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Changes in the Substrate Specificities of an Enzyme during Directed Evolution of New Functions†

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ABSTRACT: Wild-type *ebg* enzyme, the second β-galactosidase of *Escherichia coli* K12, does not permit growth on lactose. As part of a study of the evolution of new enzymatic functions, I have selected, from a *lacZ* deletion strain, a variety of spontaneous mutants that grow on lactose and other β-galactoside sugars. Single point mutations in the structural gene *ebgA* alter the enzyme so that it hydrolyzes lactose or lactulose effectively; two mutations in *ebgA* permit galactosylarabinose hydrolysis, while three mutations are required for lactobionic acid hydrolysis. Wild-type *ebg* enzyme and 16 functional mutant *ebg* enzymes were purified and analyzed kinetically to determine how the substrate specificities had changed during the directed evolution of these new functions. The specificities for the biologically selected substrates generally increased by at least an order of magnitude via increased V_{max} and decreased K_m for the substrate. These changes were very specific for the selected substrate, often being accompanied by decreased specificities for other related substrates. The single, double, or triple substitutions in the enzymes did not detectably alter the thermal stability of *ebg* enzyme.

The *ebg* system of *Escherichia coli* K12 is being used as a model to study the acquisitive evolution of new metabolic functions. Beginning with a strain of *E. coli* K12 bearing a large deletion within the *lacZ* (β-galactosidase) gene, I have selected a series of spontaneous mutant strains that have evolved the ability to utilize lactose and other β-galactoside sugars as sole carbon and energy sources (Hall & Hartl, 1974; Hall, 1976a,b, 1977, 1978; Hall & Zuzel, 1980). The spontaneous mutations occur in the *ebg* (evolved β-galactosidase) operon located at 66 min on the *E. coli* map (Hall & Hartl, 1975; Bachmann & Low, 1980). The operon is under negative control by the product of the tightly linked *ebgR* gene (Hall & Hartl, 1975). Mutations in *ebgR* are important to the evolutionary process (Hall & Clarke, 1977) but are outside the scope of this paper. The structural gene of interest is *ebgA* which specifies a 120 000 molecular weight polypeptide that is the subunit of the homohexameric *ebg* β-galactosidase (Hall, 1976a). The wild-type enzyme is designated *ebg*°, "o" standing for original (Hall, 1976a). *ebg*° enzyme has a very low activity toward lactose, and even constitutive strains, which synthesize nearly 5% of their soluble protein as *ebg*° enzyme, fail to grow on lactose or on the other β-galactoside sugars discussed in this paper (Hall & Hartl, 1975; Hall, 1978).

Spontaneous single point mutations in *ebgA* alter the properties of *ebg* enzyme so that it hydrolyzes lactose well enough to permit growth (Hall, 1977). I have isolated a large series of spontaneous *ebgA* mutants that have evolved the ability to utilize lactose (4-*O*-β-D-galactopyranosyl-D-glucose), lactulose (4-*O*-β-D-galactopyranosyl-D-fructose), galactosylarabinose (3-*O*-β-D-galactopyranosyl-D-arabinose), or lactobionic acid (4-*O*-β-D-galactopyranosyl-D-gluconic acid). The mutant strains have been classified according to the β-galactoside sugars which they can use and according to their growth rates on those sugars (Hall, 1978; Hall & Zuzel, 1980).

Class I mutants carry a single point mutation in region I of the *ebgA* gene (Hall & Zuzel, 1980). They are isolated by selecting for lactose utilization, and they grow rapidly on lactose while failing to utilize lactulose, galactosylarabinose, or lactobionic acid (Hall, 1978). Class II mutants carry a single point mutation in region II of the *ebgA* gene, about one

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Table I: Enzyme Sources and Purities[a]

enzyme	class	strain	*ebgA* allele	sp act. of purified preparation	% pure	estimated pure sp act.
O	O	SJ16/F'1B1	0	12200	93.8	13000
A	I	SJ8/F'A2	2	8800	94.8	9300
D	I	D2	168	7100	95.1	7500
E	I	R61	144	8000	95.4	8400
B	II	SJ12/F'5A2	52	9800	98.2	10000
C	II	5A11	51	10200	96.5	10600
F	II	R42	143	9300	80.9	11500
AB	IV	RT522	107	2700	95.7	2800
AC	IV	RT512	108	3000	88.5	3400
DB	IV	RT52168	106	2400	68.1	3500
DC	IV	RT51168	105	2400	81.5	3000
AF	IV	A23	134	3000	96.8	3100
AG	IV	A27	138	3900	96.3	4000
DH	IV	D21	116	2600	92.7	2800
CI	IV	5A11GA	204	3000	94.7	3200
BJ	IV	SJ60	205	5100	93.5	5500
AGN	V	A272	198	1600	94.7	1700

[a] Specific activities are in units mg^{-1} on 5 mM ONPG.

kilobase away from region I of the *ebgA* gene. They are isolated by selecting for lactulose utilization, and they grow fairly slowly on lactose and moderately well on lactulose and fail to utilize either galactosylarabinose or lactobionate (Hall, 1978; Hall & Zuzel, 1980). Class II mutants are isolated about 10% of the time during selection for lactose utilization (Hall, 1977, 1978).

Class IV mutants carry two point mutations, one in region I and the other in region II of *ebgA* (Hall & Zuzel, 1980). They grow rapidly on lactose, moderately on lactulose, and slowly on galactosylarabinose and fail to grow on lactobionate. These double mutants may be obtained in several ways: (1) from class I mutants by selection for lactulose utilization and (2) from class II mutants by selection for galactosylarabinose utilization. Both (1) and (2) involve selection of a *second* spontaneous mutation within *ebgA*. Class IV mutants have never been isolated directly from the wild-type (*ebgA*°) allele. Alternatively, class IV mutants may be obtained by selecting galactosylarabinose-utilizing recombinants from crosses between class I and class II mutants. It should be emphasized that the class IV mutants so obtained are phenotypically indistinguishable from those obtained by sequential selection. Likewise, class IV mutants can be resolved into class I and class II mutants by crosses with wild-type (*ebgA*°) strains (Hall & Zuzel, 1980).

Finally, class V mutants carry three mutations in *ebgA*. They have been isolated *only* from class IV mutants (Hall, 1978) by selecting for lactobionic acid utilization. They grow on lactose, lactulose, and lactobionate, but growth rates and utilization of galactosylarabinose vary widely with the class V mutant being examined (Hall, 1978).

These substrates used to select functional mutant enzymes have the general structure galactosyl-1-*O*-β-R where the "R substituent" is either glucose, fructose, D-arabinose, or gluconic acid.

The availability of these mutants has provided an unusual opportunity to study an array of enzymes, all of which are functional, "evolved" under known conditions, and differ from each other by one, two, or three substitutions. The ability to generate class IV mutants by intragenic recombination between class I and class II mutants provides a unique opportunity to study the effects of two different substitutions individually and together in the same polypeptide chain.

This study was initiated with four purposes in mind: (1) to determine whether the mutants classified on the basis of growth parameters would be identically classified on the basis of kinetic parameters of the enzymes in vitro, (2) to determine exactly how the specificities of *ebg* enzyme had altered during selection directed at different β-galactoside substrates, (3) to determine whether the glycon specificities had altered in any regular fashion during this selection, and (4) to determine whether there was any significant alteration in thermostability as one, two, or three amino acid replacements were present in the polypeptide chain.

Materials and Methods

E. coli K12 Strains. All strains are *ebgR*⁻ and bear *lacZ* deletion W4680. Except as noted below, all have been previously described (Hall, 1978; Hall & Zuzel, 1980; Rolseth et al., 1980). Strains SJ16/F'1B1, SJ8/F'A2, and SJ12/F'5A2 are *recA*⁻ merodiploid strains that are homozygous for the alleles *ebgA*°, *ebgA2*, and *ebgA52*, respectively. These same alleles are carried by strains 1B1, A2, and 5A2 referred to in Hall & Zuzel (1980).

Enzyme Designations. Each enzyme specified by a different allele of *ebgA* is given a letter code. Enzyme "O" is wild-type enzyme specified by the *ebgA*° allele. Enzymes specified by a single letter, i.e., "A" or "B" enzymes, differ from the wild-type enzyme by a single amino acid substitution, those designated by two letters differ from wild type by two substitutions, etc. A particular letter designates the same substitution in all enzymes. Thus enzyme A carries a particular substitution, while the enzyme AG carries the same substitution as A plus a second substitution "G". Enzymes A and B carry different substitutions, while enzyme AB carries the same two substitutions in a single polypeptide chain. The *ebgA* allele numbers associated with each enzyme are given in Table I in order that the reader may compare enzyme properties with the growth rates given in Hall (1978) and in Hall & Zuzel (1980).

Buffers. All buffers are potassium phosphate, pH 7.5. The phosphate concentrations of the buffers are given in the text.

Enzyme Assays. All assays were in 125 mM buffer containing 5 mM Mg^{2+} and 25 μM α,α-bipyridyl. All assays were performed at 37 °C. Hydrolysis of nitrophenyl substrates was determined from the rate of change in absorbance at 420 nm (Hall, 1976a). Hydrolysis of disaccharides was determined from the rate of release of galactose as measured by a coupled assay employing galactose dehydrogenase and NAD^+ (Hall, 1976a). For all assays, 1 unit of activity is the hydrolysis of

1 nmol of substrate per min. Protein concentrations were determined from the absorbance at 225 nm as previously described (Hall, 1976a). A Gilford Model 250 recording spectrophotometer was used for all determinations.

Enzyme Purifications. *ebg* enzymes were purified from the constitutive strains listed in Table I. Mass cultures (16–30 L) were grown to saturation in succinate minimal medium (Hall, 1976a) and harvested by continuous flow centrifugation at 4 °C, and the cells were disrupted by grinding with alumina. All subsequent steps were carried out at 4 °C. The cell paste was suspended in 12.5 mM buffer, and alumina plus cell debris was removed by centrifugation at 13000*g*. The supernatant was adjusted to 27 mg/mL streptomycin sulfate, held for 15 min, and centrifuged at 35000*g* to remove nucleic acids. The supernatant was adjusted to 43% saturation by the dropwise addition of neutralized saturated ammonium sulfate, and the solution was centrifuged for 20 min at 35000*g*. The resulting pellet was dissolved in a minimum volume of 50 mM buffer containing 7 mM β-mercaptoethanol. All buffers in subsequent steps also contained 7 mM β-mercaptoethanol.

The solution was applied to a 5 × 55 cm agarose gel filtration column (Bio-Gel A1.5M) and eluted with 50 mM buffer. The most active fractions were pooled and applied to a 2.5 × 20 cm column of DEAE-Sepharose (Pharmacia) that had been equilibrated with 50 mM buffer. The column was eluted with a 1-L linear gradient from 50 mM buffer to 100 mM buffer containing 0.25 M KCl.

The fractions of highest and constant specific activity were pooled and concentrated by ultrafiltration. Concentrated preparations were stored in small aliquots at −80 °C.

Criterion of Purity. Forty-microgram samples of purified enzymes were applied to $NaDodSO_4$–polyacrylamide gels as previously described (Hall, 1976a). Following electrophoresis, the gels were stained with Coomassie brilliant blue dye, destained, and scanned on a Gilford Model 2420 tube gel scanner. The areas of the protein peaks were determined by weighing of the graph paper, and the purities of the preparations were estimated from the relative peak areas. Purities are given in Table I. The preparations were not subjected to native gel electrophoresis, because the hexameric enzyme dissociates to give multiple bands under these conditions. However, several preparations were subjected to two-dimensional gel electrophoresis according to the method of O'Farrell (1975). Under these conditions, where the first dimension is isoelectric focusing and the second dimension is $NaDodSO_4$ electrophoresis, those preparations exhibited a single protein spot.

Kinetic Analyses. Purified enzymes were used for all kinetic analyses. Enzyme concentrations were estimated from the activity of the preparation under standard conditions (5 mM ONPG) immediately prior to kinetic determinations.

Substrate concentrations employed were determined by (1) solubility of the substrate and (2) contamination of the disaccharide substrates by galactose. Concentration ranges employed were the following: *o*-nitrophenyl β-galactoside (ONPG) and *p*-nitrophenyl β-galactoside (PNPG), 0.039–20 mM; *o*-nitrophenyl β-fucoside (ONPF), 0.3125–10 mM; *p*-nitrophenyl β-fucoside (PNPF), 0.625–20 mM; *o*-nitrophenyl α-L-arabinoside (ONPA), 0.3125–10 mM; *p*-nitrophenyl α-L-arabinoside (PNPA), 0.15625–5 mM; lactose, 0.195–100 mM; lactulose, 0.78–100 mM; galactosyl-D-arabinose, 0.39–50 mM; lactobionic acid, 0.78–25 mM.

K_m and V_{max} were calculated by a computer program that weights velocities according to the hypothesis that each velocity has a standard error proportional to its true value (Cornish-Bowden, 1976a). Tables II and IV show the best estimate for each parameter ± the standard error. V_{max} can be converted to K_{cat} (moles of substrate hydrolyzed per mole of active sites per second) by the equation $K_{cat} = 0.002V_{max}$, assuming one active site per M_r 120000 subunit (Hall, 1976a).

Reagents. Lactose, lactulose, ONPG, ONPF, PNPG, and PNPF were obtained from Sigma Chemical Co. Lactobionic acid was obtained from Aldrich Chemical Co. Galactosyl-D-arabinose was from Pfanstiehl Chemical Co. ONPA and PNPA were prepared by condensation of the sodium salt of the nitrophenol with acetobromoarabinose as described by Sinnott & Souchard (1973). The melting point range of ONPA was 138.5–140 °C (lit. mp 141–143 °C; Marshall et al., 1977) and that of PNPA was 203–204 °C with decomposition (lit. mp 197–199 °C; Marshall et al., 1977).

Thermal Inactivations. Enzyme concentrations were adjusted to 1.0 mg/mL in 125 mM buffer containing 7 mM β-mercaptoethanol, and the solution was incubated at 50 °C. Samples were removed at 2-min intervals for at least 12 min and diluted appropriately into chilled buffer.

Results and Discussion

The striking property of enzymes that distinguishes them from ordinary chemical catalysts is their high degree of specificity for their substrates. The importance of that high specificity is obvious where, within a cell, an enzyme must be able to clearly distinguish among an enormous number of potential substrates. Specificity, on the other hand, is fortunately not absolute, or there could be no such thing as a synthetic substrate. This study is concerned with changes in specificity during evolution of an enzyme. Intuitively, specificity means "the ability to discriminate among substrates"; however, if we are to discuss changes in specificity, we must be able to express specificity in quantitative terms. For that reason, I shall use the value V_{max}/K_m to mean specificity. That definition is chosen because, at low substrate concentrations, the velocity of an enzymatic reaction is determined almost entirely by V_{max}/K_m. However, V_{max}/K_m is clearly not the whole story in terms of biologically meaningful activity since under some conditions the intracellular substrate concentration may greatly exceed the K_m, in which case the velocity is determined essentially by the V_{max}. For that reason the tables show V_{max}, K_m, and their ratio (V_{max}/K_m) for each enzyme and substrate.

V_{max} and K_m as shown in the tables are "best estimate" values. While it is formally correct to refer to the observed value as the "apparent K_m", I shall simply use the term K_m to mean apparent K_m.

Changes in Disaccharide Specificity. Table II shows that the wild-type enzyme, *ebg*^{o}, exhibits a primary specificity for ONPG, with PNPG as a fairly close second. The specificity for disaccharides is over 400-fold lower than the specificity for nitrophenyl galactosides. The low specificity is consistent with the observation that strains synthesizing the wild-type enzyme are unable to utilize any of those disaccharides. The substrates can be ranked in the order of specificity as ONPG > PNPG ≫ lactose > lactulose > galactosylarabinose ≫ lactobionate.

The class I enzymes A, D, and E are the result of single point mutations in the *ebgA* gene. Their activities are similar to each other on all six substrates, and Table III gives the mean ± standard error of the mean for the class. With respect to the synthetic substrates, the *relative* specificities are virtually unchanged: class I enzymes still prefer ONPG to PNPG. On both substrates class I enzymes have a dramatically reduced V_{max}. An even more dramatic reduction in the K_m for these

Table II: β-Galactoside Series of Substrates[a]

enzyme		ONPG	PNPG	lactose	lactulose	Gal-Ara	lactobionate
O	V_{max}	192 000 ± 30 000	76 800 ± 13 700	620 ± 85	270 ± 56	52 ± 9.8	no detectable
	K_m	70 ± 11.7	50 ± 9.9	150 ± 24	180 ± 43	64 ± 14	activity
	V/K	2730 ± 49	1530 ± 43	4.0 ± 0.13	1.5 ± 0.06	0.81 ± 0.04	
A	V_{max}	17 000 ± 364	10 300 ± 871	4200 ± 334	113 ± 6.96	340 ± 33	no detectable
	K_m	4.19 ± 0.15	3.52 ± 0.53	22.1 ± 2.96	57 ± 4.7	23.5 ± 3.3	activity
	V/K	4040 ± 78	2930 ± 247	190 ± 13	2.01 ± 0.06	14.2 ± 0.78	
D	V_{max}	12 100 ± 635	8400 ± 462	3600 ± 322	52.2 ± 1.61	129 ± 10.6	no detectable
	K_m	3.49 ± 0.33	3.44 ± 0.34	25.4 ± 3.63	26.3 ± 1.30	9.0 ± 1.3	activity
	V/K	3500 ± 183	2400 ± 137	142 ± 10	1.98 ± 0.048	14.3 ± 1.16	
E	V_{max}	13 400 ± 2600	11 300 ± 277	2900 ± 240	42.8 ± 0.85	86.6 ± 8.8	no detectable
	K_m	(3.0 ± 0.88)	3.65 ± 0.13	19.2 ± 2.7	18.7 ± 0.65	9.1 ± 1.6	activity
	V/K	4500 ± 680	3100 ± 54	149 ± 11.8	2.29 ± 0.041	9.5 ± 0.95	
B	V_{max}	11 200 ± 149	8000 ± 220	2700 ± 371	2200 ± 184	460 ± 32	no detectable
	K_m	0.65 ± 0.017	0.36 ± 0.02	72 ± 13	33.7 ± 4.2	34.4 ± 3.1	activity
	V/K	17 400 ± 300	22 300 ± 882	36.9 ± 2.1	66 ± 3.7	13.3 ± 0.40	
C	V_{max}	13 100 ± 295	8400 ± 126	1760 ± 104	1560 ± 65	340 ± 29.4	no detectable
	K_m	0.51 ± 0.024	0.38 ± 0.013	45.7 ± 3.8	22.3 ± 1.56	22.1 ± 2.75	activity
	V/K	25 800 ± 852	22 300 ± 560	38 ± 1.27	69.7 ± 2.5	15.2 ± 0.76	
F	V_{max}	12 800 ± 1170	8100 ± 198	2600 ± 210	1900 ± 88	270 ± 14	no detectable
	K_m	0.54 ± 0.018	0.34 ± 0.021	59 ± 6.5	22.7 ± 1.76	18.2 ± 1.44	activity
	V/K	23 700 ± 561	20 800 ± 842	43.7 ± 1.67	83.6 ± 3.33	14.7 ± 0.51	
AB	V_{max}	2900 ± 64	1800 ± 36	1270 ± 35	385 ± 10	630 ± 21	82 ± 12
	K_m	0.059 ± 0.005	0.073 ± 0.005	0.68 ± 0.04	8.3 ± 0.33	2.75 ± 0.16	13.6 ± 2.7
	V/K	49 800 ± 3800	24 500 ± 1200	1880 ± 68	46.5 ± 0.83	230 ± 6.9	6.0 ± 0.44
AC	V_{max}	3400 ± 66	2170 ± 42	1560 ± 32	440 ± 13	730 ± 31	156 ± 15
	K_m	0.047 ± 0.004	0.072 ± 0.004	0.81 ± 0.033	8.5 ± 0.38	2.84 ± 0.20	22 ± 2.9
	V/K	71 900 ± 5300	30 000 ± 1500	1920 ± 48	51.7 ± 1.0	256 ± 9.6	6.93 ± 0.25
DB	V_{max}	3600 ± 51	2220 ± 17	1550 ± 48	520 ± 39	900 ± 88	180 ± 14
	K_m	0.049 ± 0.003	0.064 ± 0.002	0.69 ± 0.045	8.2 ± 0.92	3.1 ± 0.50	23 ± 2.3
	V/K	73 500 ± 3900	35 000 ± 740	2230 ± 92	63.8 ± 3.2	290 ± 23.2	7.85 ± 0.22
DC	V_{max}	3140 ± 76	1860 ± 32	1020 ± 46	280 ± 13	540 ± 34	61 ± 10
	K_m	0.061 ± 0.006	0.076 ± 0.004	0.63 ± 0.062	5.7 ± 0.45	2.2 ± 0.25	10.5 ± 2.5
	V/K	51 400 ± 4000	24 500 ± 1000	1600 ± 104	50 ± 2.0	245 ± 15.6	5.8 ± 0.56
AF	V_{max}	3110 ± 51	2010 ± 29	1600 ± 13	470 ± 13	940 ± 25	67 ± 8
	K_m	0.055 ± 0.003	0.073 ± 0.003	0.82 ± 0.016	6.2 ± 0.29	2.8 ± 0.14	15.4 ± 2.4
	V/K	56 800 ± 2800	27 400 ± 1000	1960 ± 30	76 ± 2.0	330 ± 11	4.3 ± 0.23
AG	V_{max}	4100 ± 200	2660 ± 32	1710 ± 86	480 ± 23	840 ± 54	74 ± 17
	K_m	0.094 ± 0.013	0.11 ± 0.004	0.89 ± 0.086	10.7 ± 0.81	4.3 ± 0.42	8.9 ± 3.0
	V/K	43 400 ± 4800	23 400 ± 560	1900 ± 110	45 ± 1.5	195 ± 8.4	8.3 ± 1.3
DH	V_{max}	2800 ± 50	1800 ± 25	1200 ± 40	400 ± 19	400 ± 46	70 ± 6.9
	K_m	0.057 ± 0.003	0.073 ± 0.003	1.21 ± 0.069	8.2 ± 0.58	2.2 ± 0.44	11.8 ± 1.6
	V/K	48 800 ± 2500	24 700 ± 900	1000 ± 32	49 ± 1.6	183 ± 21	5.9 ± 0.32
CI	V_{max}	3240 ± 85	1960 ± 51	1240 ± 25	380 ± 15	740 ± 24	114 ± 14
	K_m	0.060 ± 0.005	0.076 ± 0.006	0.76 ± 0.03	7.5 ± 0.44	3.5 ± 0.18	18.4 ± 3.0
	V/K	54 100 ± 4100	25 500 ± 1600	1610 ± 41	51 ± 1.4	211 ± 5	6.2 ± 0.32
BJ	V_{max}	5700 ± 92	2950 ± 100	2000 ± 48	514 ± 17	909 ± 38	140 ± 17
	K_m	0.106 ± 0.004	0.069 ± 0.008	0.93 ± 0.042	7.8 ± 0.39	3.5 ± 0.23	15.2 ± 2.5
	V/K	53 600 ± 1800	42 900 ± 3800	2150 ± 57	66 ± 1.5	261 ± 8.4	9.0 ± 0.52
AGN	V_{max}	1730 ± 68	1180 ± 19	590 ± 24	215 ± 4.0	349 ± 24	370 ± 10
	K_m	0.081 ± 0.009	0.079 ± 0.004	0.69 ± 0.06	6.5 ± 0.19	4.9 ± 0.51	3.0 ± 0.17
	V/K	21 500 ± 2000	14 800 ± 570	850 ± 45	33 ± 0.48	70 ± 3.0	123 ± 4.2

[a] V_{max} is in units mg^{-1}; K_m is in mM substrate concentration.

Table III: Summary of Data in Table II by Class[a]

		ONPG	PNPG	lactose	lactulose	galactosyl-arabinose	lactobionate
class O (n = 1)	V_{max}	192 000	76 800	620	270	52	no detectable
	K_m	70	50	150	180	64	activity
	V/K	2730	1530	4.0	1.5	0.81	
class I (n = 3)	V_{max}	14 166 ± 1465	10 000 ± 850	3566 ± 375	69 ± 22	185 ± 78	no detectable
	K_m	3.56 ± 0.345	3.54 ± 0.061	22 ± 1.8	34 ± 12	13.9 ± 4.8	activity
	V/K	4013 ± 288	2810 ± 210	160 ± 15	2.1 ± 0.1	12.7 ± 1.6	
class II (n = 3)	V_{max}	12 367 ± 590	8166 ± 120	2353 ± 298	1887 ± 184	356 ± 55	no detectable
	K_m	0.56 ± 0.043	0.360 ± 0.012	59 ± 7.6	26 ± 3.7	25 ± 4.9	activity
	V/K	23 300 ± 2524	21 800 ± 500	39.5 ± 2.1	73.1 ± 5.4	14.4 ± 0.6	
class IV (n = 9)	V_{max}	3554 ± 297	1958 ± 256	1461 ± 101	430 ± 26	737 ± 61	105 ± 15
	K_m	0.065 ± 0.007	0.076 ± 0.004	0.824 ± 0.058	7.9 ± 0.48	3.02 ± 0.22	15.4 ± 1.6
	V/K	55 922 ± 3414	28 655 ± 2154	1805 ± 122	55 ± 3.5	244 ± 15	6.7 ± 0.5
class V (n = 1)	V_{max}	1730	1180	590	215	349	370
	K_m	0.081	0.079	0.69	6.5	4.9	3.0
	V/K	21 500	14 800	850	33	70	123

[a] Means ± standard error of the mean.

substrates leads to about a 2-fold increase in the specificity for ONPG and PNPG. More dramatic, and certainly more important to the cell, is a 40-fold increase in the specificity for lactose, resulting from an increased V_{max} and a decreased K_m. The specificity for lactulose is virtually unchanged because a drop in the K_m is accompanied by a parallel drop in V_{max}. The specificity of class I enzymes for galactosylarabinose is also improved over 10-fold by increased V_{max} and decreased K_m values. The specificity for galactosylarabinose remains fairly low but is apparently at the threshold of utility to the cell since class I strains exhibit marginal growth on galactosylarabinose.

It is clear that class I enzymes represent a specific adaptation to lactose since the specificity for that substrate is vastly improved while the specificity for other substrates (except galactosylarabinose) remains virtually unchanged. Particularly interesting is the observation that the V_{max} for three substrates (ONPG, PNPG, and lactulose) is reduced, while the V_{max} for lactose and galactosylarabinose is increased. The affinity of class I enzymes for all five substrates (as reflected by apparent K_m) is increased, but the extent of that increase ranges from 20-fold (ONPG) to 4.6-fold (galactosylarabinose). The ranking of substrates according to specificity is ONPG > PNPG > lactose > galactosylarabinose > lactulose $\gg$ lactobionate.

The class II enzymes B, C, and F are also the result of single point mutations in the *ebgA* gene. Again, the activities of the three enzymes are similar with each other on all six substrates. Class II enzymes show no preference for ONPG over PNPG. The V_{max} for both substrates is greatly reduced from that of wild-type enzyme, and that decrease is accompanied by better than 2 order of magnitude decreases in K_m, leading to an order of magnitude increase in the specificities for ONPG and PNPG. V_{max} for lactose is increased and K_m is decreased, leading to a 10-fold increase in lactose specificity. Class II enzymes also show a dramatic increase in V_{max} and a dramatic decrease in K_m for lactulose, leading to a 50-fold increase in lactulose specificity. The specificity of these enzymes for lactose and lactulose is closely paralleled by the relative rates at which class II strains grow on those disaccharides. The specificity for galactosylarabinose is increased over 10-fold by an increased V_{max} and a decreased K_m.

Class II enzymes are as different from class I enzymes as they are from the wild-type enzyme *ebg*^{o}. The K_m of class II enzymes for synthetic substrates is an order of magnitude lower than that of class I enzymes. Among the disaccharides, class II enzymes prefer lactulose to lactose and have biologically meaningful activity toward both, while class I enzymes have meaningful activity toward only lactose. The two classes have some properties in common: both have substantially lower V_{max}'s on ONPG and PNPG than does the wild-type enzyme, both classes have increased affinities for all substrates, and neither class exhibits detectable activity toward lactobionate.

The nine class IV enzymes, each designated by a double letter, differ by two amino acid substitutions from the wild-type enzyme. They have 4-fold lower V_{max}'s on ONPG and PNPG than either class I or class II enzymes, but K_m's that are 3 orders of magnitude lower than those of wild-type enzyme lead to very high specificities for these substrates.

The class IV enzymes have a specificity for lactose that is 450-fold greater than that of the wild-type enzyme. Most of the increased specificity comes from the 2 orders of magnitude reduction in the K_m for lactose. Low K_m results in a specificity that is an order of magnitude higher than that of either class I or class II enzymes. The specificity for lactulose is not similarly increased. Although the K_m for lactulose is lower than that of class II enzymes, the substantially lower V_{max} results in a specificity that is slightly lower than that of class II enzymes.

The specificity of class IV enzymes for galactosylarabinose is 17-fold higher than that of either class I or class II enzymes. The increased specificity results from a lowered K_m and an increased V_{max}. The increased specificity permits class IV strains to grow on galactosylarabinose.

Class IV is the first class of *ebg* enzymes to exhibit detectable activity toward lactobionate. The specificities are low, on the same order as those of the wild-type enzyme for lactose and lactulose. As would be expected from these specificities, class IV strains do not utilize lactobionate.

The class V enzyme AGN shows a reduced V_{max} on lactose, lactulose, ONPG, PNPG, and galactosylarabinose, without significant changes in the K_m for those substrates. The specificity for lactobionate, in contrast, is increased 15-fold compared with the "parental" enzyme AG. This increase results from a 5-fold increase in V_{max} and a 3-fold decrease in K_m.

During selection directed at the "R substituent" of β-galactoside sugars, mutations resulted in amino acid substitutions that altered the kinetic properties of *ebg* enzyme in very specific ways. These mutations always decreased the K_m for β-galactoside compounds. The extent of the decrease was very specific to the class and to the substituent. Changes in V_{max} were likewise dramatic and depended upon the enzyme class and the substituent being considered. The strong specificity of these changes is borne out by the frequency with which V_{max} for one substrate is decreased while that for another substrate is increased when an enzyme is compared with its parental enzyme (see, for instance, enzyme AGN). For the single point substitutions, there is always an increase in V_{max} for the "selected" sugar (lactose for class I and lactulose and lactose for class II). The class IV enzymes are particularly interesting in this respect since they can be selected in two ways: (1) they can be derived from class I enzymes by lactulose selection, in which case there is a 6-fold increase in V_{max} for lactulose, or (2) they may be derived from class II enzymes by selection for galactosylarabinose utilization, in which case there is a 2-fold increase in V_{max} for galactosylarabinose. Class IV enzymes can also be obtained by recombination between a class I and a class II *ebgA* gene, and the enzymes so obtained (AB, AC, DB, and DC) are clearly typical of their class. The enzyme AB, for instance, carries the substitution present in enzyme A and also that present in enzyme B, yet it resembles neither A nor B in its properties. The substitutions in enzymes A and B, each of which profoundly alters the properties of *ebg*^{o} enzyme, when present together in the same polypeptide chain confer a completely new set of specificities on the enzyme. Particularly noticeable are (1) a very high specificity for ONPG and PNPG, (2) a high specificity for galactosylarabinose, and (3) a completely new activity for lactobionate.

We can group the class IV enzymes according to the substitutions they have in common; thus the "A" enzymes are AB, AC, AG, and AF, while the "B" enzymes are AB, DB, BJ, etc. Comparisons of the specificities of class IV enzymes so grouped fail to show any significant differences between groups by *t* tests. Thus we cannot predict from the fact that the class I enzyme A has a higher specificity for lactose than does enzyme D that "A-containing" class IV enzymes will have higher lactose specificities than "D-containing" enzymes. Similarly, we cannot conclude that class I substitutions are more important than class II substitutions in determining the

Table IV: Glycon Series of Substrates[a]

enzyme		ONP-Gal	PNP-Gal	ONP-Fuc	PNP-Fuc	ONP-Ara	PNP-Ara
O	V	192 000 ± 30 000	76 800 ± 13 700	(2500 ± 1900)	(2000 ± 500)	(1500 ± 660)	(8500 ± 4800)
	K	70 ± 11.7	50 ± 9.9	(230 ± 180)	(110 ± 28)	(39 ± 19)	(760 ± 430)
	V/K	2730 ± 49	1530 ± 43	11.0 ± 0.2	18.5 ± 0.4	38 ± 1.8	11.2 ± 0.03
A	V	17 000 ± 364	10 300 ± 871	(940 ± 480)	600 ± 39	920 ± 59	(1300 ± 740)
	K	4.19 ± 0.15	3.52 ± 0.53	(60 ± 32)	43 ± 3	28.5 ± 2	(74 ± 48)
	V/K	4040 ± 78	2930 ± 247	15.5 ± 0.5	13.7 ± 0.16	32 ± 0.3	16.8 ± 0.58
D	V	12 100 ± 635	8400 ± 462	300 ± 53	(886 ± 226)	800 ± 155	*b*
	K	3.49 ± 0.33	3.44 ± 0.34	18 ± 4	(71 ± 20)	31 ± 6.5	*b*
	V/K	3500 ± 183	2400 ± 137	16 ± 0.6	12.5 ± 0.37	26 ± 0.66	21.9 ± 0.8
E	V	13 400 ± 2600	11 300 ± 277	(450 ± 167)	(1200 ± 550)	(760 ± 240)	*b*
	K	(3.0 ± 0.88)	3.65 ± 0.13	(2.5 ± 10)	(85 ± 42)	(28 ± 9.7)	*b*
	V/K	4500 ± 680	3100 ± 54	17 ± 1	13.8 ± 0.6	28 ± 1.3	11 ± 0.12
B	V	11 200 ± 149	8000 ± 220	3200 ± 470	2250 ± 134	1200 ± 160	*b*
	K	0.65 ± 0.017	0.36 ± 0.02	16 ± 2.7	9.6 ± 0.76	23 ± 3.4	*b*
	V/K	17 400 ± 300	22 300 ± 882	203 ± 7	234 ± 6.7	52 ± 1.2	35.5 ± 1.2
C	V	13 100 ± 295	8400 ± 126	3200 ± 132	2440 ± 86	1500 ± 96	450 ± 74
	K	0.51 ± 0.024	0.38 ± 0.013	15 ± 0.7	9.3 ± 0.5	25 ± 1.8	9.0 ± 1.6
	V/K	25 800 ± 852	22 300 ± 560	216 ± 2.1	260 ± 5	59.3 ± 0.6	51 ± 1.7
F	V	12 800 ± 1170	8100 ± 198	3100 ± 172	2050 ± 140	1020 ± 165	540 ± 52
	K	0.54 ± 0.018	0.34 ± 0.021	17 ± 1.12	8.7 ± 0.8	18 ± 3.3	13.1 ± 1.4
	V/K	23 700 ± 561	20 800 ± 842	176 ± 2	235 ± 9.2	56 ± 1.9	41 ± 0.6
AB	V	2900 ± 64	1800 ± 36	1500 ± 167	890 ± 40	780 ± 57	300 ± 50
	K	0.059 ± 0.005	0.073 ± 0.005	4.5 ± 0.7	6.0 ± 0.43	9 ± 0.8	14.4 ± 2.7
	V/K	49 800 ± 3800	24 500 ± 1200	330 ± 20	147 ± 4	87 ± 2.2	20 ± 0.5
AC	V	3400 ± 66	2170 ± 42	1570 ± 99	980 ± 30	900 ± 125	430 ± 77
	K	0.047 ± 0.004	0.072 ± 0.004	3.7 ± 0.34	5.1 ± 0.26	8.1 ± 1.4	17.2 ± 3.3
	V/K	71 900 ± 5300	30 000 ± 1500	425 ± 17	190 ± 4.7	112 ± 5.9	25 ± 0.5
DB	V	3600 ± 51	2220 ± 17	1620 ± 42	1150 ± 47	750 ± 44	(470 ± 150)
	K	0.049 ± 0.003	0.064 ± 0.002	3.7 ± 0.15	6.1 ± 0.4	6.0 ± 0.5	(17 ± 6)
	V/K	73 500 ± 3900	35 000 ± 740	430 ± 7.2	188 ± 5.6	125 ± 3.5	27 ± 1
DC	V	3140 ± 76	1860 ± 32	1490 ± 19	950 ± 50	590 ± 31	(510 ± 118)
	K	0.061 ± 0.006	0.076 ± 0.004	4.3 ± 0.07	6.6 ± 0.5	5.8 ± 0.42	(26 ± 6)
	V/K	51 400 ± 4000	24 500 ± 1000	342 ± 2.5	143 ± 5	101 ± 2.6	19.8 ± 0.4
AF	V	3110 ± 51	2010 ± 29	1380 ± 63	860 ± 14	800 ± 57	288 ± 23
	K	0.055 ± 0.003	0.073 ± 0.003	3.8 ± 0.25	5.0 ± 0.14	8.3 ± 0.8	14 ± 1.2
	V/K	56 800 ± 2800	27 400 ± 1000	360 ± 10	171 ± 2.3	95 ± 2.5	21 ± 0.24
AG	V	4100 ± 200	2660 ± 32	1600 ± 61	1040 ± 30	900 ± 68	400 ± 56
	K	0.094 ± 0.013	0.11 ± 0.004	4.7 ± 0.25	6.2 ± 0.27	7.3 ± 0.7	15.2 ± 2.4
	V/K	43 400 ± 4800	23 400 ± 560	338 ± 7	167 ± 3.4	126 ± 4	26 ± 0.5
DH	V	2800 ± 50	1800 ± 25	1300 ± 96	800 ± 19	680 ± 35	420 ± 36
	K	0.057 ± 0.003	0.073 ± 0.003	4.0 ± 0.43	5.5 ± 0.2	8.0 ± 0.5	21 ± 2
	V/K	48 800 ± 2500	24 700 ± 900	320 ± 14	146 ± 2.8	85 ± 1.7	19.4 ± 0.16
CI	V	3240 ± 85	1960 ± 51	1210 ± 55	830 ± 37	740 ± 74	430 ± 80
	K	0.060 ± 0.005	0.076 ± 0.006	3.5 ± 0.23	5.5 ± 0.4	8.0 ± 1.0	22 ± 4
	V/K	54 100 ± 4100	25 500 ± 1600	347 ± 10	150 ± 5.1	93 ± 3.5	20 ± 0.3
BJ	V	5700 ± 92	2950 ± 100	2380 ± 80	1450 ± 42	1530 ± 140	720 ± 93
	K	0.106 ± 0.004	0.069 ± 0.008	4.5 ± 0.2	5.7 ± 0.3	8.5 ± 0.98	20 ± 2.7
	V/K	53 600 ± 1800	42 900 ± 3800	522 ± 10	254 ± 6	180 ± 6	36.5 ± 0.5
AGN	V	1730 ± 68	1180 ± 19	960 ± 22	608 ± 15	596 ± 13	360 ± 69
	K	0.081 ± 0.009	0.079 ± 0.004	1.3 ± 0.06	1.8 ± 0.1	3.6 ± 0.1	10.8 ± 0.23
	V/K	21 500 ± 2000	14 800 ± 570	706 ± 19	340 ± 13	165 ± 2.2	33 ± 1.1

[a] $V = V_{max}$ in units mg^{-1}, and $K = K_m$ in mM substrate concentration. Values in parentheses have a standard error greater than 20% of the value. [b] The standard error is so great that the value is meaningless.

Table V: Summary of Data in Table IV by Class[a]

		ONP-Gal	PNP-Gal	ONP-Fuc	PNP-Fuc	ONP-Ara	PNP-Ara
class O ($n = 1$)	V_{max}	192 000	76 800	2500	2000	1500	8500
	K_m	70	50	230	110	39	760
	V/K	2730	1530	11	18.5	38	11.2
class I ($n = 3$)	V_{max}	14 166 ± 1465	10 000 ± 850	563 ± 193	895 ± 173	827 ± 48	*b*
	K_m	3.56 ± 0.345	3.54 ± 0.061	34 ± 13	66 ± 12	29 ± 1.1	*b*
	V/K	4013 ± 288	2810 ± 210	16 ± 0.4	13 ± 0.4	29 ± 1.8	29 ± 1.8
class II ($n = 3$)	V_{max}	12 367 ± 590	8166 ± 120	3166 ± 33	2246 ± 112	1240 ± 140	796 ± 302
	K_m	0.56 ± 0.043	0.360 ± 0.012	16 ± 0.6	9.2 ± 0.3	22 ± 2	21 ± 9.7
	V/K	23 300 ± 2524	21 800 ± 500	198 ± 11.8	243 ± 8.5	55.7 ± 2.1	42.5 ± 4.5
class IV ($n = 9$)	V_{max}	3554 ± 297	1958 ± 256	1561 ± 112	994 ± 68	852 ± 91	440 ± 42
	K_m	0.065 ± 0.007	0.076 ± 0.004	4.0 ± 0.14	5.7 ± 0.17	7.7 ± 0.3	18.5 ± 1.3
	V/K	55 922 ± 3414	28 655 ± 2154	379 ± 22	172 ± 12	111 ± 10	24 ± 1.9
class V ($n = 1$)	V_{max}	1730	1180	960	608	596	360
	K_m	0.081	0.079	1.3	1.8	3.6	10.8
	V/K	21 500	14 800	706	340	165	33

[a] Means ± standard error of the mean. [b] Data in Table IV were not adequate for calculation of mean and standard error of the mean.

Table VI: Mean Specificities for Arabinosides and Fucosides Relative to Galactosides

	o-nitrophenyl		*p*-nitrophenyl	
class	fucoside	arabinoside	fucoside	arabinoside
O	0.004	0.014	0.012	0.007
I	0.004	0.007	0.005	0.006
II	0.009	0.003	0.011	0.002
IV	0.007	0.002	0.006	0.001
V	0.033	0.008	0.022	0.002

properties of class IV enzymes.

Changes in the Glycon Specificity. Tables IV and V show the activities of these enzymes on the *o*- and *p*-nitrophenyl β-D-galactosides, β-D-fucosides, and α-L-arabinosides. These substrates represent three substitutions at carbon 5 of the glycon: galactose = $-CH_2OH$, fucose = $-CH_3$, and α-L-arabinose = $-H$.

In general, the changes in V_{max}, K_m, and specificity for fucosides and arabinosides roughly parallel the changes for galactosides.

The K_m of wild-type enzyme is very high for all these substrates. Class I and class II enzymes have lowered K_m's for all substrates, with class II enzymes having lower K_m's than class I enzymes. Wild-type enzyme has a strong preference for galactosides, with 100-fold lower specificities for fucosides and arabinosides. For class I enzymes, that same relationship is evident, despite changes in both K_m and V_{max} for all substrates. Class II enzymes exhibit an increased specificity for fucosides. Unlike the case for galactosides, where the class II enzymes have a 10-fold lower V_{max} than does the wild-type enzyme, the V_{max} of class II enzymes for fucosides is virtually unchanged. Thus, although class II enzymes have increased their specificities for galactosides and for fucosides to roughly the same extent, the means by which these changes were accomplished were quite different.

We can inquire whether any glycon-specific changes occurred during the evolutionary sequence by comparing, for *o*-nitrophenyl and *p*-nitrophenyl substrates, the specificities for fucoside and arabinoside relative to galactoside. Those comparisons (Table VI) suggest that only the class V enzyme AGN has a really significant shift in its glycon specificity. It should be noted that the extent of change in the glycon specificity depends very much upon the aglycon being considered.

Thermal Inactivation. It might be expected that as enzymes accumulate substitutions which alter their substrate specificities, those substitutions would lead to increased thermolability. Studies of thermal inactivation rates at 50 °C using purified enzymes suggest that this is not necessarily the case. For replicate inactivations of A enzyme, the inactivation rate was -0.163 ± 0.017 min^{-1} (mean ± 95% confidence interval), while the rate was -0.142 ± 0.015 min^{-1} for wild-type enzyme. *t* tests revealed no significant differences between any of the classes, and the mean rate for all enzymes considered together was -0.160 ± 0.016 min^{-1}.

Conclusions

It is evident that classification of these *ebg* enzymes based upon V_{max}, K_m, and specificity for a variety of substrates is unambiguous and identical with classification based upon growth parameters. The specificities for disaccharides clearly changed in direct response to the selective pressure applied. Whenever a new physiological function "growth on disaccharide X" was selected, the resulting mutation altered the progenitor enzyme so that V_{max} was increased and K_m was decreased on disaccharide X. It was often the case, however, that when V_{max} for the selected disaccharide X was increased, V_{max} for a synthetic substrate or for another disaccharide was significantly decreased.

Selection directed at the R substituent of β-galactosides did result in alterations in glycon specificity, but in most cases there was little change in the *relative* specificities for the various glycons. There were, however, some shifts in glycon specificity: the wild-type enzyme strongly prefers galactose but makes little distinction between fucose and arabinose as glycons. Class II and class IV enzymes show a distinct preference for fucose over arabinose, and that preference is slightly more exaggerated for the class V enzyme. There was no evidence that these mutations affected the stability of *ebg* enzyme.

From the growth studies, it was obvious that the specificities of the enzymes for the *selected* disaccharides must always have increased. Those studies could not have told us, however, the means by which those specificities were increased. In some cases, increased specificities revealed by this study would not have been predicted from the growth data. In particular, it is generally the case that class IV strains grow about 80% as fast as their class I progenitors on lactose; thus their 10-fold higher specificity for lactose is unexpected. In retrospect, it is clearly explainable since the physiological concentration of lactose inside the cell under our growth conditions is about 20–50 mM. Thus class I enzymes are operating at roughly 50% of V_{max}, where V_{max} is about 3600 units/mg, and class IV strains are operating essentially at a V_{max} of about 1500 units/mg. These data suggest that class I strains would have an advantage over class IV strains under conditions of lactose excess but that the situation would be reversed under conditions of lactose limitation. That, in turn, suggests that the exact nature of the change in an enzyme as new functions are selected may well depend on the amount as well as the availability of the substrate in the environment. The array of *ebg* strains available is sufficient to test those hypotheses directly in the laboratory.

On the basis of an analysis of free-energy changes in enzyme catalysis, Cornish-Bowden (1976b) has predicted that where the attainment of high rates is the primary evolutionary objective enzymes are expected to have K_m values close to the physiological substrate concentrations. This prediction is borne out by these experiments. The enzymes selected for lactose utilization have K_m (lactose) values very close to the physiological 20–50 mM level. The physiological levels of the other disaccharides have not been measured, but correlations between growth rates and the kinetic parameters of these enzymes make it seem likely that they are in the range 5–20 mM. It should be noted that the selective pressure was exactly that mentioned by Cornish-Bowden, maximization of the rate of substrate hydrolysis.

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Characterization of Purified Epstein–Barr Virus Induced Deoxyribonucleic Acid Polymerase: Nucleotide Turnover, Processiveness, and Phosphonoacetic Acid Sensitivity†

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ABSTRACT: The Epstein–Barr (EB) virus induced DNA polymerase has been further purified and characterized with respect to nucleotide turnover activity, processiveness of synthesis, and interaction with phosphonoacetic acid (PAA). The polymerase as purified through denatured DNA–cellulose chromatography was inseparable from a labile nuclease activity associated with an equally labile DNA-dependent nucleotide turnover function. The EB virus induced DNA polymerase even in the absence of detectable nuclease or nucleotide turnover activity was less processive in its synthesis than were lymphocyte α polymerase or procaryotic polymerases, and this processiveness decreased with increasing purity of the enzyme. PAA was shown to inhibit nucleotide incorporation by the EB virus induced DNA polymerase in the presence of nuclease-activated native DNA template in the manner of a pyrophosphate analogue. Under conditions in which the concentration of 3′-hydroxyl termini in the template was more limited, PAA was not inhibitory. PAA likewise failed to significantly decrease the processiveness and the nucleotide turnover function of the polymerase.

The Epstein–Barr (EB) virus induced DNA polymerase found in certain EB virus transformed lymphocytes has been associated with productive viral DNA replication (Goodman et al., 1978; Ooka et al., 1979; Datta et al., 1980) and resembles DNA polymerase activities induced by other herpes viruses (Weissbach et al., 1973; Boezi et al., 1974; Huang, 1975; Allen et al., 1977; Knopf, 1979). The EB virus DNA polymerase has also been shown to copurify through initial chromatography steps with an EB virus induced DNase activity containing inseparable exo- and endonuclease functions (Clough, 1979, 1980). Similar nuclease activities have been reported to copurify partially with the herpes simplex virus induced DNA polymerase (Weissbach et al., 1973; Hoffman & Cheng, 1978, 1979; Knopf, 1979; Ostrander & Cheng, 1980). We report here the further purification of the EB virus DNA polymerase by using chromatography with denatured DNA–cellulose. The resultant enzyme preparation represents a high level of recovery and degree of purity, enabling us to undertake studies not previously feasible on various aspects of enzyme function. Also, polymerase characteristics at varying levels of enzyme purity have been compared.

This study characterizes the purified EB virus induced DNA polymerase with regard to nucleotide turnover, processiveness of synthesis, and inhibition by phosphonoacetic acid (PAA).[1] The purified polymerase was observed to mediate nucleotide turnover from triphosphate to monophosphate in the presence of activated DNA template and more extensively in the presence of poly[d(A-T)] template. Such DNA-dependent turnover of nucleotide is characteristic of procaryotic DNA polymerases, which are thought to mediate this process with an associated nuclease (Huang & Lehman, 1972). The nucleotide turnover exhibited by the EB virus induced DNA polymerase, however, showed certain differences from the procaryotic polymerase associated turnover. The processiveness of a DNA polymerase refers to the average number of nucleotides incorporated with each polymerase–template binding event. When this parameter was determined for the EB virus induced DNA polymerase in the presence or absence of associated turnover and nuclease activities, the number of nucleotides added per polymerase–template binding event appeared to decrease with increasing purity of the enzyme preparation and in all cases was a lower number than the processiveness of lymphocyte α polymerase or procaryotic polymerases. Studies of PAA inhibition showed that PAA was most inhibitory to the EB virus induced polymerase under assay conditions where a template with a high concentration of 3′-hydroxyl termini was present. PAA had little effect on polymerase processiveness or nucleotide turnover. From these data, we concluded that PAA did not always function as a pyrophosphate analogue as previously suggested (Leinbach et al., 1976).

Materials and Methods

Materials. [*methyl*-^{3}H]Thymidine 5′-triphosphate (55 Ci/mmol) was from Schwarz/Mann, and [α-^{32}P]deoxy-

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[1] Abbreviations used: PAA, phosphonoacetic acid; EBV, Epstein–Barr virus; PEI, poly(ethylenimine); Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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