefore use 53 mM, the estimated intracellular concentration of lactose in a growing bacterial cell, as the unit of concentration for the whole profile, as previously (Hall *et al.*, 1983); our thermodynamic standard state is thus 53 mM and 37 °C, the temperature at which the selection was carried out.

Reference to Fig. 1 reveals that the experimental data required for the construction of the profile are $k_{cat.}$ and K_m for the disaccharide, k_{+3} the degalactosylation rate, K_i for galactose, the rate of the virtual reaction with galactose, detected by ^{18}O-labelling, and the overall equilibrium constant. Data for the kinetics of hydrolysis of lactose are taken from Hall (1981).

Standard free energy of hydrolysis of lactose and lactulose. As there is as yet no reliable literature measurement of the equilibrium constant for hydrolysis of lactose, we measured it, using *lacZ* β-galactosidase to establish the equilibrium, and h.p.l.c. with refractive-index detection to analyse the equilibrium mixture. Use of *lacZ* β-galactosidase to establish the equilibrium results also in the establishment of the equilibrium between all possible β-galactopyranosylglucoses, and also, in principle, galactosylgalactoses as well. However, from 1.5 M-glucose and 0.25 M-galactose (i.e. an excess of glucose) in practice we detected only lactose and allolactose [the β(1→6) isomer]. Four independent measurements starting from the monosaccharides gave an equilibrium constant for lactose hydrolysis of 152 ± 19 M and for allolactose hydrolysis of 45 ± 16 M, at 37 °C. From 0.15 M-lactose a whole range of disaccharide products were initially formed, but lactose and allolactose slowly predominated (cf. Huber & Hurlburt, 1986), and the composition of two such reaction mixtures at apparent equilibrium, gave equilibrium constants of 175 ± 51 M for lactose hydrolysis and 43 ± 7 M for allolactose hydrolysis. The value of the free energy of hydrolysis of lactose that we used previously, essentially a structurally informed guess, corresponded to an equilibrium constant of 167 M (Hall *et al.*, 1983): our continuing use of this value is now fully experimentally justified.

Goldberg & Tewari (1989) also used *lacZ* β-galactosidase to establish the hydrolytic equilibrium between lactose, glucose and galactose, but did not address the isomer problem. Their value of −8.74 kJ/mol for the hydrolysis of lactose in fact corresponds quite closely to our value for the hydrolysis of total disaccharide (lactose + allolactose) of −9.1 kJ/mol.

Table 1. Michaelis–Menten parameters for the hydrolyses of β-D-galactopyranosyl derivatives by *ebg*^{ab} in 0.125 M-potassium phosphate buffer, pH 7.5, containing 5 mM-$MgCl_2$ and 54 μM-2,2-bipyridyl at 25.0 °C or (*) 37 °C.

Sources of pK data are given in Burton & Sinnott (1983).

Leaving group	pK_a	$k_{cat.}$ (s^{-1})	K_m (mM)
4-Nitrophenol	7.15	3.1	0.10
2-Nitrophenol	7.17	4.0	0.056
3,4-Dinitrophenol	5.42	3.3	0.047
		10.2*	0.049*
4-Cyanophenol	7.95	1.7	0.21
3,5-Dinitrophenol	6.69	3.9	0.082
Phenol	9.99	1.2	0.64
3-Nitrophenol	8.39	4.4	0.28
4-Bromophenol	9.34	2.7	0.21
2-Naphthol	9.51	1.5	0.18
3-Methylphenol	10.07	1.5	0.32
Water	15.7	16*	15* (K_i value)
2,4-Dinitrothiophenol		0.56	0.16
2,4,6-Trinitrothiophenol		2.8	0.26
3-Chloropyridine	2.81	3.1	0.34
3-Bromopyridine	1.85	4.0	0.38
4-Bromoisoquinoline	3.31	3.0	0.22
Pyridine	5.22	0.12	2.3
Isoquinoline	5.44	0.11	2.3
3-Methylpyridine	5.70	0.022	0.7

We also made measurements of the equilibrium constant for lactulose hydrolysis by the same technique. Five independent measurements starting from galactose and fructose gave a value of 39 ± 10 M, and two measurements starting from lactulose gave a value of 40 ± 11 M. In the reaction mixture we also detected a second disaccharide. However, without extensive data on structures produced by *lacZ*-β-galactosidase-catalysed transgalactosylation of lactulose, similar to that which exists on the analogous reaction of lactose (e.g. Huber & Hurlburt, 1986), we cannot be completely confident that β-D-galactopyranosylfructoses other than lactulose do not co-chromatograph with lactulose in our analytical system, and thereby give rise to an erroneously low value for the equilibrium constant.

Degalactosylation step. Rate-limiting degalactosylation in the hydrolysis of certain aryl glycoside substrates by *ebg*^{b} was demonstrated by two techniques, nucleophilic competition with methanol and pre-steady-state measurements with stoichiometric concentrations of enzyme (Li *et al.*, 1983), and degalactosylation has also been shown to limit the rate of hydrolysis of 3,4-dinitrophenyl galactoside by *ebg*^{a} (Li *et al.*, 1983) and *ebg*^{ab} (Elliott *et al.*, 1988) by nucleophilic competition with methanol.

The pre-steady-state measurements with *ebg*^{b} were, however, performed with 2-naphthyl galactoside, with the use of fluorescence to monitor aglycone liberation. This method involved fitting the time course of the appearance of fluorescence to burst amplitude, burst rate constant and steady-state rate. By a series of assumptions, a molecular mass per active site of 176 ± 26 kDa was calculated. However, it is desirable to have spectrophotometric data to estimate molecular mass per active site. The data in Table 1, in which it is seen that galactosides of acidic aglycones have the same $k_{cat.}$ values, suggest that for these substrates, as well as for 3,4-dinitrophenyl galactoside, $k_{cat.}$ in fact represents k_{+3}. We therefore performed pre-steady-state measurements of the size of the burst of liberated *p*-nitrophenol in the hydrolysis of *p*-nitrophenyl galactoside by *ebg*^{ab} (our equipment did not

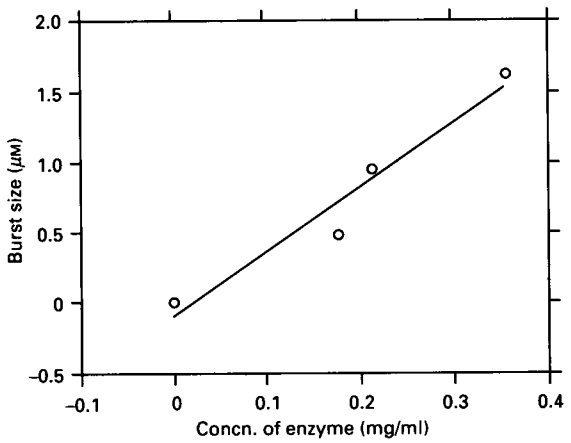

Fig. 4. Size of the pre-steady-state burst of *p*-nitrophenol aglycone observed on rapidly mixing various concentrations of enzyme with 1 mM-*p*-nitrophenyl galactoside in a stopped-flow mixing device, monitored by a conventional spectrometer

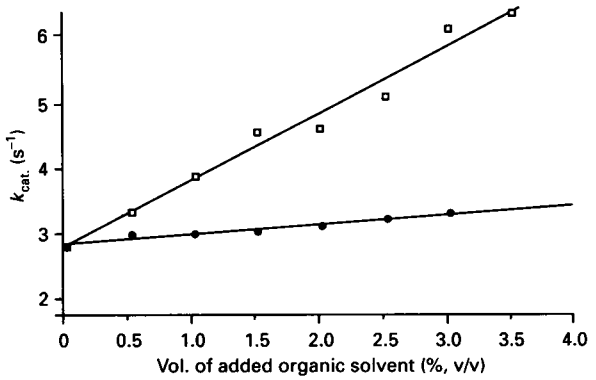

Fig. 5. $k_{cat.}$ for galactosyl thiopicrate as a function of volume of added organic solvent (□, methanol; ◆, dioxan)

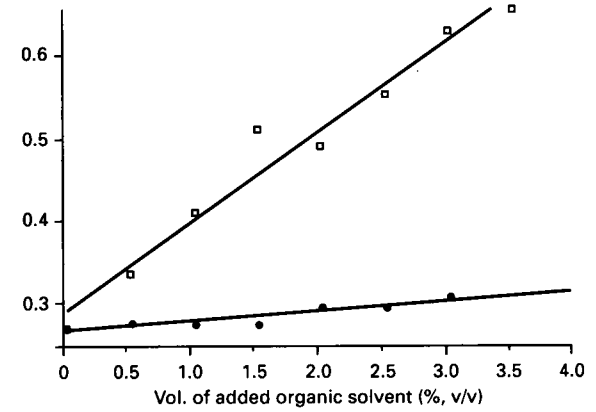

Fig. 6. K_m for galactosyl thiopicrate as a function of volume of added organic solvent (□, methanol; ◆, dioxan)

allow us to measure the rate of the transient phase). Fig. 4 displays the size of the pre-steady-state burst of aglycone in the hydrolysis of 1 mM-*p*-nitrophenyl galactoside (10 K_m) by various concentrations of *ebg*^{ab}: the least-squares line indicates its size to be 4.5_6 μM/mg. Burst size (Π) is given by:

$$\Pi = [k_{+2}/(k_{+2}+k_{+3})]^2[[S]/([S]+K_m)]^2[E]_0$$

Since [S] = 10 K_m, the burst size corresponds to 76% of that predicted on the assumptions of four independent active sites per $\alpha_4\beta_4$ aggregate, and the most active protein isolated being 100% active, if also $k_{+2} \gg k_{+3}$. This last condition may not be completely met: if $k_{+2} = 6.7\,k_{+3}$ the discrepancy in burst size would be accounted for. From the $k_{cat.}$ values in Table 1 it is clear that for galactosides of aglycones only a couple of pK units less acidic than *p*-nitrophenol $k_{+2} \sim k_{+3}$, so it is indeed likely that the condition that $k_{+2} \gg k_{+3}$ is not met.

From the data in Table 1 it appears that degalactosylation is now so slow that even a thioglycoside, galactosyl thiopicrate, is a sufficiently good substrate for degalactosylation to be apparently rate-limiting. This is a useful finding in the context of future attempts to apply selection pressure to the degalactosylation step. All previous selection pressure has been applied to the first step, and in the resulting evolvants an improvement in $k_{cat.}/K_m$ has been invariably accompanied by a kinetic deterioration in k_{+3}. Attempts to improve k_{+3} by selection on nitrophenyl galactosides are likely to encounter complications from the toxicity of nitrophenols to the bacterium; however, under aerobic conditions nitrothiophenolate anions are readily oxidized to water-insoluble diaryl disulphides. We therefore now report methanol competition data (Figs. 4 and 5), establishing that indeed k_{+3} does limit the hydrolysis of galactosyl thiopicrate.

In the presence of methanol the kinetic scheme for the enzyme becomes:

$$E+\beta GalX \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\cdot\beta GalX \xrightarrow{k_{+2}} E\cdot\alpha Gal \xrightarrow{k_{+3}} E+\beta GalOH$$
$$\downarrow k_{+4}[MeOH]$$
$$E+\beta GalOMe$$

so that now:

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

and

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

so that if $k_{+2} \gg k_{+3}+k_{+4}[MeOH]$ there is a linear increase in both $k_{cat.}$ and K_m with methanol concentration. The data in Figs. 5 and 6 show that for galactosyl thiopicrate this is indeed the case.

The lines shown are the linear least-squares best fit, and, if uncorrected for the non-specific medium effects caused by the addition of an organic solvent, give values of k_{+4}/k_{+3} of 1.5 M^{-1} from Fig. 5 and 1.7 M^{-1} from Fig. 6. If the non-specific medium effect of adding methanol can in fact be modelled by dioxan, which in this context is non-nucleophilic, then these values become 1.2 M^{-1} and 1.5 M^{-1} respectively, so that there is fair agreement between the value of k_{+4}/k_{+3} measured kinetically and that measured from the products (1.04 M^{-1}).

The selectivity of the galactosyl-*ebg*^{ab} between water and methanol on a molar basis is 57, that for galactosyl-*ebg*^{a} is 102 and that for galactosyl-*ebg*^{b} is 63 (Li *et al.*, 1983). The values for the single mutants are much higher than that for the wild-type (7: Burton & Sinnott, 1983); however, the preferences are not additive, *ebg*^{ab} behaving much like *ebg*^{b}.

Measurement of the virtual reaction of galactose. In previous studies with *ebg*^{o}, *ebg*^{a} and *ebg*^{b} (Hall *et al.*, 1983) we used the technique of isotopic washout of ^{18}O, monitored by natural-abundance ^{13}C-n.m.r. spectroscopy, to measure $k_{cat.}$ for this process. Because unlabelled material competes with labelled material in the exchange, the wash-out is first-order, the observed first-order rate constant being given by:

$$k_{obs.} = k_{blank} + k_{cat.}\cdot[E]_0/(K_m + [galactose])$$

Fig. 7 illustrates the anomeric ^{13}C resonances of the labelled

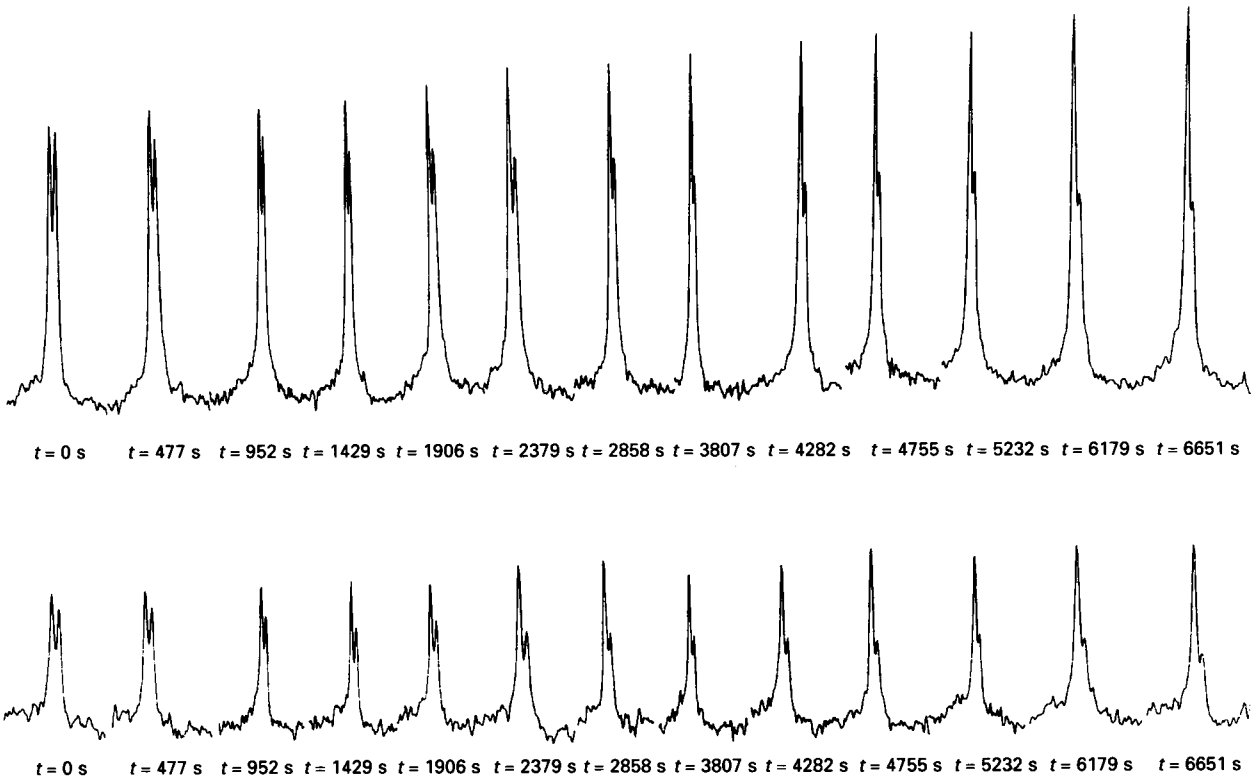

Fig. 7. ^{13}C-n.m.r. spectra of the anomeric carbon atoms of galactose (β top, α bottom; total concentration 0.679 M) in the presence of *ebg*^{ab} (2.9 mg/ml), in standard buffer containing 10% ^2H_2O as lock signal

galactose during the wash-out. Mutarotation was fast compared with the reaction monitored, so that the rate of isotope wash-out could be obtained in duplicate from a single run, by examination of the resonances for both the α anomer and the β anomer of the sugar.

The derived value for $k_{cat.}$ is somewhat higher than that for k_{+3}, but we consider this to be a consequence of the large errors of the method, and that in fact the two values are the same, as is also the case with *ebg*^{b}. This means that, as with *ebg*^{b}, we cannot locate the E·galactose complex, since k_{+3}, not cleavage of the C–OH bond, limits the hydrolysis of galactose. Measured K_m (or K_i) values cannot be converted into K_s values without a knowledge of k_{+2} for hydroxy as a leaving group.

Evolution of the free-energy profile for hydrolysis of lactose by *ebg* enzymes

In Table 2 are set out the free energies of various intermediates and transition states for the *ebg*^{o}-, *ebg*^{a}-, *ebg*^{b}- *and ebg*^{ab}-catalysed hydrolysis of lactose, data for the wild-type and the single-mutant enzymes being taken from Hall *et al.* (1983). The diffusional barriers (transition states 1 and 4) are included for purely illustrative purposes, their energies being calculated on the assumption that enzyme–small ligand diffusion takes place with a rate constant of 10^7 M^{-1}·s^{-1}. It is clear they are unlikely to be kinetically significant.

It is apparent from the last column in Table 2 that the free-energy profile for *ebg*^{ab} cannot be completely deduced by adding the *ebg*^{o}→*ebg*^{a} and *ebg*^{o}→*ebg*^{b} changes together at each point of the profile.

Additivity does apparently appear to operate for transition state 2. Hall (1976) deduced a linear relationship between the growth rate of *lacZ*$^-$ *ebg*-containing evolvants and $k_{cat.}/K_m$ for lactose hydrolysis. This means that the transition state 2 is the free-energy barrier to which selection pressure has been applied.

Additivity predicts an energy of transition state 3 only 1.5_3 kJ/mol different from that observed, but changes are small anyway, so additivity of free energy changes cannot really be said to apply.

A noteworthy feature of the profile for *ebg*^{ab} is that transition states 2 and 3 are closer in energy than with other forms of the enzyme. This means that the chance of the galactosyl-enzyme, formed from another glycosyl donor in the presence of glucose, synthesizing lactose are greater than that of other forms of the enzyme. This may be related to the *ebg*^{ab} enzyme being the only form of the enzyme that can synthesize allolactose from lactose (Hall, 1982*b*).

The energy of the E·lactose complex with *ebg*^{ab} cannot be even approximately predicted by the additivity assumption. Because degalactosylation is not kinetically accessible with *ebg*^{o}, it is possible only to put an upper bound on the predicted energy of the galactosyl-enzyme, but this is sufficient to demonstrate that additivity does not apply here either.

The fact that additivity applies to some points on the free-energy profile but not others implies immediately that the generalizations of Albery & Knowles (1976) about the evolutionary history of enzymic free-energy profiles might be misleading. These authors suggested that the easiest change to bring about would be a lowering of the free energies of all bound states, that the next would be a selective change in the energies of intermediates and the most difficult would be the selective acceleration of an individual step. These ideas suggest a picture of the evolution of a free-energy profile in which changes of a certain type occur smoothly and cumulatively until their limits of optimization are reached, and then the more difficult changes occur. If changes in the free-energy profile are non-additive, however, then the consequences of a given evolutionary change on the profile will depend on the evolutionary history of the enzyme.

Table 2. Free energies of intermediates and transition states in the hydrolysis of lactose by *ebg* enzymes

Data refer to a standard state of 53 mM and 37 °C; transition states 1 and 4 are calculated with an arbitrary illustrative estimate of 10^7 $M^{-1} \cdot s^{-1}$ for the diffusion of enzyme and substrate.

Quantity	Free energy (kJ/mol)				
	ebg^o	*ebg*^{a}	*ebg*^{b}	*ebg*^{ab}	*ebg*^{ab} (predicted*)
E + Lactose	0.0	0.0	0.0	0.0	
Transition state 1	42.0_5	42.0_5	42.0_5	42.0_5	
E·Lactose	2.6_8	-1.5_1	1.1_7	-10.2_9	-3.0_1
Transition state 2	77.1_5	67.6_6	71.8_4	61.7_6	62.3_1
E·αGal	$> -19.4_1$	-21.2_5	-23.6_8	-24.3_5	$< -29.2_9$
Transition state 3	40.7_1	45.1_0	42.5_9	45.4_4	46.9_9
E·GalOH	-22.1_8	-25.4_0	$> -23.6_8$	$> -23.5_6$	
Transition state 4	21.5_9	21.5_9	21.5_9	21.5_9	
E + GalOH	-20.4_2	-20.4_2	-20.4_2	-20.4_2	

* The values were predicted by adding the differences *ebg*^{a}-*ebg*^{o} and *ebg*^{b}-*ebg*^{o} to the *ebg*^{o} value.

Apart from the continual erosion of transition state 2, to which selection pressure has been applied, changes elsewhere in the profile are for the most part random and non-additive. However, the changes do conform to the refined ideas of Burbaum *et al.* (1989), which take into account whether the enzyme maintains reactants and products at close to equilibrium (as with triose phosphate isomerase) or whether the enzyme catalyses a thermodynamically irreversible reaction (as appears to be the case with β-galactosidase when the organism is growing on lactose; Dean, 1989). In the latter case the selective alteration in the energies of bound states will result in post-rate-determining steps becoming slower, as is observed. Nonetheless, the changes that are observed as a consequence of individual quanta of evolutionary change are also in line with the ideas of Pettersson (1989), who suggested that evolutionary pressure was directed towards improvement of $k_{cat.}$ with the energies of bound intermediates irrelevant because of the differing standard states necessary to account for the different physiological concentration of each metabolite, which alters as a consequence of each evolutionary change.

It is not possible at present to construct free-energy profiles for the hydrolysis of lactulose, using our present data for the free energy of hydrolysis of this sugar and the kinetic data of Hall (1981), because the kinetics of lactulose uptake by growing *E. coli* have not been studied, so that there is no estimate of its intracellular concentration.

Transition-state characterization

Information about the structure of transition state 2 for the various evolvants has been obtained from structure–reactivity correlations and for transition state 3 from secondary kinetic isotope effects.

As is the case with most enzymic structure–reactivity correlations, adventitious interactions of the substituents with the protein cause Brønsted-type plots to be very noisy. Nonetheless, if a wide enough leaving-group pK range is taken, information can emerge that is not hidden by the noise. A further complication, however, arises if structure–reactivity parameters are calculated from $k_{cat.}$; this parameter may represent k_{+3}. In the case of *ebg*^{ab}, considerations of the low value of k_{+3} are therefore essential for interpretation of $\beta_{lg}(V)$ values derived from the data in Table 1. All but the glycosides of the least acidic phenols have essentially similar $k_{cat.}$ values, and this value is the same as that for the glycosylpyridinium ions of the two less basic pyridines. It is thus clear that $\beta_{lg}(V)$ values (-0.096 ± 0.034 for the aryl galactosides and -0.69 ± 0.07 for the pyridinium salts) will be closer to zero than the true value for the bond-breaking process. The values derived from the second-order rate constants, $\beta_{lg}(V/K)$, however, reflect only the first chemical step and binding processes; their values, -0.30 ± 0.05 for the aryl galactosides and -0.95 ± 0.09 for the pyridinium salts, are identical with those for *ebg*^{b} (-0.31 ± 0.04 and -0.99 ± 0.09) (Li *et al.*, 1989). Detectable differences in β_{lg} values for *ebg*^{o}- and *ebg*^{a}-catalysed hydrolysis of pyridinium salts were consistent with less C–N bond cleavage at the transition state in the evolvant: likewise, differences in β_{lg} values for *ebg*^{o}- and *ebg*^{b}-catalysed hydrolysis of aryl galactosides were consistent with more efficient application of electrophilic or acidic catalysis to the leaving aglycone (Li *et al.*, 1989). We therefore conclude that the more effective application of acid catalysis to the leaving aglycone, which by as-yet-undetermined mechanisms results from evolutionary change of Trp-977 to Cys in the α-subunit, survives the additional Asp-92→Asn change sensibly unaltered. However, the earlier transition site in the hydrolysis of pyridinium salts arising from the Asp-92→Asn change by itself is reversed by the second amino acid change.

The structure of transition state 3 was addressed by measurement of α- and β-deuterium kinetic isotope effects. α-Deuterium kinetic isotope effects, arising from substitution at the reaction centre, have their origin in the weakening of a C–H bending vibration as the hybridization of the reaction centre changes from sp^3 to sp^2. In the case of a simple heterolytic dissociation (S_N1 or $D_N + A_N$ reaction) they thus correlate with the charge development at the transition state. If an incoming nucleophile is present, to some extent it will repel the hydrogen atom of the bending C–H bond, thereby increasing the effective force constant for the bending vibration and decreasing the effect. β-Deuterium kinetic isotope effects arise from substitution of deuterium for hydrogen vicinally to the reaction centre. They have their origin in two effects. One is a small geometry-independent inductive effect, in which deuterium behaves as if it were slightly more electron-donating than hydrogen, the other a larger geometry-dependent hyperconjugation. This hyperconjugative weakening of the C–H or C–^{2}H bond is at a maximum when the dihedral angle between the bond and the electron-deficient p orbital on the adjacent carbon atom is 0° and zero when it is 90°.

The values of secondary deuterium kinetic isotope effects on the degalactosylation of *ebg*^{ab} [$^{\alpha^2H}(V)$ and $^{\beta^2H}(V)$ for 3,4-dinitrophenyl galactoside] are 1.077 ± 0.017 and 0.996 ± 0.016. These values are the same, within experimental error, as those

found for the degalactosylation of *ebg*^{a} and *ebg*^{b} (Li *et al.*, 1989), and reinforce the conclusion that the pyranose ring of the galactosyl-enzyme intermediate cannot be in the $^4C_1$ conformation, since the α-deuterium kinetic isotope effect indicates significant charge development at the transition state, and in the $^4C_1$ conformation the C-2–L bond exactly eclipses the electron-deficient *p* orbital, so that β-deuterium kinetic isotope effects will be at a maximum (1.07–1.10).

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