Distinct Sites for Deoxyguanosine and Deoxyadenosine Phosphorylation on a Monomeric Kinase from *Lactobacillus acidophilus*†

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ABSTRACT: Base-line separation of two paired deoxynucleoside kinase activities (deoxycytidine/deoxyadenosine and deoxyguanosine/deoxyadenosine kinase), previously resolved as overlapping peaks from Blue Sepharose, has now been achieved. The improved separation and recovery in relatively small volumes were accomplished by eluting Blue Sepharose with a bisubstrate mixture: 0.5 mM dCyd plus 1 mM ATP released dCyd/dAdo kinase, and 1 mM dGuo plus 5 mM ATP eluted dGuo/dAdo kinase. The latter pair of activities showed copurification through UDP–Sepharose affinity chromatography and HPLC anion-exchange chromatography. The HPLC preparation appeared to be homogeneous, on the basis of nondenaturing polyacrylamide gel electrophoresis at several gel concentrations and pH values. Both dGuo and dAdo kinase activities coincided with the protein band. A single band of protein was also observed upon sodium dodecyl sulfate gel electrophoresis. The estimated molecular weight of the denatured protein (56 000) agrees closely with values obtained for native activity by sedimentation equilibrium or gel permeation chromatography. The rate of dAdo phosphorylation was found to be stimulated more than 3-fold by the presence of dGuo, and dGuo kinase was also slightly activated by the presence of dAdo. This mutual activation indicates that dGuo and dAdo kinase activities do not share a common site. Selective chemical inactivation of dGuo kinase by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine eliminated the ability of dGuo to stimulate dAdo kinase in parallel with the loss of dGuo kinase activity. These lines of evidence strongly suggest that dGuo and dAdo kinase activities are functions of separate sites on a monomeric polypeptide and that these sites may be in allosteric communication.

Lactobacillus acidophilus R-26 exhibits an unusual dependency on deoxynucleoside salvage to satisfy its DNA precursor requirements, since it lacks a ribonucleotide reductase essential for deoxyribonucleotide synthesis de novo. Although a single deoxynucleoside is sufficient to meet this requirement, due to deoxyribosyl transfer between bases, all four deoxyribonucleoside kinases are necessary to generate the four deoxynucleotide precursors of DNA synthesis.

Three of the four deoxynucleoside kinases of *Lactobacilli* appear to be organized pairs of activities. In previous work from this laboratory (Deibel & Ives, 1977), a deoxycytidine/deoxyadenosine kinase pair was resolved from linked deoxyguanosine/deoxyadenosine kinases, whereas thymidine kinase was distinct and separable from the other three deoxynucleoside kinase activities (Durham & Ives, 1971). Unlike the deoxynucleoside kinases of *Bacillus subtilis*, in which two nucleosides appear to share a common active site, the *Lactobacillus* enzymes have separate sites for each of two nucleosides which each enzyme phosphorylates. This conclusion is based on evidence for kinetic interactions between sites—saturation of one site by its nucleoside substrate or nucleotide products stimulates the turnover of the other nucleoside (Deibel et al., 1977)—and on preliminary evidence for selective chemical inactivation of one of the two active sites (Deibel & Ives, 1977).

It is now desirable to learn if these paired enzymes are truly examples of multifunctional enzymes, which, in the simplest case, can be defined as a polypeptide chain having two or more different catalytic sites and which is the product of a single gene (Kirshner & Bisswanger, 1976). In this paper, we will present several lines of evidence which imply that separate sites for dGuo and dAdo phosphorylation reside on a single polypeptide chain and that these sites may interact allosterically.

Experimental Procedures

Materials. Nucleosides and nucleotides were from Sigma Chemical Co. and P-L Biochemicals, except that ATP used for column elution was purchased from Plenum Scientific Research. Tritiated nucleosides were from ICN and Amersham Corp. 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine (FSO_2BzAdo)[1] was obtained from Sigma. Agarose beads (Sepharose CL-6B) were produced by Pharmacia, UDP–Sepharose was supplied by P-L Biochemicals, and Cibacron Blue 3GA was a generous gift from Ciba-Geigy (Basel). Reagents for polyacrylamide gel electrophoresis and Bradford protein determinations were supplied by Bio-Rad, and fluorescamine was purchased from Roche.

Bacterial Cultures and Enzyme Extraction. Lactobacillus acidophilus cells (ATCC 11506) were grown as described previously (Deibel & Ives, 1978), but with glucose reduced to 1%. Cells were harvested in mid-log phase (A_{550} = 1.3), 6 h after addition of 500 mL of inoculating culture to a 24-L fermentation mixture, with a yield of about 93 g. Cells were stored frozen in 10-g portions. Cells were disrupted by suspending 16 g in 100 mL of extraction buffer (0.1 M Tris-HCl, pH 8.0, 3 mM EDTA, and 10 μM phenylmethanesulfonyl fluoride) and flailing in a Bead-Beater (Biospec Products) containing about 200 mL of glass beads for a total of 15 min. The container was jacketed with ice, and the contents were allowed to cool for 5 min after running 5 min. The homogenized bacterial extract was decanted, centrifuged, and subjected to streptomycin and ammonium sulfate fractionation,

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[1] Abbreviations: FSO_2BzAdo, 5′-[*p*-(fluorosulfonyl)benzoyl]-adenosine; dAK, deoxyadenosine kinase activity; dGK, deoxyguanosine kinase activity; dGp_4A, 5′-deoxyguanosine 5′-adenosine P^1,P^4-tetraphosphate; SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; DMF, dimethylformamide; HEPES, *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid; HPLC, high-pressure liquid chromatography; Bis-Tris-HCl, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane hydrochloride.

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as described previously (Deibel & Ives, 1978), and stored at −20 °C in 50% glycerol until needed for further purification.

Cibacron Blue 3G-A substituted agarose was prepared by a modification of the method of Böhme et al. (1972), increasing the concentration of dye to produce substitutions of 4–5 μmol/mL of gel (Sepharose CL-6B, Pharmacia).

Gel Electrophoresis. Two discontinuous buffer systems were used for polyacrylamide slab gel electrophoresis: the Tris–glycine/Tris-HCl system used by Laemmli (1970), which has a pH of 8.8 at room temperature (actually about 9.5 at the 4 °C temperature used), and a Tris–diethylbarbiturate/Tris-HCl system (Williams & Reisfeld, 1964), pH 7.5 at room temperature. These buffers were used both in a nondenaturing mode (no detergent or thiol present), in conjunction with activity measurements, and in a denaturing mode for subunit analysis. The R_f's of the enzyme activity were determined by cutting nondenaturing gel channels into 2-mm slices and assaying as described by Deibel & Ives (1977). Protein bands were visualized by silver staining (Sammons et al., 1981).

Determination of Molecular Weight. The molecular weights of the combined dCyd, dAdo, and dGuo kinase activities partially purified on a Blue Sepharose column (fraction IV, Results) were determined by sedimentation equilibrium in a Beckman Airfuge (Pollet et al., 1979). Samples consisted of 150 μL of fraction IV (4 mg/mL) in 0.1 M Tris-HCl (pH 8.0) containing 1 mM MgATP and 0.5% dextran T40, and standard proteins (bovine serum albumin, ovalbumin, and α-chymotrypsinogen A, 5 mg/mL), in the same buffer, were centrifuged in separate tubes. Centrifugation was carried out for 18 h at 45 000 rpm (7.5 psig air pressure) using a 30° fixed-angle rotor and cellulose propionate tubes. The air supplied to the drive was chilled by passage through copper coils immersed in ice. The depletion of protein from the top of the stabilization gradient was analyzed in eight successive 10-μL samples withdrawn from the meniscus. A rack and pinion device was used to follow the meniscus more accurately while sampling with a capillary pipet. The kinase activity or protein concentration was determined in each fraction, and the natural logarithm of this was plotted against the square of the radial distance from the axis of rotation. The slopes of these plots were related to the molecular weights of the standard proteins and used to estimate the molecular weights of the kinase activities (Pollet et al., 1979).

Reaction of FSO_2BzAdo with dGK/dAK. Eight microliters of 25 mM FSO_2BzAdo in DMF was added to 192 μL of dGK/dAK (63 μg of fraction IVB protein, 0.142 unit of dGK, and 0.033 unit of dAK) in 50 mM HEPES, pH 8.0. From this reaction mixture incubated at 4 °C, 20-μL aliquots were withdrawn at 0, 40, 100, and 180 min, diluted with 80 μL of 20 mM Tris-HCl (pH 8.0) containing 15% glycerol, and assayed for residual activities.

Determination of Enzyme Activities and Protein Concentrations. Enzyme assays were carried out as described by Deibel & Ives (1978) except that Whatman DE-81 anion-exchange paper and an improved washing device (Ives, 1984) were used. It was confirmed that the reaction velocity was constant throughout the 30-min fixed-time assay period. A unit of activity is defined as the amount of enzyme phosphorylating a nanomole of deoxynucleoside in 1 min at 20 °C. Protein determinations employed the Bradford (1976) or fluorescamine (Udenfreund, et al., 1972) method.

Results

Purification of Deoxyguanosine/Deoxyadenosine Kinase. Improved Blue Sepharose Fractionation. A step 3 enzyme fraction (precipitated twice with ammonium sulfate) was prepared as described by Deibel & Ives (1977, 1978). Five milliliters of this fraction (160 mg) was diluted to 300 mL with 15 mM potassium phosphate (pH 6.6) containing 15% glycerol and applied to a 35-mL column (25 mm diameter × 71 mm) of Cibacron Blue 3GA substituted Sepharose. After the column was washed with 500 mL of equilibration buffer, the column pH was readjusted to pH 8.0 with elution buffer (15 mM potassium phosphate, pH 8.0, containing 15% glycerol). Lactate dehydrogenase was then eluted with 350 mL of 2 mM NADH in elution buffer. This was followed by 300 mL of 1 mM AMP in elution buffer, a procedure which was found to eliminate another persistent impurity in the electrophoretic pattern. We have discovered that the *Lactobacillus* deoxynucleoside kinases can be eluted with much greater specificity, and with less tailing, by use of both substrate components of the ternary enzyme–substrate complex. Thus, dCyd/dAdo kinase was eluted with 1 mM MgATP plus 0.5 mM dCyd in the elution buffer, and dGuo/dAdo kinase was eluted with 5 mM MgATP plus 1 mM dGuo (see Figure 1). Approximately 1 L of elutrient was required to bring off the last traces of dCyd/dAdo kinase, but the bulk of the activity was contained in about 250 mL. In contrast to the use of ATP as the only elutrient (Deibel & Ives, 1977, 1978), this bisubstrate elution procedure affords base-line separation of the two deoxynucleoside kinase pairs with recovery in substantially smaller volumes. The peak fractions were concentrated to about 10 mL on an Amicon YM10 membrane and are designated fractions IVA and IVB, respectively. Fraction IVB contained 2.2 mg of protein and 59% of the dGK activity applied to the column (48% overall recovery). This preparation phosphorylates mainly dGuo and dAdo. The only other nucleosides phosphorylated at a detectable rate were dCyd and Guo, each at only about 2% of the rate of dGuo phosphorylation, when assayed at 20 μM concentrations. Thus, it can be assumed that none of the dAK activity in our subsequent preparations is contributed by the dCyd/dAdo kinase.

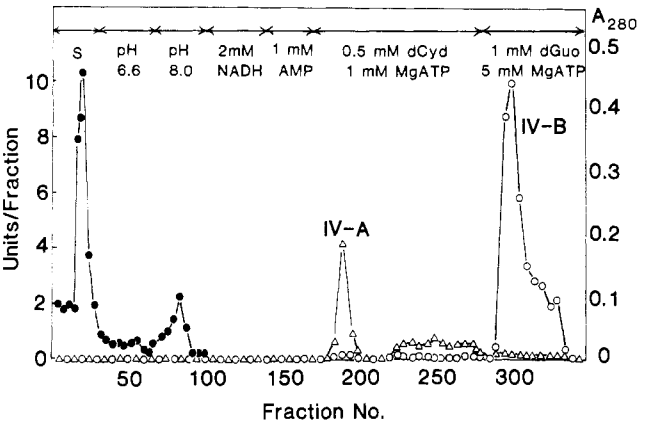

FIGURE 1: Bisubstrate elution of Blue Sepharose to separate dGuo kinase from dCyd kinase and from bulk protein. The column was prepared as described under Experimental Procedures, and elution was carried out as shown (20-mL fractions). Of the total protein applied, 65% emerged prior to addition of UV-absorbing elutrients. (●) A_{280}; (△) dCK activity; (○) dGK activity.

UDP–Sepharose Affinity Chromatography. UDP strongly inhibits the *Lactobacillus* deoxynucleoside kinases, particularly dGuo kinase (Durham & Ives, 1971). A commercial preparation of UDP–Sepharose, in which UDP was linked through the ribose moiety, was found to retain dGuo/dAdo kinase activities quantitatively. A 1-mL column of this medium was equilibrated with 20 mM Tris-HCl (pH 8.0) containing 25 mM $MnCl_2$ and 10% glycerol. A 200-μL sample of fraction IVB (42 μg) was diluted 15-fold with equilibration buffer and

Table I: Purification of dGuo/dAdo Kinases from *L. acidophilus* R-26

fraction	protein (mg)	sp act.[b] dGK	sp act.[b] dAK	yield of dGK (%)
(I) supernatant extract	5900	0.19	0.05	100
(II) streptomycin supernatant	2030	0.48	0.13	87
(III) 65% $(NH_4)_2SO_4$ ppt	800	1.13	0.31	81
(IVB) Blue Sepharose[a]	2.2	52.	12.5	48
(V) UDP–Sepharose[a]	2[c]	320.	30.	47
(VI) HPLC anion exchange[a]	0.11[c]	850.	100.	2.

[a] Only a portion of the preceding fraction was applied as detailed in the text; yields are corrected to the entire amount. [b] Specific activities are expressed as nanomoles per minute per milligram of protein. [c] Values in micrograms.

applied to the column. The column was washed with 30 mL of 0.3 M NaCl dissolved in the equilibration buffer, and then the dGuo/dAdo kinase activity was eluted with 5 mM EDTA in 20 mM Tris (pH 8.0) and 10% glycerol. The chelation of the Mn^{2+} resulted in quantitative desorption of the enzyme in a volume of only 5–6 mL. The active fractions were concentrated to 1 mL (2 μg) on an Amicon YM10 membrane (fraction V).

Anion-Exchange HPLC Fractionation. The few remaining impurities in fraction V were removed from the enzyme protein by anion exchange at two pHs, using a Pharmacia FPLC system fitted with a Mono-Q column. Fraction V concentrate (250 μL) was diluted to 500 μL with 20 mM Bis-Tris-HCl, pH 5.4, containing 10% glycerol, and injected onto the column equilibrated with the same buffer. After the column was washed with 2.5 mL of equilibration buffer, elution was brought about with a 25-mL linear gradient of NaCl (0–0.30 M) dissolved in equilibration buffer, pumping at rate of 0.75 mL/min. Activity peaks from four such column runs were combined, concentrated, and applied to the Mono-Q column equilibrated at pH 8.0 with 20 mM Tris-HCl containing 10% glycerol. Gradient elution was carried out as before, except at pH 8.0. The activity peak was concentrated on an Amicon YM10 membrane (fraction VI). The results of these purification procedures are summarized Table I. The purification factor of 4500 must be regarded as a very conservative estimate, since both activities (particularly dAK) become very labile in the course of the final ion-exchange procedures, and because any traces of Tris buffer remaining in the final dialyzed sample would inflate the estimated protein in the fluorescamine assay.

Purity Analysis of Fraction VI. The criteria chosen for proof of purity had to take into account the very limited amount of protein available from the final purification steps. Thus, it was necessary to use silver staining (Sammons et al., 1981) to visualize submicrogram quantities of protein on electrophoretic gels. Isoelectric focusing proved to be impractical due to persistent interference with the staining by traces of ampholyte. We therefore sought to demonstrate that enzyme activity was associated with the single electrophoretic band after separation on nondenaturing gel electrophoresis, varying both gel concentration and pH. Hedrick & Smith (1968) have shown that two proteins having different charges and different molecular weights can be expected to exhibit identical electrophoretic R_f's at only one gel concentration. Therefore, any significant protein impurities can be expected to be resolved from the enzyme by altering the acrylamide concentration of the separating gel. In fact, only one band was detectable on gels varied from 10–16% acrylamide. Figure 2 shows the congruence of dGK and dAK activities and a

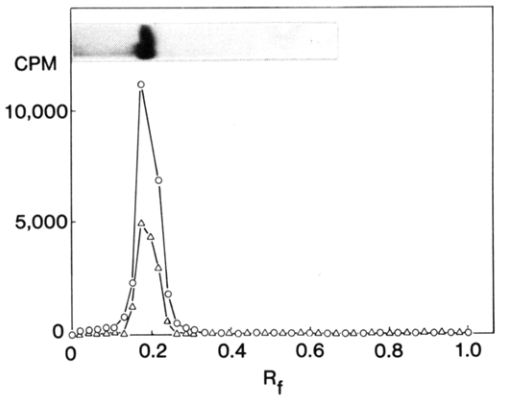

FIGURE 2: Congruent electrophoretic migration of protein, dGK, and dAK in a 16% polyacrylamide gel. Electrophoresis and enzyme assays were carried out as described under Experimental Procedures. One channel was cut into 2-mm slices for assays, and a parallel channel was stained as shown, $R_f = 0.17$. No stainable material was seen beyond $R_f = 0.6$. (○) dGK activity; (△) dAK activity.

stained protein band, developed on a 16% polyacrylamide gel; similar comigration of activities with a single protein band was obtained with 12% and 14% gels. Further confirmation of enzyme homogeneity is found in the fact that deoxyadenosine and deoxyguanosine kinase activities comigrate with a single band of protein at pH 7.5 (not shown), as well as at pH 8.8. Assuming that the enzyme and a major impurity could both have the same molecular weight and net charge at one pH, it is very doubtful that their charges could change by an identical degree at the other pH. These lines of evidence, together with the evident homogeneity of the denatured protein (below), make it very likely that the deoxyguanosine and deoxyadenosine kinase activities are still associated in a homogeneous protein.

Comparison of the Molecular Weight of the Native Protein with That of Denatured Polypeptide. The molecular weights of the three deoxynucleoside kinase activities of *Lactobacillus acidophilus* R26 have been found to be identical by two substantially different procedures. In an earlier report from this laboratory, gel permeation chromatography of the unresolved dCyd, dAdo, and dGuo kinase activities on Bio-Gel P-150 yielded a molecular weight of 50000 ± 4000 for all three activities (Deibel & Ives, 1977). The molecular weights of the mixed fraction IV kinase activities have also been determined by a sedimentation equilibrium method described by Pollet et al. (1979). With standard proteins of known molecular weights, this method affords an approximate accuracy of ±5% and an approximate reproducibility of ±3% and should be considered the more reliable estimate. The molecular weights of the dCyd, dAdo, and dGuo kinase activities were found to be identical by this method also, 60000. Considering the different variables involved in the gel permeation and sedimentation equilibrium methods (Stokes radius vs. partial specific volume, respectively), the results of the two molecular weight determinations agree quite well.

Figure 3 shows reduced and denatured enzyme run on discontinuous SDS–polyacrylamide gel electrophoresis, using the procedure of Laemmli (1970). Only a single band is seen (M_r ~56000), again attesting to the homogeneity of the fraction VI preparation. Further, the molecular weight of the "subunit" is equal to that of the native enzyme, within experimental error. It is apparent, then, that this enzyme consists of but one polypeptide chain. Since the homogeneous protein exhibits both dAdo and dGuo kinase activities, the enzyme must be considered bifunctional if these activities are not catalyzed by a common active site.

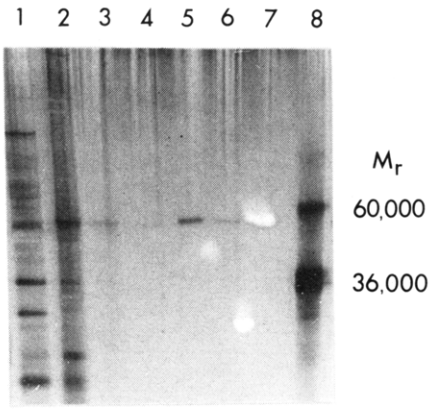

FIGURE 3: SDS–polyacrylamide gel electrophoresis of enzyme at various stages of purification. Using a discontinuous buffer system and a 12% gel, development was carried out as described under Experimental Procedures. Lane 1 contains fraction III; 2, fraction IV; 3, fraction V; 4, buffer only; 5, fraction VI; 6, fraction VI (another preparation); 7, buffer only; 8, catalase and glyceraldehyde phosphate dehydrogenase molecular weight markers. Other markers used include bovine serum albumin, ovalbumin, and ribonuclease (lane not shown).

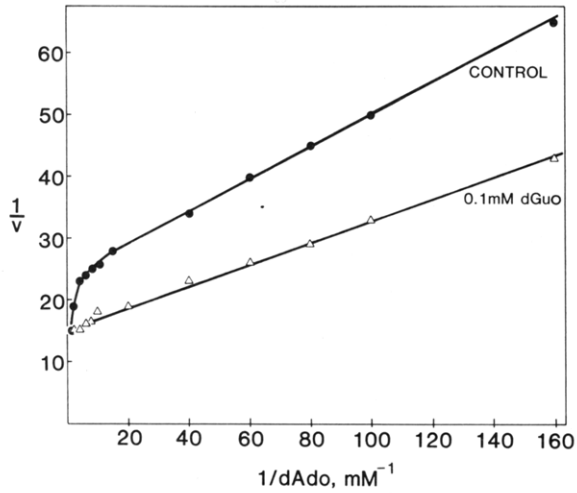

FIGURE 4: Effect of varying dAdo concentration on the reaction rate; stimulation by an effector, dGuo. MgATP concentration was 10 mM, and dGuo concentration was 0.1 mM. The dAdo concentration was varied from 6.25 to 1000 μM. Fraction IVB protein (1.1 μg) was used, and other conditions were as described under Experimental Procedures. Apparent K_m's for the control and stimulated dAK plots were 11.3 and 11.7 μM, respectively, and are considered identical within experimental error.

Kinetic Interaction of dGuo and dAdo Sites. Figure 4 shows that the stimulation of dAdo phosphorylation by dGuo is a noncompetitive process. In general, noncompetitive kinetics suggests, but do not prove, that different sites are involved in the binding of two ligands. However, the activation of dAdo phosphorylation by dGuo clearly indicates that both substrates bind to the enzyme simultaneously, at multiple, probably distinct sites. If both were phosphorylated by a common catalytic site, these substrates should be mutually competitive. The concentration of dGuo required to produce half-maximal activation, 9 μM, is approximately equal to the K_m apparent [K_m(app)] for dGuo phosphorylation, 11 μM. The most likely explanation is that occupancy of the dGuo site is accompanied by a conformational change which alters the catalytic activity at the dAdo site, whereas binding of dAdo is unaffected by dGuo (K_m is unchanged). At dAdo concentrations higher than about 100 μM, the control line curves (or bends) downward and tends to converge near the ordinate with the straight line generated by dGuo-stimulated enzyme. A homotropic effect of increasing dAdo concentrations was also seen in the case of the dCyd/dAdo kinase by Deibel et al. (1977). In that example, it appeared that dAdo could be producing the curvature by binding also to the dCyd site, thereby producing the same stimulatory effect as dCyd exerts on dAdo phosphorylation. In the present case, on the other hand, it seems most unlikely that dAdo binds to the dGuo site, since not even millimolar concentrations of dAdo compete with dGuo phosphorylation. We could envision a separate nucleoside binding regulatory site, but the following chemical evidence is more consistent with the activating effect of dGuo, at least, being mediated through the dGuo phosphorylation site. This leaves the homotropic activation by dAdo unexplained at present.

Table II: Additive Rates of dAdo and dGuo Phosphorylation under Saturating Conditions[a]

labeled nucleoside (each 100 μM)	unlabeled nucleoside (each 100 μM)	activity (pmol of [^{3}H]dNMP/min)
dAdo		0.60
dAdo	dGuo	1.40
dGuo		4.0
dGuo	dAdo	4.4
dAdo + dGuo		5.9

[a] MgATP concentration was 10 mM. Fraction IVB enzyme (230 ng) was used.

In the opposite sense, the activity of dGK is activated slightly in the presence of dAdo. However, this stimulation (about 20%, maximum) is too small to produce a significant kinetic slope effect. Therefore, a reciprocal plot of dGuo activation, patterned after Figure 4, did not reveal clearly whether or not activation by dAdo is also noncompetitive.

Additive Turnover of dAdo and dGuo. The experiment summarized in Table II reveals additive rates of phosphorylation when both nucleosides are present. Each substrate stimulates the turnover of the other—a substantial effect of dGuo or dAdo phosphorylation and a slight effect of dAdo on dGuo phosphorylation. If both were phosphorylated at a common site, these nucleosides should be mutually inhibitory, and if they were phosphorylated by different enzymes, mutual stimulation would be unlikely. Moreover, since each substrate is present at a concentration which exceeds the apparent K_m by about 10-fold (unpublished results), the stimulation cannot be due to first-order kinetic effects. Finally, dGuo does not stabilize dAdo kinase, so stabilization of dAdo kinase during assay cannot be its effect. The most reasonable explanation, then, is that both sites turn over simultaneously on the same protein molecule and are stimulated when the opposite site is occupied. It should be mentioned here that the stimulation factors vary somewhat with the age of the enzyme preparation; generally dGuo stimulates dAK about 3-fold, and dAdo stimulates dGK about 20%.

Selective Modification of One Site by Affinity Probes. (Fluorosulfonyl)benzoyl nucleoside derivatives have proven to be very useful in the labeling of nucleotide binding sites of a wide variety of enzymes (Colman, 1983). We have tested the effects of 5′[*p*-(fluorosulfonyl)benzoyl]adenosine, a probe which mimics the structure of ATP, on dAdo/dGuo kinase. The experiment shown in Figure 5, showing the progressive effect of FSO_2BzAdo on these activities, was carried out at 4 °C to avoid thermal denaturation of this labile protein. After 3 h of exposure to this reagent, 65% of the dGK activity was lost (Figure 5A), compared with only 20% of the dAK activity (Figure 5B), indicating that these two nucleosides are phosphorylated at different sites. Upon addition of 10 mM MgATP to the reaction mixture, dAdo kinase was completely

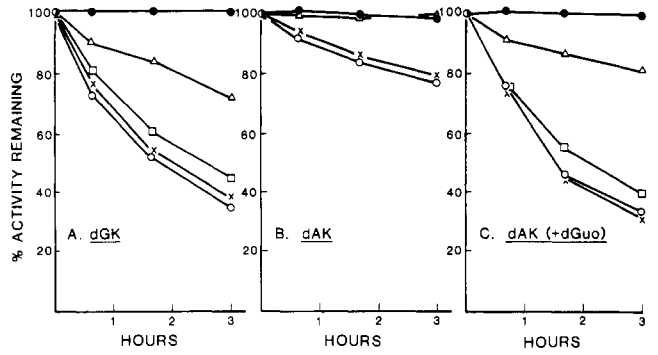

FIGURE 5: Selective modification of the dGK domain by FSO_2BzAdo and concurrent loss of dGuo activation of dAK. dGK/dAK (fraction IVB) was incubated as described under Experimental Procedures with the additives shown. Aliquots were withdrawn at intervals, diluted, and assayed for residual enzyme activities as shown. (●) Untreated control; (○) added 1 mM FSO_2BzAdo; (△) added 1 mM FSO_2BzAdo + 10 mM MgATP; (□) added 1 mM FSO_2BzAdo + 0.2 mM dGuo; (×) added 1 mM FSO_2BzAdo + 0.2 mM dAdo. (A) Residual dGK activity; (B) residual dAK activity; (C) residual dAK activity, measured in the presence of 100 μM dGuo. (Initial activity was 324% of unstimulated activity.)

protected from inactivation, whereas dGK was only partially protected, probably because its unusually large K_m for MgATP (1.7 mM at dGK, 0.11 mM at dAK; unpublished results) practically prevents saturation of the ATP subsite of dGK. Deoxynucleoside substrates, on the other hand, provided no significant protection in either case, which indicates that the probe is not attacking the deoxynucleoside subsites. If random sequential kinetic mechanisms apply to these activities, as has been observed previously with the dCyd/dAdo kinase (Deibel et al., 1977), the nucleoside should not block the binding of an ATP analogue. On the other hand, covalent modification of the dGK site by the ATP analogue does interfere with the ability of dGuo to stimulate dAdo phosphorylation. Figure 5C shows the rapid loss of this capability, paralleling the loss of dGK activity in panel A. Since the proportional losses of dGK and of dGuo-stimulated dAK activity are about equal, and are much greater than the loss of unstimulated dAK activity, it may be inferred that dAK stimulation is produced by the binding of dGuo to the dGK site (in the presence of MgATP). Chemical modification of the dGuo phosphorylating site abolishes communication with the dAK site by preventing the conformational changes associated with the normal ternary complex, or possibly by preventing the binding of dGuo.

It is interesting that FSO_2BzAdo attacks dGK selectively, in contrast to MgATP, which has the greater affinity for dAK. The replacement of negatively charged phosphates by the relatively hydrophobic benzoyl ester group may account for this reversal of affinities.

Discussion

It now seems highly probable that the deoxyguanosine and deoxyadenosine kinase activities of *Lactobacillus acidophilus* R-26 are the functions of separate active sites on a single polypeptide chain. This hypothesis is supported by the following lines of evidence: (i) After several thousand-fold purification, these activities remain associated, being electrophoretically coincident with a single protein band. Nearly equal enrichment factors for the two activities were observed at each purification step, except that there was an unexplained differential loss of dAK activity during concentration after the UDP–Sepharose chromatography. Further evidence for association of dAK and dGK on the same protein was recently obtained in this laboratory with a powerful new bisubstrate affinity column whose ligand, 5-deoxyadenosine 5′-adenosine P^1,P^4-tetraphosphate (dAP_4A), is directed very specifically toward the dAK catalytic site (Ives et al., 1984). Either dCyd/dAdo or dGuo/dAdo kinases were retained, presumably by virtue of their dAK sites, and each pair of activities was coeluted from the column with dAdo plus ATP. By contrast, a column directed toward dGuo kinase, with the bisubstrate inhibitor dGp_4A as a ligand, retained most of the dGK, half of the dAK, but less than 1% of the dCK (S. Ikeda, unpublished experiment). Thus, these highly specific columns appear to bind only their homologous kinase activities, and the retention and coelution of both dAK and dGK by either column are a clear indication that both activities reside on the same protein molecule. (ii) The molecular weights of the native enzyme and the denatured subunit are equal within a small experimental error, indicating that the two activities are linked through a single polypeptide chain. (iii) Each of these two nucleosides noncompetitively *stimulates* the turnover of the other without altering its binding, whereas turnover at the two sites is additive. This rules out competition for a common site and points to distinct catalytic sites for the phosphorylation of the two deoxynucleosides. (iv) Further support for this concept is provided by selective chemical inactivation of the dGK site by an affinity probe, 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine. In parallel with the loss of dGuo kinase activity, the ability of dGuo to activate dAK is also lost, whereas most of the basal dAK activity is retained. Besides indicating the existence of separate sites, this experiment also suggests (but does not prove) that the activation of dAK is mediated through the dGuo kinase site. At present, however, little is known of the mechanism of communication between the dGK and dAK sites.

Taken together, the experimental evidence we have presented makes it seem most unlikely that we are dealing with very similar, but separate, enzymes for dGuo and dAdo phosphorylation. If we are to consider this alternative possibility, we must envision each such separate enzyme as having a regulatory site capable of binding the opposite nucleoside, and then such a site would not be expected to bind strongly to the bisubstrate columns described above. The FPLC ion-exchange methods used in purification and the electrophoretic procedures used in purity analysis are each capable of resolving proteins differing by only a few charged groups, so we believe that these activities are much more likely to be associated with a multifunctional enzyme than with separate, but similar, proteins. Further proof that the dGuo/dAdo kinase consists of a single polypeptide chain might be obtained by end-group analysis or peptide mapping, but this is not feasible with the limited amounts of purified protein currently available. For the same reason, the stoichiometry of affinity labeling by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine has not been analyzed yet. This would be useful in determining whether this agent attacks only the dGuo kinase active site, or also a putative regulatory site which exerts positive control of dAdo phosphorylation. Such studies will probably have to await the cloning of the *Lactobacillus* deoxynucleoside kinase genes.

Multifunctional enzymes generally catalyze sequential steps within a catalytic pathway. In the present example, however, we have a very unusual case of the first committed steps of *parallel* pathways being catalyzed by one enzyme protein. [The closest functional analogy might be the ribonucleotide reductase of *Lactobacillus leichmannii* (Panagou et al., 1972), which, though it does not have multiple catalytic domains, does supply all four deoxynucleoside triphosphates through product-controlled specificity adjustments of a single polypeptide.] Curiously, dAK activity is divided between two bifunctional

enzymes, dGuo/dAdo kinase and dCyd/dAdo kinase, and has a specific activity on either protein which is less than one-fourth that of the opposite domain. However, when the dGuo and dCyd sites are saturated, activity at the dAdo kinase site increases severalfold, so that the regulated combined output of the two bifunctional pairs may be envisioned as the approximately equal quantities of dAMP, dCMP, and dGMP needed for DNA synthesis. A separate thymidine kinase, also subject to both positive and negative controls (Durham & Ives, 1971), supplies the fourth deoxynucleotide, dTMP.

Registry No. dGuo, 961-07-9; dAdo, 958-09-8; FSO_2BzAdo, 57454-44-1; dGuo/dAdo kinase, 89618-28-0; dCyd/dAdo kinase, 75302-35-1.

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Chorismate Mutase/Prephenate Dehydrogenase from *Escherichia coli* K12: Purification, Characterization, and Identification of a Reactive Cysteine†

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ABSTRACT: The bifunctional enzyme involved in tyrosine biosynthesis, chorismate mutase/prephenate dehydrogenase, has been isolated from extracts of a regulatory mutant of *Escherichia coli* K12. The pure enzyme is a homodimer of total molecular weight 78 000 and displays Michaelis–Menten kinetics for both activities. Fingerprinting and amino acid sequencing of tryptic and thermolytic peptides of the S-[^{14}C]carboxymethylated enzyme allowed the identification of three unique cysteine-containing sequences per subunit. Chemical modification of the native enzyme with 5,5′-dithiobis(2-nitrobenzoate) or iodoacetamide showed that one sulfhydryl group per subunit was particularly reactive, and the integrity of this group was essential for both enzymic activities. This work supports previous proposals for a close spatial relationship between the active sites.

Chorismate mutase/prephenate dehydrogenase (EC 5.4.99.5/1.3.1.12) is a bifunctional enzyme (Cotton & Gibson, 1965; Koch et al., 1971a) catalyzing two consecutive steps in the biosynthesis of tyrosine in *Escherichia coli* K12 and other enteric bacteria (Figure 1). Chorismate, the product of the common aromatic pathway, is rearranged to form prephenate, which subsequently undergoes oxidative decarboxylation to yield (4-hydroxyphenyl)pyruvate, the keto acid equivalent of tyrosine. The activity of this enzyme is regulated by repression of its synthesis (Brown & Somerville, 1971; Im et al., 1971) and end-product inhibition (Koch et al., 1971a). The enzyme was first purified from *E. coli* and *Aerobacter aerogenes* by Koch et al., who showed it to be a bifunctional dimer of closely similar or identical subunits (Koch et al., 1970a,b, 1971a,b). Chorismate mutase/prephenate dehydratase, a similar enzyme involved in the biosynthesis of phenylalanine (Figure 1), has also been characterized as a bifunctional dimer of identical subunits (Davidson et al., 1972; Gething & Davidson, 1976).

Chemical modification studies have been used to examine both the nature of the groups involved in the functions of these enzymes and the relationship between the active sites of the two activities of each enzyme. For chorismate mutase/prephenate dehydratase, Gething and Davidson (1977a,b) established that the reaction of approximately 1 mol of sulfhydryl group/subunit with either 5,5′-dithiobis(2-nitrobenzoate) (Nbs_2)[1] or *N*-ethylmaleimide caused the loss of all of the prephenate dehydratase activity but of only a small percentage of the chorismate mutase activity. It was concluded that a sulfhydryl group is essential for the prephenate dehydratase

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[1] Abbreviations: Cmc, 5-(carboxymethyl)cysteine; dansyl chloride, 5-(dimethylamino)naphthalene-1-sulfonyl chloride; Nbs_2, 5,5′-dithiobis(2-nitrobenzoate); SDS, sodium dodecyl sulfate.

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