Catalysis by the large subunit of the second β -galactosidase of *Escherichia coli* in the absence of the small subunit

Sergei V. CALUGARU*, Barry G. HALL† and Michael L. SINNOTT*‡

*Department of Chemistry (M/C 111), University of Illinois at Chicago, 845 West Taylor Street, Chicago, IL 60607-7061, U.S.A., and †Department of Biology, River Campus, University of Rochester, Rochester, New York 14627, U.S.A.

Plasmids containing the *ebgA*^o and *ebgA*^a genes of *Escherichia coli* under the control of the *lac* repressor and promoter have been constructed and inserted into *Salmonella typhimurium* CH3. This system expresses the large subunit of the *ebg*^o and *ebgA*^a β -galactosidase in high yield (20–60 % of total protein). The large subunits have been purified to homogeneity. As isolated they are tetramers of significant catalytic activity; the N-terminal amino acid residue is Met, but it is not formylated. The k_{cat} values for a series of aryl galactosides were 6–200-fold reduced from the corresponding values for the holoenzymes. k_{cat}/K_m values for glycosides of acidic aglycones, though, were unchanged, whilst k_{cat}/K_m values for galactosides of less acidic aglycones showed a modest (up to 10-fold) decrease. The k_{cat} values for glycosides of acidic aglycones hydrolysed by *ebg*^o and *ebgA*^a large subunits were essentially invariant with aglycone *pK*, suggesting that hydrolysis of the galactosyl-enzyme intermediate had become rate-determining for these substrates. Rate-determining hydrolysis of the glycosyl-enzyme intermediate was confirmed by pre-steady-state measurements and nucleophilic competition with methanol.

Absence of the small subunit was thus estimated to cause a 200-fold decrease in degalactosylation rate for *ebg*^o and a 20-fold one for *ebgA*^a. $\beta_{\text{lg}}(V/K)$ values of -0.57 ± 0.08 for *ebg*^o and -0.54 ± 0.08 for *ebgA*^a isolated subunits were significantly more negative than for holoenzymes. It is suggested that the small subunit is associated with the optimal positioning of the electrophilic Mg^{2+} ions in these enzymes. Use of PCR in the construction of the plasmid also inadvertently led to the production of ψebg ^o large subunit in which there was a PCR-introduced Leu⁹ → His change. Values of k_{cat} for aryl galactosides, calculated on the assumption that the ψebg ^o large subunit, like the *ebg*^o and *ebgA*^a large subunits, was 100 % active as isolated, were about an order of magnitude lower than for true *ebg*^o large subunit, whilst K_m values were similar. The very significant kinetic effect of this inadvertent site-undirected mutagenesis indicates that quite large kinetic effects of amino-acid replacements in enzymes may have no obvious mechanistic significance.

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*, also under negative control [2]. The wild-type enzyme, *ebg*^o, is too catalytically feeble to permit growth on lactose or other β -galactoside disaccharides, but selection of *lacZ*[−] mutants on various β -galactoside disaccharides results in a series of experimental evolvants of *ebg* containing various mutations which render the enzyme more catalytically efficient [3,4].

The *ebg* enzymes have an $\alpha_4\beta_4$ quaternary structure in which the holoenzyme contains four *ebgA* gene products (*M_r* 117927) and four *ebgC* gene products (*M_r* 19917) [5,6]. Determination of the sequences of these genes [6] revealed that the start of *ebgC* overlapped by four nucleotides the end of *ebgA*. This complex quaternary structure stands in contrast with the simple tetrameric structure of the *lacZ* enzyme; the contrast is the more striking when the 50 % nucleotide identity of the *lacZ* and *ebgA* genes, which results in a 33.7 % amino acid homology in the gene products, is considered.

Yet more remarkable is the result of estimates of sequence similarity of active-site residues: of the 15 amino acid residues forming the active site of the *lacZ* enzyme, 13 are conserved in the wild-type *ebgA*-gene product. The commonest type of amino acid change resulting in a lactose-positive phenotype, Asp⁹² → Asn in the *ebgA* gene-product, as seen for example in the *ebgA*^a

enzyme, results in the number of conserved active-site residues increasing to 14 (Table 1).

The function of the small subunit of *ebg* enzymes is not then immediately apparent, yet its presence in the holoenzyme is required for a lactose-positive phenotype in the organism [6]. We therefore now report an investigation of catalysis by *ebg* enzymes containing only the large subunit. Three such enzymes have been investigated: the wild-type (*ebg*^o) large subunit, the large subunit of a 'first-generation' experimental evolvant (*ebgA*^a, containing only the Asp⁹² → Asn change) and a large subunit for which kinetic data had been obtained on the incorrect assumption that it was the *ebg*^o large subunit, before N-terminal protein sequencing revealed a PCR-induced Leu → His change at residue 9.

MATERIALS AND METHODS

Enzymes

To aid in the preparation of the large amounts of isolated large subunits, plasmids based on the pUF 1000 vector were constructed. The pUF vector was derived from pSE380 [7] by cutting at the single *NcoI* site, filling in the overlapping strands with the Klenow fragment of DNA polymerase I, and ligating the blunt ends with T4 DNA ligase to destroy a translation start site at the position of the *NcoI* site. In these constructs, the *ebgA* gene was under the control of the *tac* repressor and the very-high-efficiency *lac* promoter. *Salmonella typhimurium* strain CH3

‡To whom correspondence should be addressed.

Table 1 Active-site residues of *lacZ* β -galactosidase conserved in the *ebg* sequence

The fifteenth residue at the *lacZ* active site is Asn⁶⁰⁴; in *ebg*^o the corresponding residue is Asp⁵⁷⁹.

<i>ebg</i> ^o residue number	92	341	372	375	400	402	449	489	490	512	515	543	576	977
Residue	Asn*	His	Arg	His	Glu	His	Glu	Met	Tyr	Glu	His	Trp	Phe	Trp [§]
<i>lacZ</i> residue number	102	357	388	391	416	418	461	502	503	537	540	562	601	999

*In wild-type *ebg*^o is Asp, but changes to Asn as a consequence of a Class I evolutionary change.

§Changes to Cys as a consequence of a Class II evolutionary change.

was used as host; this organism does not have a chromosomally encoded *ebg* enzyme. Unfortunately, PCR amplification was used in the construction, and this was subsequently shown to have introduced errors in some of these and other related plasmids. The following procedures were therefore required to verify the sequences of the genes encoding the proteins on which kinetic measurements were made.

Plasmid construction

The *ebgA* genes were amplified in two segments, using the PCR protocol based on the thermostable *Pfu* polymerase (Stratagene, La Jolla, CA, U.S.A.). The 5' segment included bp 1283–2961 and the 3' segment bp 2913–4331. The two segments were digested with restriction enzyme *Bam*HI, which cuts at bp 2938 (present on both fragments), and ligated with T4 DNA ligase into the vector, which had been digested with the same enzymes.

5' Segment

Primer 1 included bp 1283–1307 of the *ebg* operon, plus an additional 22 bases at the 5' end. The *ebg* operon bases include the ribosome-binding site and the first three codons of *ebg* enzyme. The 5' end bases included stop codons in all three frames to ensure that no inadvertent 'gene fusion' products were formed, and included a site for the *Eco*R1 restriction enzyme. Primer 2 encompassed bp 2913–2932 of the *ebg* operon.

3' Segment

Primer 1 encompassed bp 2913–2932 of the *ebg* operon, and primer 2 included bp 4407–4431 of the *ebg* operon, and an additional 10 bp at the 5' end that contained a *Hind*III site.

Construction of plasmid for large (α) subunit of *ebg*^o (pUF 1003)

The 5' segment was amplified from strain A2 [6] DNA, and the 3' segment from strain 1B1 (wild-type) DNA, as described above. The sequence was confirmed as authentic by DNA sequencing.

Construction of plasmid for large (α) subunit of *ν**ebg*^o (pUF 1004)

Both segments were amplified from strain 1B1 DNA. The sequence was shown to have an error at bp 1318 (T → A), originally detected as a Leu⁹ → His change in the N-terminal peptide sequence.

Construction of plasmid for large (α) subunit of *ebg*^o (pUF 1026)

The fragment from bp 1283 to 2938 in pUF 1004 was replaced by that region from plasmid pUF 1024, coding for *ebg*^o holoenzyme. The nucleotide sequence of the *ebgAC* genes of this plasmid had been verified by sequencing.

Verification of plasmid sequences

The sequences of the *ebg* genes on all constructed plasmids were verified by sequencing with the 'fmol' kit from Promega (Madison, WI, U.S.A.). To ensure that all PCR-induced misincorporations were detected, a set of samples in which the same sequencing primer was used was loaded on to the sequencing gel twice: once in the normal fashion and once with all A's in adjacent lanes, all the C's in adjacent lanes, etc. This method has been shown to detect all differences among the samples [8]. A range of *ebg* alleles (not confined to the 'large-subunit-only' species described here) had been cloned and contained several differences that had been determined when they had originally been sequenced [6]. Each of these differences was detected in a 'blind' examination of autoradiograms.

Preparation of crude *ebg* enzyme extracts

S. typhimurium strain CH3 containing the appropriate plasmid was grown in mineral-salts medium (2 litres) [9] containing Difco Casamino acids (20 g) and ampicillin (200 mg). After growth with vigorous aeration at 37 °C to a cell density of $8 \times 10^9 \cdot \text{litre}^{-1}$, 2'-propyl 1-thio- β -D-galactopyranoside was added to a final concentration of 0.2 mM, additional ampicillin (25 mg) was added, and aeration was continued overnight (typically 17 h). The cells were then harvested by centrifugation, and ground with twice their wet weight of alumina (type 305) in a mortar prechilled to 4 °C. When the cells had been disrupted, they were suspended in 12.5 mM potassium phosphate buffer, pH 7.5, at 4 °C (2 ml/g wet weight of cells) and a solution of streptomycin sulphate (300 mg $\cdot \text{ml}^{-1}$) was added to a final concentration of 27 mg $\cdot \text{ml}^{-1}$. The well-mixed solution was stored on ice for 0.5 h, then centrifuged at 25000 *g* for 20 min. The supernatant was recovered and stored at -85 °C until required. Extracts contained 10–80 mg of protein $\cdot \text{ml}^{-1}$, 20–60 % of which was *ebg* enzyme.

Isolation of enzymes

The enzymes were isolated from crude extracts by medium-pressure chromatography on a Waters 650 system (Milford, MA, U.S.A.) at room temperature.

A Protein PAK Glass DEAE-5PW column (10 μ ; 8.0 mm $\times$ 7.5 cm) was used for isolation of *ebg*^o α -subunit. The crude extract (0.6–0.9 ml) was applied to the column equilibrated with 50 mM potassium phosphate buffer, pH 7.5. The column was washed at a rate of 1.0 ml/min with the initial buffer (25 ml), then KCl gradient was used (0–1 M, 25 ml) in the same buffer. Active fractions were combined and dialysed against the initial buffer.

The large subunit of the Leu⁹ → His mutant was isolated with the use of Protein PAK Glass HIC (Phenyl-5PW) column (10 μ ; 8.0 mm $\times$ 7.5 cm). The crude extract (0.6–1 ml) was brought to 1 M KCl by the addition of solid salt and loaded on to the column equilibrated with 50 mM potassium phosphate buffer,

pH 7.5, containing 1 M KCl. The column was washed at a rate of 1.0 ml/min with the equilibration buffer (20 ml), then descending gradient of KCl (1–0 M, 30 ml) was performed. Active fractions were combined and dialysed against 50 mM potassium phosphate buffer, pH 7.5.

The large subunit of *ebg^a* was obtained by gel filtration of the crude extract (0.5–0.6 ml) through Protein Pak Glass 300SW column (10 μ ; 8.0 mm $\times$ 30 cm) in 50 mM potassium phosphate buffer, pH 7.5. The flow rate was 0.8 ml/min.

The purity of the enzymes was demonstrated by SDS/PAGE in PhastGel Gradient 8-25 on PhastSystem (Pharmacia–LKB Biotechnology, Piscataway, NJ, U.S.A.) and (in the case of α -subunits of *ebg^a* and *Leu⁹ → His* mutant) by N-terminal sequencing. The sequences were determined on Applied Biosystems model 477A sequencer by Dr. A. Steinschneider (Research Resources Center, University of Illinois at Chicago, IL, U.S.A.).

Substrates

p-Nitrophenyl β -D-galactopyranoside was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.); other galactosides were as described previously, or re-synthesized by the same methods [10].

Methods

The sequence alignment of the *lacZ* and *ebg* protein sequences was performed using CLUSTAL V [11].

Protein concentration was determined by phenol reagent method (Sigma Diagnostics Procedure no. 690).

Molecular masses of the enzymes were estimated by gel-permeation chromatography on a Sephacryl S-300 column (1.0 cm $\times$ 50 cm). The column was equilibrated and washed with 50 mM potassium phosphate buffer, pH 7.5, at a flow rate of 0.29 ml/min. The following proteins were used as M_r standards: BSA (66000), alcohol dehydrogenase (150000), β -amylase (200000), apoferritin (443000), *lacZ* β -galactosidase (465000) and thyroglobulin (669000). All the standard proteins were purchased from Sigma, with the exception of *lacZ* β -galactosidase, which was from Cambrian Chemicals (Croydon, Surrey, U.K.).

All kinetic data refer to 0.125 M potassium phosphate buffer, pH 7.5, containing 25 μ M 2,2'-bipyridyl and 5 mM MgCl_2 at 25 °C.

Michaelis–Menten parameters for the hydrolysis of β -D-galactopyranosyl derivatives were determined spectrophotometrically in a Lambda 6 spectrophotometer (Perkin–Elmer, Norwalk, CT, U.S.A.) fitted with a thermostatically controlled cell block. Liberation of 3,4-dinitrophenol and 3,5-dinitrophenol was monitored at 390 nm ($\Delta\epsilon$ 14990 and 1850 $\text{M}^{-1}\cdot\text{cm}^{-1}$ respectively), of 4-nitrophenol and 3-nitrophenol at 400 nm ($\Delta\epsilon$ 14330 and 251 $\text{M}^{-1}\cdot\text{cm}^{-1}$ respectively), of 4-bromophenol at 288 nm ($\Delta\epsilon$ 749 $\text{M}^{-1}\cdot\text{cm}^{-1}$), of phenol at 277 nm ($\Delta\epsilon$ 1170 $\text{M}^{-1}\cdot\text{cm}^{-1}$) and of 3-methylphenol at 279 nm ($\Delta\epsilon$ 893 $\text{M}^{-1}\cdot\text{cm}^{-1}$).

Individual K_m and k_{cat} values were calculated by fitting of the experimental data to the Michaelis–Menten equation using the program Kaleidagraph (Synergy, Philadelphia, PA, U.S.A.). k_{cat}/K_m values were estimated by linear least-squares treatment of double-reciprocal plots ($1/v$ versus $1/[S]$) with the use of the same program; values did not differ significantly from the quotient of the separate values.

The pre-steady-state burst of 4-nitrophenol during hydrolysis of 2.5 mM 4-nitrophenyl β -D-galactopyranoside was measured

by using a SFA-12 rapid kinetics accessory (Hi-Tech Scientific, Salisbury, Wiltshire, U.K.) connected to a Perkin–Elmer Lambda 3 spectrophotometer and R 100A recorder. Liberation of aglycone was monitored at 400 nm.

For determination of the selectivity of the galactosyl-enzymes between water and methanol, mixtures were prepared (200 μ l) containing 50 mM 4-nitrophenyl β -D-galactopyranoside and various methanol concentrations in the standard buffer used for kinetic measurements (see above). Dioxan was used instead of methanol in control experiments. After the addition of 50 μ l of the enzyme solution (1.1 mg/ml or 1.0 mg/ml for the α -subunits of *ebg^a* and *ebg^a* respectively) the reaction mixtures were kept at room temperature for 6 h, then they were heated at 65–70 °C for 20 min and centrifuged in Eppendorf microcentrifuge for 8 min. The supernatants were analysed by HPLC on a Spectra-Physics (San Jose, CA, U.S.A.) SP8800 system with an SP 8430 refractive-index detector and an SP 4270 integrator. An Econosyl NH_2 column (5 μ ; 4.6 mm $\times$ 250 mm; Alltech Associates, Deerfield, IL, U.S.A.) was washed isocratically with 75, 80 or 85 % (v/v) acetonitrile in water at a rate of 2.0 ml/min.

RESULTS AND DISCUSSION

Gel-permeation chromatography on Sephacryl S-300 indicated that the isolated large subunit of wild-type enzyme had an M_r of about 520000 and that of *ebg^a* about 510000. Since the *ebgA*-gene product has an M_r of around 120000 on SDS/PAGE [3] and 117927 from its amino acid sequence [6], it is clear that the large subunits of the enzyme aggregate into tetramers.

With a protein preparation containing only large subunits, it was possible to enquire whether the N-terminal methionine indicated from the gene sequence was present in the mature protein or cleaved off in a processing step. In fact N-terminal sequencing of the *Asp⁹² → Asn* mutant indicated a sequence:

Met-Asn-Arg-Trp-Glu-Asn-Ile-Gln-Leu-Thr-

and of the supposed wild-type α -subunit:

Met-Xaa-Arg-Trp-Glu-Asn-Ile-Gln-His-Thr-

(Xaa was identified as Asn or Cys or a modified amino acid), revealing that the terminal methionine was still present, but not as the *N*-formyl derivative.

In Table 2 are set out Michaelis–Menten parameters for hydrolysis of a series of aryl galactosides by the true wild-type (*ebg^a*) large subunit and the *Leu⁹ → His* mutant, together with data for the wild-type holoenzyme [10]. In Table 3 the data are set out for the *Asp⁹² → Asn* mutant large subunit, and for the corresponding *ebg^a* holoenzyme [12]. In all cases k_{cat} values were calculated on the assumption that the isolated enzyme was a 100 %-pure and 100 %-active protein of M_r 120000 per active site. This was confirmed as essentially correct by pre-steady-state measurements (see below).

It is immediately apparent that, as anticipated from the biological work, k_{cat} values are lower for the isolated large subunits than for the holoenzyme. However, if the wild-type large subunit and holoenzyme are compared, it is seen that the k_{cat} values for the isolated subunit are independent of leaving group, at least with the more acidic leaving groups ($\text{pK} < \approx 9$).

This behaviour is commonly found for two-step hydrolases proceeding through substituted-enzyme intermediates. In the case of the *ebg* enzymes, the initial sugar product is β -galactopyranose [10], and their action involves the intermediacy of a covalent (presumably α) galactosyl-enzyme intermediate [10]. Non-covalent processes are kinetically insignificant in all cases so far examined. The plateau value of k_{cat} observed with all

Table 2 Hydrolysis of β -D-galactopyranosyl derivatives by ebg^o β -galactosidase of *E. coli* and by large subunits of ebg^o and ψebg^o at 25 °C in 0.125 M potassium phosphate buffer (pH 7.5)/5 mM $MgCl_2$ /25 μ M 2,2'-bipyridyl

The variation ranges indicated correspond to a confidence limit of 0.95.

Leaving group	pK_a [10]	ebg^o Holoenzyme [10]				ebg^o Large subunit				ψebg^o Large subunit			
		k_{cat} (s^{-1})	$10^3 \times K_m$ (M)	$10^{-3} \times k_{cat}/K_m$ ($s^{-1} \cdot M^{-1}$)		k_{cat} (s^{-1})	$10^3 \times K_m$ (M)	$10^{-3} \times k_{cat}/K_m$ ($s^{-1} \cdot M^{-1}$)		k_{cat} (s^{-1})	$10^3 \times K_m$ (M)	$10^{-3} \times k_{cat}/K_m$ ($s^{-1} \cdot M^{-1}$)	
3,4-Dinitrophenol	5.42	190	9.7 ± 1.4	18	0.76	0.044 ± 0.002	16	0.098	0.039 $\pm$ 0.003	2.4			
3,5-Dinitrophenol	6.69	150	7.5 ± 1.4	14	0.90	0.048 ± 0.012	22	0.12	0.042 ± 0.005	2.7			
4-Nitrophenol	7.15	66	25 ± 3	2.5	0.67	0.35 ± 0.02	1.8	0.095	0.29 ± 0.02	0.34			
3-Nitrophenol	8.39	51	30 ± 3	1.9	0.72	0.72 ± 0.05	0.98	0.096	0.49 ± 0.04	0.20			
4-Bromophenol	9.34	16	150 ± 10	0.11	0.81	3.4 ± 0.5	0.23	0.086	2.4 ± 0.5	0.040			
Phenol	9.99	38	160 ± 30	0.27	0.41	9.6 ± 1.1	0.041	0.036	5.4 ± 0.8	6.5×10^{-3}			
3-Methylphenol	10.09	2.6	9.6 ± 2.6	0.27	0.42	5.9 ± 0.7	0.077	0.064	7.5 ± 2.6	9.7×10^{-3}			

Table 3 Hydrolysis of β -D-galactopyranosyl derivatives by ebg^o β -galactosidase of *E. coli* and by the large subunit of this enzyme at 25 °C in 0.125 M potassium phosphate buffer (pH 7.5)/5 mM $MgCl_2$ /25 μ M 2,2'-bipyridyl

The pK_a values were the same as those given in Table 2. The variation ranges indicated correspond to a confidence limit of 0.95. Abbreviation: n.d., not determined.

Leaving group	ebg^o Holoenzyme [12]		ebg^o Large subunit		
	k_{cat} (s^{-1})	$10^3 \times K_m$ (M)	k_{cat} (s^{-1})	$10^3 \times K_m$ (M)	$10^{-3} \times k_{cat}/K_m$ ($s^{-1} \cdot M^{-1}$)
3,4-Dinitrophenol	15	1.0	0.60	0.036 ± 0.002	17
3,5-Dinitrophenol	15	1.3	0.81	0.045 ± 0.003	17
4-Nitrophenol	16	3.0	0.61	0.43 ± 0.02	1.4
3-Nitrophenol	14	7.1	0.66	0.44 ± 0.04	1.5
4-Bromophenol	7.5	17	0.51	2.2 ± 0.2	0.22
Phenol	10	17	0.25	5.1 ± 0.4	0.044
3-Methylphenol	n.d.	n.d.	0.29	2.6 ± 0.8	0.11

three isolated large subunits then probably represents hydrolysis of the galactosyl-enzyme intermediate. This was shown to be the case when similar behaviour was seen with ebg^a , ebg^b [12], ebg^{ab} [5] and ebg^{abcd} and ebg^{abcde} (K, S. and Sinnott, M. L., unpublished work) holoenzymes: the degalactosylation rate became lower as the enzyme became more highly evolved.

The kinetic methods used previously [5,12,13] do indeed establish that the plateau k_{cat} values for isolated large subunits represent hydrolysis of galactosyl-enzyme intermediates. In the presence of methanol (MeOH), galactosyl- ebg enzymes preferentially react to yield methyl β -galactoside, according to the following kinetic scheme:

so that now:

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

and

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

Figure 1 Variation of Michaelis-Menten parameters for hydrolysis of p -nitrophenyl β -D-galactopyranoside by ebg^o large subunit with the concentration of organic solvent (○) methanol and (□) dioxan

Data refer to 0.125 M potassium phosphate buffer (pH 7.5)/5 mM $MgCl_2$ /25 μ M 2,2'-bipyridyl at 25.0 °C. (a) Variation of k_{cat} ; (b) variation of K_m .

Figure 2 Variation of Michaelis-Menten parameters for hydrolysis of p -nitrophenyl β -D-galactopyranoside by ebg^a large subunit with the concentration of organic solvent (○) methanol and (□) dioxan

Data refer to 0.125 M potassium phosphate buffer (pH 7.5)/5 mM $MgCl_2$ /25 μ M 2,2'-bipyridyl at 25.0 °C. (a) variation of k_{cat} ; (b) variation of K_m .

A linear increase in k_{cat} or K_m in the presence of methanol therefore indicates that hydrolysis of the galactosyl-enzyme is rate-determining, provided the increase is in accord with the selectivity of the galactosyl-enzyme between water and methanol, as measured in a separate experiment, and also that a non-nucleophilic organic solvent (dioxan is used) does not cause a similar increase.

The variation of Michaelis-Menten parameters for p -nitrophenyl galactoside with the concentration of methanol and of dioxan, when this substrate is hydrolysed by the ebg^o and ebg^a large subunits, is displayed in Figures 1 and 2; similar data (not

Figure 3 Variation of pre-steady-state burst with protein concentration when *ebg*[°] large subunit (◇) and *ebg*^a large subunit (○) is rapidly mixed with *p*-nitrophenyl galactoside

shown) were obtained for the Leu⁹ → His mutant. The selectivity between water and methanol (k_{+4}/k_{+3}) indicated from the data for k_{cat} is 1.1, 1.4 and 0.72 M⁻¹ for the large subunits of wild-type enzyme (*ebg*[°]), the Asp⁹² → Asn mutant (*ebg*^a) and the Leu⁹ → His (*ψebg*[°]) mutants respectively. The selectivity indicated from analysis of the methyl galactoside formed from hydrolysis of *p*-nitrophenyl galactoside in the presence of methanol is 0.70 and 1.8 for the large subunits of *ebg*[°] and *ebg*^a, indicating the kinetic effect of methanol does indeed arise from nucleophilic interception of the galactosyl-enzyme. This selectivity can be compared with one of 0.13 M⁻¹ for *ebg*[°] [10] and 1.9 M⁻¹ for *ebg*^a [13]. The absence of the small subunit therefore appears to alter the selectivity of *ebg*[°] between water and methanol, but not *ebg*^a.

A burst of *p*-nitrophenol before the steady state was established was observed on rapid mixing of *ebg*[°] and *ebg*^a large subunits with *p*-nitrophenyl galactoside (to give a final substrate concentration of 2.5 mM), in accord with the conclusions of the methanol-competition work. Plots of burst size against protein concentration are shown in Figure 3. The least-squares best lines through these plots indicate, on the assumption of 100% pure and 100% active protein, a burst size of 0.78[E]₀ for the *ebg*[°] large subunit and one of 0.70[E]₀ for the *ebg*^a large subunit. When non-covalent events are kinetically insignificant, the burst size (π) is given by [14]:

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

From the K_m values in Tables 2 and 3, a burst of 0.77[E]₀ for the *ebg*[°] large subunit and one of 0.73[E]₀ for the *ebg*^a large subunit would be expected if the proteins were 100% pure and 100% active, and $k_{+2} \gg k_{+3}$. This is therefore clearly the case.

Comparison with the data for enzyme containing both small and large subunits indicates that the major effect of the absence of the small subunit appears to be to decrease the degalactosylation rate. Rate-limiting degalactosylation is not readily demonstrated with aryl galactoside substrates hydrolysed by *ebg*[°], because of the lower preference of the wild-type galactosyl-enzyme for methanol and the high k_{cat} values, which preclude stopped-flow measurements with our equipment. However, the similarity of k_{cat} for the best aryl galactoside substrate, 3,4-dinitrophenyl galactoside, and that of galactosyl fluoride [15], when the fluoride undergoes spontaneous, pH-independent hydrolysis some 10⁴ times faster than the 3,4-dinitrophenyl galactoside [16], strongly suggest that degalactosylation has

become rate-determining with these substrates with very good leaving groups. If this is so, then the absence of the small subunit decelerates degalactosylation by over two orders of magnitude. The effect on the first-generation evolvant, *ebg*^a, is less pronounced, degalactosylation being reduced in rate by a factor of around 20.

This lowering of the degalactosylation rate could have many causes, but a precedent from work of Withers' group is suggestive [17]. The acid-catalytic Glu¹²⁷ of the (retaining) exoglucanase/xylanase of *Cellulomonas fimi* was changed to residues which cannot act as general acids (Ala, Gly or Gln). k_{cat} . Values for all substrates decreased dramatically because of a much lower rate of hydrolysis of the glycosyl-enzyme (an α -glycosylated Glu²³³ residue). Glu¹²⁷, acting as a general acid catalyst for the departure of aglycone in the first step of the reaction, was also acting as a general base for the attack of water on the glycosyl-enzyme intermediate in the second step of the reaction, the near-microscopic reverse of the first. The effect on k_{cat}/K_m of the incapacitation of the acid-catalytic group, though, depended on the substrate. There was no effect on the 2,4-dinitrophenyl glycoside, about an order of magnitude reduction for the 4-nitrophenyl glycoside and two to three orders of magnitude reduction for the *p*-bromophenyl glycoside. In accord with chemical expectation, removal of the acid-catalytic machinery of the enzyme has little or no effect on the action of the enzyme on substrates with acidic leaving groups, but its importance increases as the leaving group becomes less acidic and harder to remove. Removal of the acid-catalytic machinery of a retaining glycosidase will therefore result in a drastically more negative β_{lg} value, calculated on k_{cat}/K_m , as well as a much-decreased rate of the second step of the reaction.

Examination of the data in Tables 2 and 3 reveals a similar, but less pronounced, pattern of behaviour. There is little or no effect of the small subunit on k_{cat}/K_m for dinitrophenyl galactosides, but an effect becomes discernible for the galactosides of less acidic phenols such as phenol itself. The derived β_{lg} (V/K) values ($\pm 95\%$ confidence limit) of -0.57 ± 0.08 for *ebg*[°] and ψebg [°] large subunits and -0.54 ± 0.08 for *ebg*^a large subunit differ from the values of -0.46 ± 0.08 and -0.36 ± 0.06 obtained for the wild-type holoenzyme *ebg*[°] [10] and the Asp⁹² → Asn holoenzyme mutant, *ebg*^a [12]. The β_{lg} value for the small subunit is indisputably more negative than that for the holoenzyme in the *ebg*^a case, and has a 95% probability of being more negative in the *ebg*[°] case. The effect of removal of the small subunit on β_{lg} (V/K) for the *ebg* enzyme is in the same direction, but less pronounced, as the effect of site-directed mutagenized incapacitation of the acid catalytic group on β_{lg} (V/K) for the *C. fimi* endoglucanase/xylanase.

All kinetic evidence (including site-directed-mutagenesis studies) is consistent with *lacZ* β -galactosidase using electrophilic catalysis by an active-site Mg²⁺ ion, rather than general acid catalysis [18], to promote the departure of the leaving group. Structural data are also consistent with this: the Mg²⁺ is directly across the active-site cleft from the nucleophilic [19] Glu⁵³⁷. Given the very high degree of conservation of the active-site residues of the *lacZ* enzyme in *ebg*, and the much greater effect of removal of magnesium on the activity of *ebg*[°] against *p*-nitrophenyl galactoside than activity against galactosyl 3-bromopyridinium ion [10], to the leaving group of which the Mg²⁺ cannot co-ordinate, it appears that the *ebg* enzymes likewise use an electrophilic Mg²⁺. Further evidence for this comes from the labelling of Glu⁴⁰⁰, a counterpart of Glu⁴¹⁶, one of the ligands of the Mg²⁺ ion in the *lacZ* enzyme, by the suicide inactivator β -D-galactopyranosyl *p*-nitrophenyl triazene [20]. In the enzyme active site this reagent decomposes to β -D-GalpCH₂N₂⁺, the

electrophilic methylene of which is in the same position as the leaving group oxygen atom of a normal substrate.

The question of the function of the small subunit of *ebg* enzymes then becomes 'how can the presence or absence of the small subunit affect the operation of electrophilic catalysis, when the Mg^{2+} -binding site is on the large subunit?'. The answer probably lies in the fewer degrees of freedom possessed by an electrophilic metal ion in a protein than by a carboxylic acid; the site at which a metal ion in a protein can exert its electrophilic action is much more rigorously defined than the site to which an aspartic or glutamic acid side chain can donate a proton. A potential role for the small subunit would then be to alter slightly the conformation of the large subunit so that the Mg^{2+} is more effective as an electrophile.

This model can also provide some rationalization for why the effect of small-subunit removal is more pronounced on k_{+3} than on k_{+2} . Because of the steric restrictions about the electrophile, and its inflexibility, it is doubtful whether electrophilic catalysis of the departure of bulky, unnatural leaving groups such as phenols is particularly effective anyway, and so further disruptions will have little effect. The hydrolysis of the glycosyl-enzyme, by contrast, by microscopic reversibility probably involves a magnesium-bound hydroxide as a nucleophile. The components of the transition state for this step of the reaction are those of the natural reaction, and in the holoenzyme their disposition with respect to the metal ion is likely to be more optimal than the components of the transition state for the first chemical step of an unnatural substrate. Misalignment of the Mg^{2+} , arising for the absence of the small subunit, is therefore likely to have a proportionately greater effect on the second chemical step than the first chemical step of these reactions.

The kinetic data for the *ψebg* large subunit serve as a salutary caution against attributing catalytic roles to amino acid residues in proteins on the basis of modest changes in kinetic parameters as a consequence of site-directed mutagenesis. The $Leu^9 \rightarrow His$

change in the *ebg* large subunit arose as a consequence of site-undirected mutagenesis. Nonetheless, with no reason for thinking that Leu^9 was a 'contributor to catalysis', change to His lowers the degalactosylation rate by a factor of around 8.

We thank the National Institutes of Health for financial support of this work through grant GM 46663.

REFERENCES

- 1 Jacobsen, R. H., Zhang, X.-J., DuBose, R. F. and Matthews, B. W. (1994) *Nature* (London) **369**, 761–766
- 2 Hall, B. G. and Hartl, D. L. (1975) *Genetics* **81**, 427–435
- 3 Hall, B. G. (1976) *J. Mol. Biol.* **107**, 71–84
- 4 Hall, B. G. (1981) *Biochemistry* **20**, 4042–4049
- 5 Elliott, A. C., K, S., Sinnott, M. L., Smith, P. J., Bommuswamy, J., Guo, Z., Hall, B. G., and Zhang, Y. (1992) *Biochem. J.* **282**, 155–164
- 6 Hall, B. G., Betts, P. W. and Wootton, J. C. (1989) *Genetics* **123**, 635–648
- 7 Brosius, J. (1989) *DNA* **8**, 759–777
- 8 Nachman, M. W., Boyer, S. N., Searle, J. B., and Aquadro, C. F. (1994) *Genetics*, **136**, 1105–1120
- 9 Hall, B. G. and Hartl, D. L. (1974) *Genetics* **76**, 391–400
- 10 Burton, J. and Sinnott, M. L. (1983) *J. Chem. Soc. Perkin Trans. 2*, 359–364
- 11 Higgins, D. G. (1994) *Methods Mol. Biol.* **25**, 307–318
- 12 Li, B. F. L., Holdup, D., Morton, C. A. J. and Sinnott, M. L. (1989) *Biochem. J.* **260**, 109–114
- 13 Li, B. F. L., Osborne, S., Sinnott, M. L. and Yates, D. W. (1983) *J. Chem. Soc. Perkin Trans. 2*, 365–369
- 14 Hiromi, K. (1979) *Kinetics of Fast Enzyme Reactions*, p. 231, Kodansha, Tokyo
- 15 K, S., Konstantinidis, A. K., Sinnott, M. L. and Hall, B. G. (1993) *Biochem. J.* **291**, 15–17
- 16 Jones, C. C. and Sinnott, M. L. (1977) *J. Chem. Soc., Chem. Commun.* 767–768
- 17 McLeod, A. M., Lindhorst, T., Withers, S. G. and Warren, R. A. J. (1994) *Biochemistry* **33**, 6371–6376
- 18 Selwood, T. and Sinnott, M. L. (1990) *Biochem J.* **268**, 317–323
- 19 Gebler, J. C., Aebershold, R. and Withers, S. G. (1992) *J. Biol. Chem.* **267**, 11126–11130
- 20 Fowler, A. V. and Smith, P. J. (1983) *J. Biol. Chem.* **258**, 10201–10207