# Heterodimeric Deoxyguanosine Kinase/Deoxyadenosine Kinase of *Lactobacillus acidophilus* R-26:† Heterotropic Activation of Deoxyadenosine Kinase Subunit Implicated by Limited Proteolysis and Affinity Labeling‡

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ABSTRACT: The deoxyguanosine (dGuo) kinase/deoxyadenosine (dAdo) kinase complex of *Lactobacillus* has been purified to homogeneity by using a newly constructed dATP-Sepharose column as a final step (2700-fold purification). A heterodimeric structure for the complex has recently been established [Ikeda et al. (1994) *Biochemistry 33*, 5328−5334]. On the basis of the kinetic and structural data accumulated so far, a model for the heterotropic activation of the dAdo kinase subunit by dGuo or dGTP is proposed: (1) there is an intrinsic difference in the enzyme conformation of the two subunits, with the dAdo kinase subunit being in a constrained (closed) state and the counterpart dGuo kinase subunit being in a relaxed (open) state, as reflected in their relative $V_{max}$ values and in the presence or absence of heterotropic activation, and (2) the conformational change induced by the binding of dGuo or dGTP to the active site of the dGuo kinase subunit causes the activation of the dAdo kinase subunit through subunit−subunit interactions. These proposed mechanisms are strongly supported by the following new findings made in this work: (1) low concentrations of chaotropic agents such as guanidine−HCl were found to increase the $V_{max}$ of dAdo kinase up to 2-fold—in the same kinetic fashion, apparently, as the activation by dGuo—while showing no effect on dGuo kinase; (2) the proteolytic inactivation of dAdo kinase by trypsin is significantly slower than that of dGuo kinase, but its rate of inactivation is stimulated by dGTP to the same level as for dGuo kinase; (3) the activating effect of dGuo on dAdo kinase was abolished in the course of differential proteolytic inactivation of the dGuo kinase by trypsin in the presence of dATP; and (4) photoaffinity labeling with [8-$^{14}$C]-8-azido-Ade produces a new species of kinase heterodimer in which the dAdo kinase subunit is permanently activated as a result of specific labeling of the dGuo kinase active site.

The quaternary structures of oligomeric proteins and the allosteric regulation of biological activity based on subunit−subunit interactions have been among the most intriguing subjects for enzymologists for many years (Klotz et al., 1970; Hung et al., 1982; Traut, 1988). *Lactobacillus acidophilus* R-26 has two paired, highly homologous, deoxynucleoside kinase complexes, namely, dGuo[1] kinase/dAdo kinase and dCyd kinase/dAdo kinase (Deibel & Ives, 1977). Earlier kinetic work indicated that each dAdo kinase activity is stimulated by the deoxynucleoside substrate for the counterpart kinase (i.e., dGuo or dCyd), implying the presence of positive allosteric interactions between heterologous phosphorylation sites in each of the complexes (Deibel et al., 1977; Chakravarty et al., 1984). Recently, we have established that unique heterodimeric structures exist for each of these kinase complexes, with each subunit of a dimer catalyzing the phosphorylation of a specific deoxynucleoside substrate (Ikeda et al., 1994). Functional assignment of subunits has been accomplished by applying limited proteolysis while protecting the heterologous subunit with its specific end-product inhibitor, dNTP. With models based on these kinetic and structural data, the mechanisms for the heterotropic activation of the dAdo kinase subunit will be examined in detail in this work, employing as probes limited proteolysis and affinity labeling. For the purpose of simplicity, this paper deals only with the dGuo kinase/dAdo kinase complex, which appears functionally and structurally to be in parallel with the other *Lactobacillus* kinase complex, dCyd kinase/dAdo kinase. The study of this new type of oligomeric enzyme may add another dimension to the understanding of the diversity of allosteric phenomena.

We have previously developed a new affinity medium for dCyd kinase, dCTP-Sepharose, where dCTP was bound to Sepharose through a phosphohexyl-spacer arm, and the dCyd kinase/dAdo kinase complex was successfully purified to homogeneity with that affinity medium (Ikeda et al., 1988). The basis for designing this affinity medium was again the remarkable specificity and strength of the binding of each dNTP to its respective kinase—in contrast to the binding of ATP by all these kinases. The binding site for dNTP appears to overlap the specific catalytic site for the phosphorylation of each corresponding deoxynucleoside (Ikeda et al., 1986,

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[1] Abbreviations: dGuo, deoxyguanosine; dAdo, deoxyadenosine; dCyd, deoxycytidine; Ade, adenine; dNTP, deoxynucleoside triphosphate; dGK, dGuo kinase; dAK, dAdo kinase; dCK, dCyd kinase; dCTP-Sepharose, dCyd 5′-tetraphosphate-bound Sepharose (previously called $dCp_4$-Sepharose (Ikeda et al., 1988)); dATP-Sepharose, dAdo 5′-tetraphosphate-bound Sepharose ($dAp_4$-Sepharose); SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; TLC, thin layer chromatography.

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1994). In this *non*allosteric model for the end-product inhibition, the deoxynucleoside moiety of dNTP is believed to fit optimally into the binding site for a deoxynucleoside substrate, while its triphosphate group interacts with the site for the phosphate donor, ATP, but in an orientation exactly opposite to that of ATP. In the current work, another new affinity medium, dATP-Sepharose, homologous to dCTP-Sepharose, has been developed and successfully applied to the purification of the dGuo kinase/dAdo kinase complex. The kinase complexes so purified have been used simultaneously for the demonstration of their heterodimeric structures (Ikeda et al., 1994) and for the present work on their allosteric properties.

## EXPERIMENTAL PROCEDURES

*Materials.* 1,1′-Carbonyldiimidazole, 6-aminohexanol 1-phosphate, and cyanogen bromide were purchased from Aldrich Chemical Co. Ethyl trifluorothiol acetate was obtained from Pierce Chemical Co. The disodium salt of dATP was from American Research Products Co. *N*,*N*-Dimethylformamide and methanol were distilled over $CaH_2$ before use. Tributylamine (Aldrich Chemical Co.) was distilled over ninhydrin. Sepharose CL-6B and Sephadex G-10 were from Pharmacia. DEAE Trisacryl M was obtained from IBF Biotechnics. dGTP, TPCK-treated trypsin from bovine pancreas, trypsin inhibitor from soybean, and guanidine−HCl (grade I) were obtained from Sigma Chemical Co. Tritiated deoxynucleosides were from ICN. [8-$^{14}$C]-2′-dAdo was obtained from Amersham Corp. Reagents for gel electrophoresis were supplied by Bio-Rad. Preparative cellulose TLC plates (Uniplate precoated with Avicel F, 0.25 mm) were supplied by Analtec, Inc. HPLC grade acetonitrile and water were from Fisher Scientific.

*Enzyme Preparation and Assays.* An ammonium sulfate fraction was prepared from an extract of *Lactobacillus acidophilus* R-26 (ATCC 11506) as described previously (Deibel & Ives, 1977). This fraction had been subjected to dCTP-Sepharose affinity chromatography to purify the dCyd kinase/dAdo kinase complex (Ikeda et al., 1988). The run-through fraction from the dCTP-Sepharose column, stored at −20 °C, was used as a source for further purification of the dGuo kinase/dAdo kinase complex, since it proved to contain nearly 100% of the dGuo kinase activity (relative to that in the original ammonium sulfate fraction), but only 1−3% of the dCyd kinase activity. dAdo kinase and dGuo kinase assays were carried out radiometrically (Deibel & Ives, 1977; Ives, 1984). One unit of activity is defined as the amount producing 1 nmol of deoxynucleoside monophosphate per minute at 20 °C. Protein determinations employed the Bradford method (Bradford, 1976).

*Preparation of the Affinity Medium, dATP-Sepharose.* A new affinity medium (dATP-Sepharose) for purifying dAdo kinase has been constructed by following the methods used for the preparation of dCTP-Sepharose (Ikeda et al., 1988) and dGTP-Sepharose (Hoffmann & Blakley, 1975). A yield of 0.29 mmol of the affinity ligand, $P^3$-[(6-aminohex-1-yl)-phosphoryl]-dATP (I), was obtained from 0.94 mmol of dATP and 1.88 mmol of *N*-(trifluoroacetyl)-6-aminohexanol phosphate (i.e., 31% based on the amount of dATP used). The compound gave 3.8 and 0.92 molar ratios of total phosphate and amine, respectively, per mole of adenine, determined from the UV absorption of the base. The compound was also found to be stable to alkaline phosphatase, confirming the proposed polyphosphate structure. Affinity ligand I has been found to inhibit *Lactobacillus* dAdo kinase activity (dCTP-Sepharose run-through fraction free of dCyd kinase/dAdo kinase) competitively *versus* ATP ($K_i$(app) = 30 $\mu$M), but noncompetitively *versus* dAdo ($K_i$(app) = 37 $\mu$M at 0.1 mM MgATP). Previous kinetic work (Ikeda et al., 1986) revealed that dATP inhibits dAdo kinase competitively *versus* ATP ($K_i$(app) = 1.0 $\mu$M irrespective of dAdo concentration), but noncompetitively *versus* dAdo ($K_i$(app) = 0.9 $\mu$M at 0.1 mM MgATP), confirming the ordered kinetic mechanism. Thus, the kinetic behavior of the affinity ligand I mimics that of dATP except that the apparent dissociation constant of the former is 1 order higher, probably due to the spacer arm attached, or to the loss of one negative charge from the $\gamma$-phosphate.

Sepharose CL-6B (20 mL) was activated with cyanogen bromide according to the simplified activation method of March et al. (1974) and was suspended in 40 mL of 0.2 M sodium bicarbonate containing 38.2 $\mu$mol of affinity ligand I. The coupling was carried out at 4 °C for 17 h with gentle shaking. The concentration of ligand bound to Sepharose was found to be 0.8−0.9 $\mu$mol/mL of Sepharose, based on the decrease in the absorbance (259 nm) of the supernatant of the coupling reaction mixture.

*Purification of the dGuo Kinase/dAdo Kinase Complex by Affinity Chromatography on dATP-Sepharose.* dATP-Sepharose (20 mL) was packed in a column (16 mm diameter) and equilibrated with buffer A (15 mM K-phosphate buffer, pH 8.0, containing 20% glycerol). *Lactobacillus* extract previously passed through dCTP-Sepharose (Ikeda et al., 1988), containing 35 units of dGuo kinase and 5 units of dAdo kinase (46.1 mg protein), was applied to the column. While more than 95% of the total protein applied ran through the column, around 90% of the dGuo kinase/dAdo kinase activities applied were retained on the column. The column was washed successively with 50 mL of buffer A, 250 mL of buffer A containing 0.3 M KCl, and 50 mL of buffer A. Then, both dGuo kinase (11.2 units) and dAdo kinase (1.46 units) were specifically eluted with 0.1 mM dATP in buffer A containing 0.3 M KCl, confirming that these two activities remain associated with each other. The optimal salt concentration to assist the biospecific elution of the enzyme with dATP has been found to be 0.3 M KCl, while almost no enzyme was eluted with dATP in the presence of either 0 or 0.5 M KCl. This suggests that it is important for the successful elution of the enzyme to balance two different types of nonspecific interactions (hydrophobic and ionic) supplementary to the biospecific interaction between enzyme and affinity ligands, as generally accepted for the mechanism for affinity chromatography (Scopes, 1994). A typical yield of about 10 $\mu$g of pure protein was obtained with this column. Active dGuo kinase/dAdo kinase fractions were combined and concentrated to a minimum volume by Centricon-10 ultrafiltration. The purified enzyme preparations were stored at −20 °C in the presence of the eluent dATP for best stability. Just before each experiment, dATP was removed from the enzyme preparation by repeated dilution with buffer A and reconcentration on a Centricon concentrator. A preparation of the dGuo kinase/dAdo kinase complex purified with dATP-Sepharose gave a single stained protein band on SDS−PAGE by the Laemmli method (Ikeda et al., 1994). The subunit molecular mass is estimated to be 27 200 Da. On nondenaturing PAGE (10% acrylamide gel), the purified dGuo kinase/dAdo kinase also showed the

congruence of dGuo kinase and dAdo kinase activities corresponding to a single stained protein band (not shown). Since the native dGuo kinase/dAdo kinase is known to have a $M_r$ of 50 000−54 000 (Deibel & Ives, 1977; Chakravarty et al., 1984), it appears that the enzyme is composed of two subunits of similar size. This is exactly consistent with the case of the dCyd kinase/dAdo kinase complex mentioned previously (Ikeda et al., 1988). In earlier work from this laboratory (Chakravarty et al., 1984), the dGuo kinase/dAdo kinase complex purified by rather conventional methods appeared to consist of a monomeric polypeptide (56 000 Da). Since the amount of pure enzyme obtained in this work is almost 100 times greater than in the previous case, it seems probable that an impurity was mistaken for the enzyme in the previous work, particularly given the fact that silver staining, with its varied response to different proteins, was used.

*Gel Electrophoresis.* The discontinuous Tris−glycine/Tris−HCl buffer system (Laemmli, 1970) was used for both nondenaturing PAGE (10% acrylamide) and SDS−PAGE (12% acrylamide), with a Bio-Rad Model 360 mini vertical slab-cell (83-mm-long gel plate). Protein bands were visualized by staining with Coomassie Blue R-250. The discontinuous MZE 3328.IV buffer system (pH 6.6 gel buffer) (Moos et al., 1988) was used for SDS−PAGE (12.0/13.2% acrylamide step gradient) to separate heterodimers as described previously (Ikeda et al., 1994).

*Differential Limited Proteolysis of dGuo Kinase/dAdo Kinase with Trypsin.* Purified dGuo kinase/dAdo kinase (0.2 $\mu$g in 20 $\mu$L of buffer A) was incubated at 20 °C with trypsin (0.2−2.0%, w/w) for up to 30 min. To protect the dGuo kinase or dAdo kinase subunit from proteolysis, 0.1 mM of dGTP or dATP, respectively, was included in the digestion mixture. During the course of proteolysis, an aliquot (2.0 $\mu$L) was withdrawn and mixed with buffer A (65 $\mu$L, 4 °C) containing 0.1 mM dGTP or dATP and soybean trypsin inhibitor (5 molar ratio over trypsin) to stop the proteolysis. From this mixture, 20 $\mu$L aliquots were taken for each kinase assay.

*Preparation of 8-Bromo-2′-dAdo (II), 8-Azido-2′-dAdo (III), and 8-Azido-Ade (IV).* 8-Bromo-dAdo (II) (1.42 mmol, 71%) was prepared by the direct bromination of 2′-dAdo (2.0 mmol) (Holmes & Robins, 1964), modified according to the procedures used for the preparation of 8-bromo-AMP and 8-bromo-cAMP (Czarnecki et al., 1979; Haley, 1977). 8-Azido-2′-dAdo (III) was prepared from II by the method of Long *et al.* (1967) as modified for the preparation of 8-azido-AMP and 8-azido-cAMP (Czarnecki et al., 1979; Haley, 1977). A mixture of 0.03 mmol of II and 10 mmol of triethylammonium azide dissolved in 0.5 mL of dry DMF was tightly sealed and heated at 75 °C for 16 h. An aliquot of the reaction mixture was analyzed by reversed-phase HPLC run on a Varian 5000 LC equipped with a 4.6 mm × 220 mm column of SPHERI-5, RP-18 (Brownlee Labs), employing a linear gradient of solvent A (10 mM K-phosphate, pH 7.0) and solvent B (100% acetonitrile), at a flow rate of 1.0 mL/min. Two major peaks absorbing at 260 nm were detected at retention times of 15.2 and 17.0 min. Both compounds exhibited a UV-absorption peak at 281 nm, characteristic of 8-azidoadenine analogs, which disappeared rapidly upon UV-irradiation. The deoxyribose content was determined by the diphenylamine method (Burton, 1956), and the deoxyribose−adenine ratio was found to be 0.04:1.00 for the compound under the first peak and 0.99:1.00 for the product under the second peak, identifying the former as the byproduct, 8-azido-Ade (IV), and the latter as the expected product, 8-azido-dAdo (III). By injecting small aliquots of reaction mixture repeatedly onto HPLC, 3−4 $\mu$mol of both compounds III and IV were obtained (10−13% yields).

[8-$^{14}$C]-8-Azido-Ade was prepared from [8-$^{14}$C]-2′-dAdo by following the methods used for the preparation of nonradioactive 8-azido-Ade (IV). [8-$^{14}$C]-2′-dAdo (11.6 $\mu$Ci, 0.97 Ci/mmol) was treated with bromine without adding carrier deoxynucleoside. The product was separated on preparative TLC (4.30 $\mu$Ci recovered, 37.1%). Unlabeled 8-bromo-dAdo (221 nmol) was added to 2.21 nmol (2.15 $\mu$Ci) of [8-$^{14}$C]-8-bromo-dAdo, and the mixture was treated with 10 $\mu$mol of triethylammonium azide. From this reaction 0.72 $\mu$Ci (74.5 nmol, 33.4%) of [8-$^{14}$C]-8-azido-Ade was recovered by HPLC.

*Photoaffinity Labeling and Autoradiography.* To the preparation of dGuo kinase/dAdo kinase (1.0 $\mu$g/10 $\mu$L of buffer B (50 mM Tris−HCl buffer, pH 8.0, containing 20% glycerol)) were added various protecting ligand(s) and nonradioactive 8-azido-dAdo or 8-azido-Ade (final concentration 0.01−0.10 mM). The enzyme solution was placed in the well of an immunoassay plate kept on ice during the irradiation. A short wave UV lamp (Mineralight, UVS.12 from Ultra-violet Products, Inc.) was placed about 1 cm from the enzyme solution. At intervals during the irradiation, 1.0 $\mu$L aliquots were taken for standard kinase assays (80 $\mu$L total assay volume). The labeling reagent and/or protecting ligand(s) remaining after photoirradiation are assumed to have no significant effect on kinase assays for the following reasons: (1) the labeling reaction mixture is diluted 80-fold upon the kinase assay; (2) saturating concentrations of deoxynucleoside (20 $\mu$M) and MgATP (10 mM) are used for the assay; and (3) the kinase activities after photoirradiation are expressed as percent relative to the activities at zero time. The kinase complex (1.0 $\mu$g) was labeled with [8-$^{14}$C]-8-azido-Ade (0.1 mM final concentration) in the presence of 10 mM ATP and 0.1 mM dAdo. After the irradiation for 3 min, the enzyme solution was treated with SDS−PAGE sample buffer and subjected to electrophoresis (pH 6.6) (Ikeda et al., 1994). After the electrophoresis was completed, the gel was treated overnight with several changes of fixing solution to remove unreacted radioactive Ade derivatives from the gel. The gel was stained with Coomassie Blue-250, and, after being soaked in methanol−glycerol−water (45:5:50) for 20 min, it was dried and exposed to Kodak X-OMAT film at −70 °C for 3 days.

## RESULTS AND DISCUSSION

*Characteristics of Homogeneous dGuo Kinase/dAdo Kinase Complex and Proposed Mechanisms for the Heterotropic Activation of the dAdo Kinase Subunit.* The dGuo kinase/dAdo kinase complex of *Lactobacillus* has been purified to homogeneity by affinity chromatography on a newly constructed dATP-Sepharose column as described in detail in the Experimental Procedures. Our previous work with partially purified preparations of the complex has shown that the $K_m$ for the deoxynucleoside substrate of each kinase falls within a range of 2−5 $\mu$M, while dGuo kinase has a much higher $V_{max}$ (5−7-fold) than the counterpart dAdo kinase, when measured *independently* (Chakravarty et al., 1984). This activity ratio of dGuo kinase to dAdo kinase

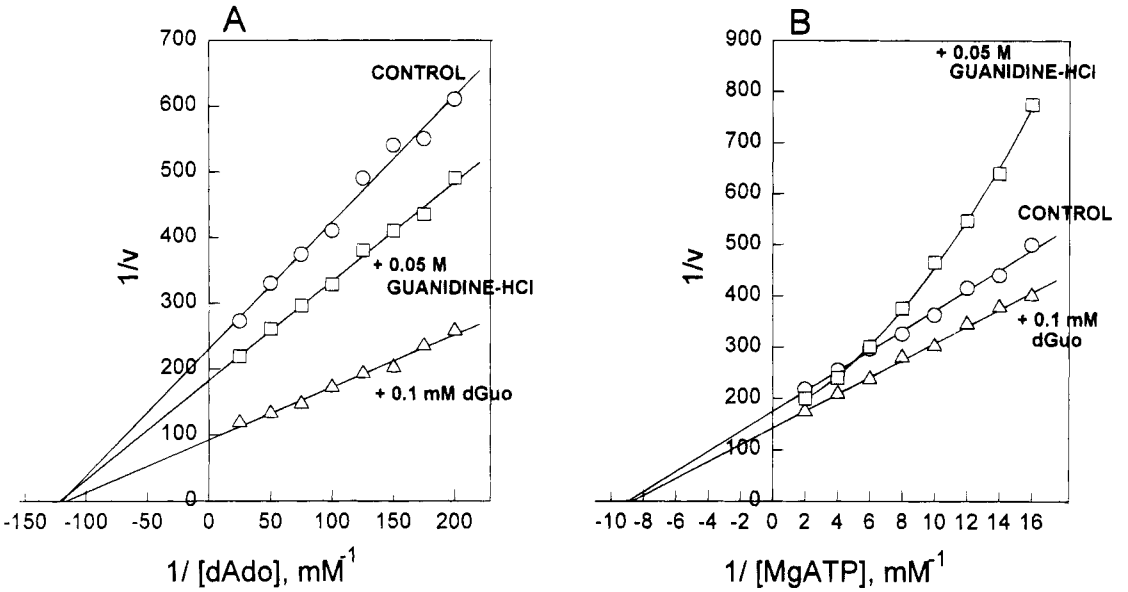


FIGURE 1: Heterotropic activation of dAdo kinase subunit by dGuo and the stimulation of dAdo kinase activity by a low concentration of guanidine−HCl (○—○, control; △—△, dGuo added (final concentration 0.1 mM); □—□, guanidine−HCl added (final concentration 0.05 M)). (A) the dAdo concentration was varied, with MgATP concentration fixed near saturation (10 mM). (B) The MgATP concentration was varied, with dAdo concentration fixed near saturation (20 $\mu$M).

remained almost unchanged through the dATP-Sepharose affinity purification, and the homogeneous preparation of the complex has a specific activity of 2150 units/mg for dGuo kinase and 280 units/mg for dAdo kinase (1700-fold purification). This may indicate that there is an intrinsic difference in enzyme conformation between two kinase subunits under assay conditions, reflected in their substantially different specific activities. Our previous kinetic work also has shown that the activity of dAdo kinase is stimulated 5−7 times by adding the deoxynucleoside substrate (dGuo) specific for the counterpart dGuo kinase to the assay mixture (Deibel et al., 1977; Chakravarty et al., 1984). The activation was found to be a purely $V_{max}$ effect, with no effect on $K_m$s for either dAdo or ATP. Furthermore, the concentration of dGuo required to produce the half maximal dAdo kinase activation (11 $\mu$M) was found to be virtually identical to the $K_m$ for dGuo phosphorylation (9 $\mu$M), indicating the possibility that dGuo binding at the active site of dGuo kinase causes the activation of dAdo kinase. dGTP, a specific end-product inhibitor for dGuo kinase, also produces a similar activation effect on the associated dAdo kinase, when assayed at subsaturating concentrations of ATP, consistent with the kinetics indicating that dNTP binding takes place at the active site of the corresponding kinase (Ikeda et al., 1986). However, such a large activation effect is not apparent in the opposite direction with regard th dGuo kinase, in which a stimulation of 20%, at most, is produced by adding dAdo or dATP. Thus, the activity of fully activated dAdo kinase is comparable to the activity of its counterpart dGuo kinase. This kinetic work has now been repeated with the homogeneous kinase complex, with results which are basically the same as those obtained previously with the partially purified preparation. Results on the heterotropic activation of dAdo kinase by dGuo are shown in Figure 1. Other aspects of this figure will be discussed below.

Taken together, these kinetic data seem to support the idea that the two subunits of the dGuo kinase/dAdo kinase heterodimer exist in two different states of conformation (roughly defined) under assay conditions, with dGuo kinase in an open (relaxed), fully active state, but with dAdo kinase in a constrained, partially active state. Figure 2 is an attempt to illustrate these different states of enzyme conformation and their interconversion upon the binding of various ligands to each kinase subunit. The shape, size, and location of the active site on each subunit carry no real dimensions, rather they represent the relationship of substrate binding sites to the sites for dNTP binding (cf. Ikeda et al., 1994). Since it has been shown (Chakravarty et al., 1984; Ikeda et al., 1994) that the dGuo kinase subunit and the dAdo kinase subunit exhibit very similar structural and functional features such as the molecular weight, the common sequence of 15 amino acids near the N-terminus including the consensus region for ATP binding, the specific inhibition by the respective dNTP and so on, the active sites for two kinase subunits are tentatively placed in a symmetrical orientation. The dimension of the interface between two subunits shown in Figure 2 is also tentative. The first assumption in this model is that the two subunits originally exist in two different enzyme conformations in the absence of any ligand, as depicted in Figure 2a. When ATP binds to the active site of each kinase, the intrinsic difference in the conformations of the two subunits is assumed to be unchanged (Figure 2b). As a result of these causes, when assayed independently using the respective [$^3$H]deoxynucleoside, dGuo kinase shows much higher activity than dAdo kinase (Figure 2c vs e). Upon the binding of dGuo to the active site of dGuo kinase, dAdo kinase seems to undergo a conformational change from a constrained form to an open form as shown in Figure 2b to c, and e to f. Thus, dAdo kinase activity is greatly increased upon the addition of dGuo to dAdo kinase assay mixture (Figure 2f). On the other hand, since the dGuo kinase subunit is originally in a fully active state, the binding of dAdo to the dAdo kinase subunit does not change the conformation of the dGuo kinase subunit significantly (Figure 2c to d). In a similar fashion, upon the binding of dGTP to the active site of dGuo kinase, dAdo kinase subunit undergoes a conformational change to the fully active state (Figure 2g). On the other hand, the binding of dATP to the active site of dAdo kinase does not produce a significant change on dGuo kinase (Figure 2h). Although the ideas

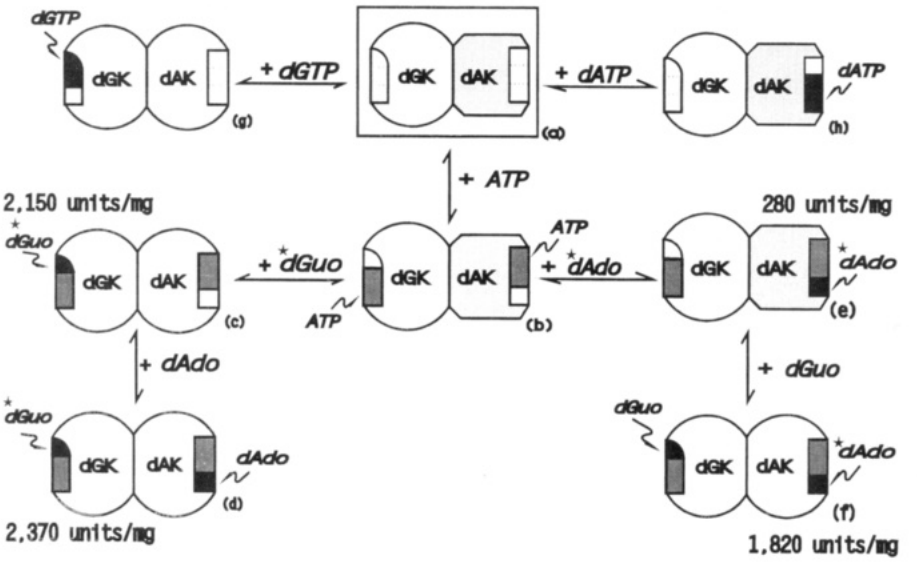


FIGURE 2: Schematic representation of a model for the subunit conformations of the heterodimeric dGuo kinase/dAdo kinase (dGK/dAK) complex from *Lactobacillus* and for the heterotropic activation of dAdo kinase subunit. The round (white) and rectangular (shaded) subunits represent the fully active (or open) and the less active (or constrained) states of enzyme conformation, respectively. *dGuo and *dAdo represent [$^3$H]deoxynucleoside used for the corresponding kinase assay.

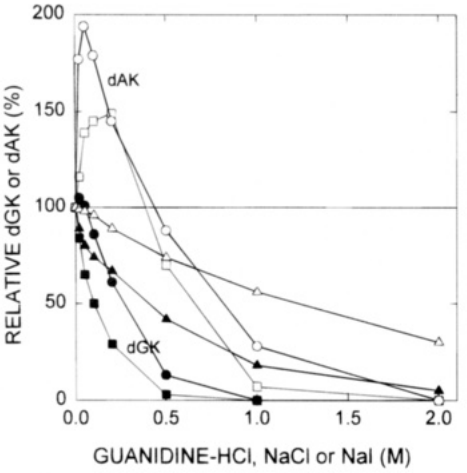


FIGURE 3: Effect of chaotropic salts on paired kinase activities of the dGuo kinase/dAdo kinase (dGK/dAK) complex (solid symbols, dGK; open symbols, dAK). Various concentrations of guanidine−HCl (●, ○) or NaI (■, □) dissolved in buffer A were mixed with dGK/dAK (6 ng in a final 40 $\mu$L of buffer A, no dNTP included) at 4 °C, and then, immediately, the kinase assay at 20 °C was started by adding 40 $\mu$L of assay mixture. As a control, NaCl (▲, △) was added instead of guanidine−HCl or NaI. The effect of varying concentrations of each salt on the capacity of DE-81 paper in disk assay method to retain [$^3$H]-dNMP formed by kinase reaction has been taken into consideration in calculating the relative percent kinase activity.

depicted in Figure 2 are still speculations based on kinetics data, the following experiments based on various more direct chemical alterations seem to fully support the concept illustrated.

*Differential Effect of Guanidine−HCl on Heterodimer.* Guanidine−HCl, frequently used as a protein denaturing agent, is supposed to disrupt noncovalent interactions in enzyme proteins. We have observed that the two subunits of the dGuo kinase/dAdo kinase heterodimer respond to this agent in opposite ways or to different degrees when the complex was assayed for each kinase activity for 30 min in the presence of various concentrations of guanidine−HCl (Figure 3). dGuo kinase activity decreased sharply as the concentrations of guanidine−HCl were increased up to 1 M, although it is not clear whether the decrease in activity is due to a reversible inhibition or to time-dependent denaturation. On the other hand, dAdo kinase activity *increased* almost 2-fold at low concentrations of guanidine−HCl (~0.05 M) and then decreased as the concentration of guanidine−HCl was elevated to 2 M. When guanidine−HCl was replaced by NaCl as a control, both kinase activities gradually fell as the concentration of NaCl was increased up to 2 M, while the activation of dAdo kinase was not seen at any concentration of NaCl. Again, dGuo kinase activity dropped at lower salt concentrations than dAdo kinase. These results seem to indicate that the dAdo kinase subunit is more resistant to the denaturing and/or inhibiting effects of guanidine−HCl and NaCl than the dGuo kinase subunit and that the activation of dAdo kinase caused by the low concentration of guanidine−HCl is not simply due to ionic effects, but is probably due to the chaotropic properties of guanidine−HCl. Another chaotropic salt, NaI, has also been found to produce an activation effect on dAdo kinase similar to that of guanidine−HCl (Figure 3). Furthermore, it has been shown (Figure 1A) that the activation of dAdo kinase by guanidine−HCl is a purely $V_{max}$ effect with no change in the $K_m$ for dAdo, exactly like the effect of dGuo. From this result, it is deduced that there is no time-dependent denaturation of dAdo kinase by low concentration of guanidine−HCl during the assay time, with MgATP near a saturating concentration. This has been confirmed in a separate experiment in which both dAdo kinase and dGuo kinase were found to maintain their original activities during the incubation with 0.05 M guanidine−HCl at 20 °C for 30 min in the presence of 10 mM ATP (not shown). However, when the effect of guanidine−HCl was examined with varying concentrations of MgATP (Figure 1B), the activation of dAdo kinase was seen only at a MgATP concentration higher than its $K_m$, while a higher degree of apparent inhibition was seen as the concentration MgATP was decreased. This may indicate that low concentrations of guanidine−HCl have two overlapping effects on dAdo kinase, i.e., the activation effect and a time-dependent denaturation effect. The latter seems to be significant when the kinase subunit has no bound MgATP. This deduction has been confirmed by another experiment in which the stability of dAdo kinase and dGuo

kinase to a low concentration of guanidine−HCl was compared by measuring each of the activities remaining after the complex was incubated at 20 °C with 0.05 M guanidine−HCl in the absence of ATP at various times. dGuo kinase or dAdo kinase was found to lose 40% or 25%, respectively, of its original activity after a 30 min incubation. These results indicate that a significant conformational difference in the two kinase subunits is also found in the absence of ATP. While the binding of ATP also undoubtedly produces significant changes in the conformations of the kinase heterodimer, the focus of this work is on the heterotropic activation of the dAdo kinase subunit, i.e., the conformational difference in the heterodimer subunits under *assay conditions*. Therefore, any conformational differences induced by ATP alone are ignored at this time, and the subunit conformations in the absence and presence of ATP are temporarily depicted by the same shape in the model (Figure 2a and b). In any event, the observed differential effects of guanidine−HCl or NaI on the heterodimeric subunits seems to suggest that the dAdo kinase subunit under assay conditions originally exists in a constrained state of enzyme conformation, but becomes more relaxed (open) in the presence of low concentrations of chaotropic salts, while dGuo kinase, which is already in an open state, is not further activated by these agents.

*Differential Rate of Tryptic Inactivation of the Heterodimer.* Proteolytic inactivation has been widely applied to detecting the conformational changes in enzymes induced by the binding of various ligands (Mihalyi, 1972; Markus et al., 1993; Kopperschlaeger et al., 1993). In many instances, the binding of substrate or inhibitor reduces the rate of proteolytic inactivation of a particular enzyme, indicating the possibility that the enzyme becomes more compact upon the binding of such a ligand. In some other rare occasions, however, the proteolytic inactivation is accelerated by the binding of a certain ligand, providing clear evidence of the conformational changes to a more open or exposed state. When the dGuo kinase/dAdo kinase complex was incubated with trypsin in the absence of any substrate or inhibitor, it has been found that dAdo kinase loses its activity significantly more slowly than dGuo kinase (Ikeda et al., 1994). The initial phase of this proteolysis has now been examined in greater detail, as shown in Figure 4. The $k_{inact}$ has been calculated to be 1.71 $min^{-1}$ for dGuo kinase and 0.73 $min^{-1}$ for dAdo kinase. Since two kinase subunits exhibit many common features in their function and structure, including the consensus region for an ATP binding site near the N-terminus of each (Ikeda et al., 1994), the difference in the rate of tryptic inactivation observed between dGuo kinase and dAdo kinase may be reflecting the difference in global enzyme conformation between two subunits in the absence of any ligand, as depicted in Figure 2a. Since the addition of ATP almost completely protects both subunits from proteolysis, probably due to the physical blocking of the digestion site (Ikeda et al., 1994), it is difficult to detect any conformational difference in the two subunits by this method in the presence of ATP (Figure 2b). On the other hand, as shown in Figure 4, dGTP, upon binding to the active site of dGuo kinase, stimulates the rate of proteolytic inactivation of dAdo kinase ($k_{inact}$ = 1.40 $min^{-1}$) nearly to the level of dGuo kinase inactivation, while protecting its homologous kinase subunit from inactivation. dATP, on the contrary, did not have any effect on the rate of proteolytic inactivation of dGuo kinase (not shown). These results clearly support

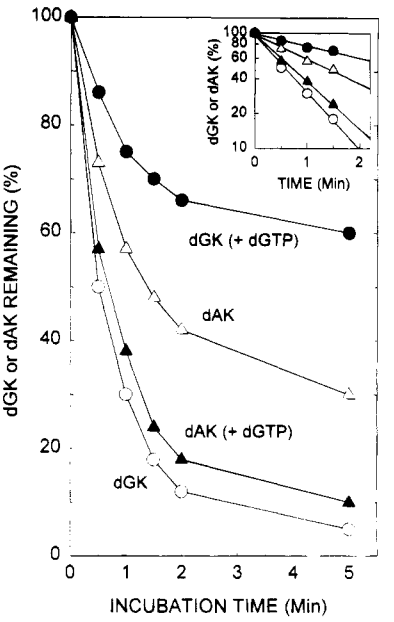


FIGURE 4: Differential tryptic inactivation of the dGuo kinase/dAdo kinase (dGK/dAK) heterodimer and the effect of dGTP. dGK/dAK was incubated with trypsin (2%, w/w) in the absence of any ligand under the conditions described in the Experimental Procedures. During the course of proteolysis, aliquots were withdrawn and mixed with buffer A containing soybean trypsin inhibitor to stop the proteolysis. The activity remaining for each kinase was measured under the standard assay conditions (○—○, dGK; △—△, dAK). In another experiment, dGTP was added to the digestion mixture (final concentration 0.1 mM) (●—●, dGK; ▲—▲, dAK).

the idea of two different states of enzyme conformation for the heterodimer and their interconversions, as depicted in Figure 2a−g or h, consistent with the kinetics showing that dGTP, upon binding to the active site of dGuo kinase, stimulates the activity of the dAdo kinase subunit, while dATP has no significant activating effect on dGuo kinase (Chakravarty et al., 1984).

*Demonstration of Intersubunit Heterotropic Activation of dAdo Kinase by Differential Limited Proteolysis.* The heterotropic activation of the dAdo kinase subunit by dGuo or dGTP has been most readily explained by the subunit−subunit interaction caused by the binding of dGuo or dGTP to the active site of dGuo kinase subunit (Figure 2e−f, and a−g). However, there is the other possibility that the activation is caused within the dAdo kinase subunit by the binding of dGuo or dGTP to an effector site. To examine the latter possibility, the following experiment was designed. It was shown in our preceding paper (Ikeda et al., 1994) that dATP differentially protects the dAdo kinase subunit from tryptic proteolysis, while leaving the counterpart dGuo kinase subunit susceptible to it. In order to see the effect of differential proteolysis of the dGuo kinase subunit on the properties of the counterpart dAdo kinase subunit, the limited proteolysis of the dGuo kinase/dAdo kinase complex in the presence of dATP was repeated using a smaller amount of trypsin and examining the time course of proteolysis over longer period (Figure 5). In this experiment, in addition to assaying the normal dAdo kinase activity, the dAdo kinase activity in a fully activated state was measured by adding dGuo to the assay mixture, and each activity was expressed in absolute units/mg instead of as activity relative to zero time activity. As shown previously (Ikeda et al., 1994), dGuo kinase not protected by dNTP loses more than 95% of its activity upon prolonged incubation, while dAdo kinase

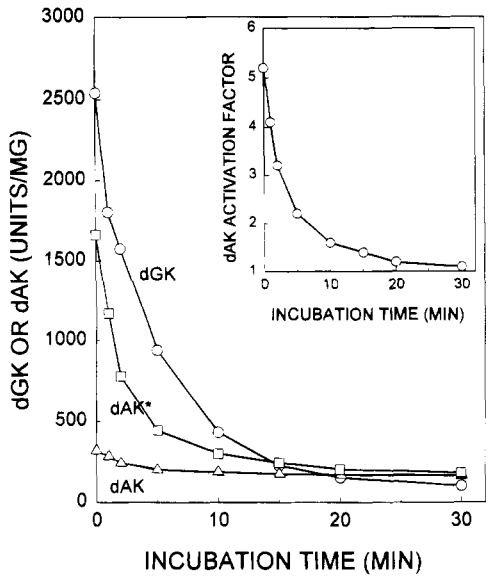


FIGURE 5: Effect of differential limited proteolysis of the dGuo kinase/dAdo kinase (dGK/dAK) heterodimer on the heterotropic activation of dAdo kinase (dAK). dGK/dAK complex was incubated with trypsin (0.2%, w/w) in the presence of 0.1 mM dATP under the conditions described in the Experimental Procedures. Each kinase activity remaining during the incubation is expressed by the absolute units/mg (○—○, dGK; △—△, dAK; □—□, dAK* (dAK fully activated by 0.1 mM dGuo added in assay mixture)). The activation factor of dAK at each incubation time (inset) was calculated from the two dAK assays.

protected by dATP retains about 50% of its original activity after an initial rapid loss of activity due to the effect of the proteolysis on the counterpart subunit. On the other hand, the dAdo kinase activity assayed in the presence of dGuo (fully activated dAdo kinase) decreased rapidly with only 10% of its original activity remaining after 30 min incubation. As a result, the extent to which dAdo kinase can be activated by dGuo drops sharply in parallel with the first-order inactivation of dGuo kinase (Figure 5, inset). After incubation for 30 min, when a relatively small amount of dGuo kinase activity remains, the activation of dAdo kinase by dGuo is also almost entirely abolished.

These results are taken as confirmation that the activation of dAdo kinase takes place through subunit—subunit interaction rather than from the binding of dGuo to the dAdo kinase subunit and that it is most likely that the binding of dGuo and ATP (or dGTP) at the active site of dGuo kinase triggers the conformational change required for the activation of the dAdo kinase subunit. Upon proteolysis of the dGuo kinase subunit, it seems that these ligands no longer bind to their active site(s), or if they do, they cannot induce such a conformational change.

*Differential Photoaffinity Labeling of dGuo Kinase/dAdo Kinase with 8-Azido-Ade.* Photoaffinity labeling with 8-azidopurine nucleotide derivatives has been successfully carried out with a number of nucleotide binding proteins and enzymes (Colman, 1983). In this work, an analogous deoxynucleoside derivative, 8-azido-dAdo, was utilized for the first time for an affinity labeling experiment in the anticipation that this compound would bind to the dAdo phosphorylation site and label the dAdo kinase subunit upon the UV irradiation.

In order to assess the complications possibly caused by nonspecific direct photoinactivation, the stability of the kinase preparation to UV-irradiation was examined prior to the photoaffinity labeling experiments. As shown in Figure 6A, both of the paired kinase activities of dGuo kinase/dAdo kinase were lost fairly rapidly upon UV-irradiation, in the absence of affinity labeling reagent. However, it was found that 10 mM ATP almost completely protects both kinases from such photoinactivation. Therefore, in all the subsequent experiments with affinity labeling reagents (deoxynucleoside substrate analogs), 10 mM ATP was included in the reaction mixture. Since dAdo kinase has been shown to follow an ordered sequential kinetic mechanism with ATP being the first substrate to bind (Chakravarty et al., 1984), it is expected that the presence of ATP is required for the binding of labeling reagent at the nucleoside site. A schematic representation of the model depicting the photoaffinity labeling of the dGuo kinase/dAdo kinase complex is shown in Figure 7.

Somewhat surprisingly, 8-azido-dAdo (0.01−0.10 mM) failed to label the targeted dAdo kinase specifically and only partially inactivated the counterpart dGuo kinase (to a maximum of 15%). It is probable that the 8-azido-derivatization forces the dAdo into an unnatural *syn* configuration incompatible with normal binding to its subsite (Lei et al., 1979; Czarnecki et al., 1982). However, 8-azido-Ade, a byproduct of 8-azido-dAdo synthesis, was found to inactivate the dGuo kinase both preferentially and stoichiometrically upon UV irradiation for only 3 min, with no apparent effect on dAdo kinase activity (Figure 6B). When 0.1 mM dGuo (final concentration) was added to the labeling mixture as a control, dGuo kinase was substantially protected from the inactivation, suggesting that the photoligand attacks the dGuo binding site. On the other hand, dAdo kinase turned out to be partially inactivated in the presence of dGuo. This unanticipated result was more clearly understood when 0.1 mM dAdo (final concentration) was added to the labeling mixture instead of 0.1 mM dGuo. As shown in Figure 6C, dAdo kinase, protected by dAdo, exhibited a concomitant *stimulated activity* over the 3-min interval required for dGuo kinase inactivation, when an aliquot was assayed upon 80-fold dilution with saturating concentrations of dAdo and ATP. The labeling of the dGuo site with 8-azido-Ade can be regarded as having activated dAdo kinase in a permanent fashion, in contrast to the reversible activation brought about by the addition of dGuo to the dAdo kinase assay mixture (Figure 7l vs Figure 2f). When 0.1 mM dGuo was included in the labeling mixture in addition to 0.1 mM dAdo, both the inactivation of dGuo kinase and the stimulation of dAdo kinase disappeared (Figure 6C). These results, altogether, indicate that 8-azido-Ade binds to the active sites of both dGuo kinase and dAdo kinase in the absence of protecting deoxynucleosides and inactivates both kinases by forming covalent bonds with the protein subunits upon UV irradiation, but with the reactivity favoring dGuo kinase. In the presence of both dGuo and dAdo, neither of the kinases is labeled. In the presence of only dAdo, dGuo kinase is preferentially labeled, causing the stimulation of dAdo kinase which is protected from the reagent by dAdo (Figure 7i−j). On the other hand, in the presence of only dGuo, dAdo kinase is preferentially inactivated. The apparent lack of effect on dAdo kinase in the absence of any protecting deoxynucleoside (Figure 6B) seems to be due to the net canceling effect of the inactivation of dAdo kinase on the stimulating of the remaining dAdo kinase by the dGuo kinase labeled with the reagent.

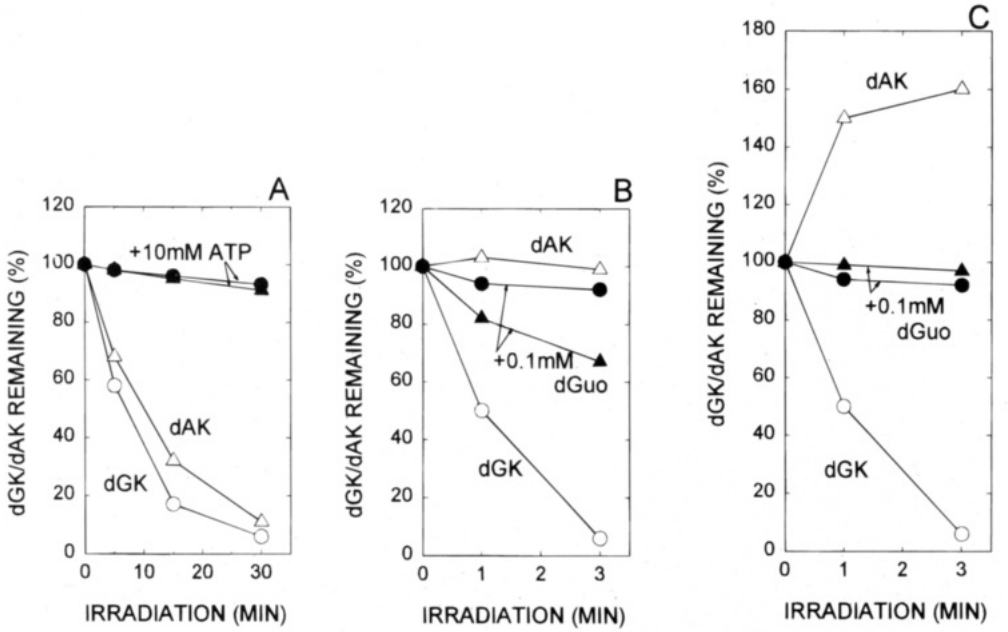


FIGURE 6: Photoaffinity labeling of dGuo kinase/dAdo kinase (dGK/dAK) with 8-azido-Ade (circles, dGK; triangles, dAK). (A) dGK/dAK (1.0 $\mu$g/10 $\mu$L of buffer B) was photoirradiated in the absence of labeling reagent under the conditions as described in the Experimental Procedures. An aliquot (1.0 $\mu$L) was taken at each irradiation time for the dGK and dAK assays (open symbols). In another experiment, 10 mM ATP (final concentration) was included in the enzyme solution (closed symbols). (B) dGK/dAK (1.0 $\mu$g/10 $\mu$L of buffer B containing 10 mM ATP) was photoirradiated in the presence of 0.1 mM 8-azido-Ade (open symbols). In a control experiment, 0.1 mM dGuo (final concentration) was included in the enzyme solution (closed symbols). (C) dGK/dAK (1.0 $\mu$g/10 $\mu$L of buffer B containing 10 mM ATP and 0.1 mM dAdo) was photoirradiated in the presence of 0.1 mM 8-azido-Ade (open symbols). In a control experiment, 0.1 mM dGuo (final concentration) was included in the enzyme solution (closed symbols).

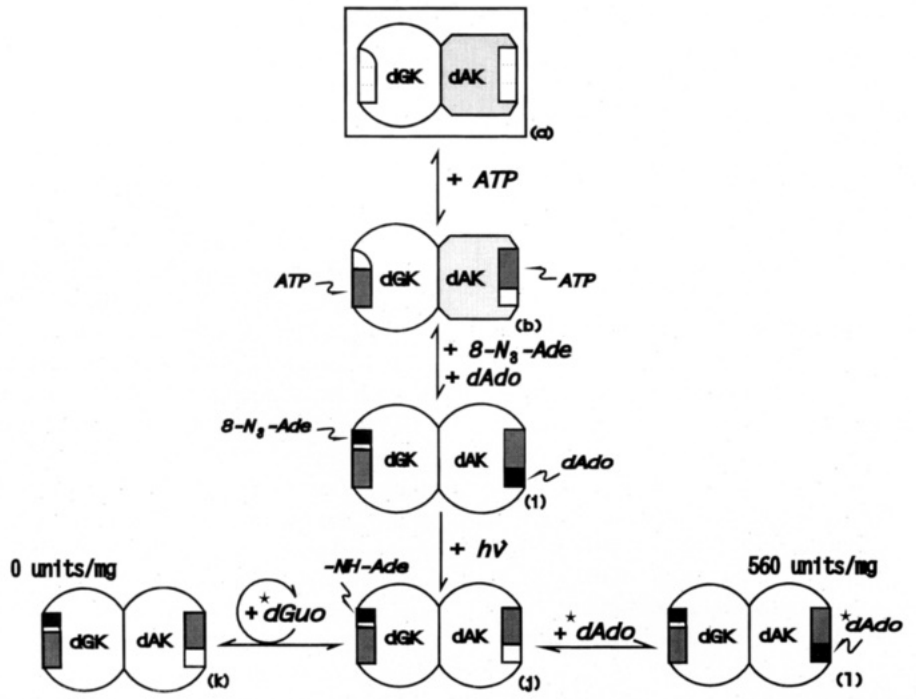


FIGURE 7: Schematic representation of the differential photoaffinity labeling of the dGuo kinase/dAdo kinase (dGK/dAK) complex with 8-azido-Ade. The scheme illustrates a model for the labeling of dGK/dAK in the complete system (in the presence of 10 mM ATP and 0.1 mM dAdo), corresponding to Figure 6 (C, open symbols). At first, 8-azido-Ade is presumed to bind reversibly to the subsite for dGuo at the active site of dGK (i). Then, upon UV-irradiation, the compound forms a covalent bond with the dGK subunit protein (j).

In order to see how strongly 8-azido-Ade binds to each kinase, its inhibition constant toward each subunit of dGuo kinase/dAdo kinase complex was measured in the absence of any protecting ligand(s) in the dark to avoid the formation of the photoactivated species. 8-Azido-Ade was found to be a fairly strong competitive inhibitor against dAdo for dAdo kinase ($K_i$ = 40 $\mu$M) (data not shown), though it produces only partial inactivation of dAdo kinase upon UV-irradiation. On the other hand, 8-azido-Ade is a much weaker competitive inhibitor against dGuo for dGuo kinase ($K_i$ = 860 $\mu$M), while it inactivates dGuo kinase more effectively and completely. It seems likely that an appropriate amino acid residue is located in a proper position at the active site of dGuo kinase and that the formation of covalent bonds with the photoactivated 8-azido-Ade species proceeds *progressively* in spite of its relatively poor affinity for the

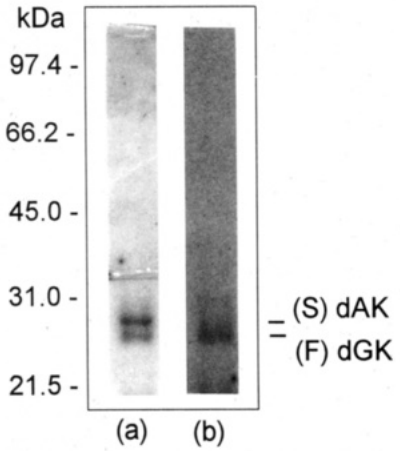


FIGURE 8: SDS−PAGE of the dGuo kinase/dAdo kinase (dGK/dAK) complex upon the differential photoaffinity labeling with [8-$^{14}$C]-8-azido-Ade. dGK/dAK (1.0 $\mu$g) was photolabeled with 0.1 mM [$^{14}$C]-8-azido-Ade in the presence of 10 mM ATP and 0.1 mM dAdo as described in the Experimental Procedures. The enzyme solution was treated with SDS−PAGE sample buffer and applied to SDS−PAGE (pH 6.6). (a) Protein bands stained with Coomassie Blue R-250 and (b) radioactive band detected on X-ray film. F and S designate the fast- and slow-moving subunit of the kinase heterodimer complex (Ikeda et al., 1994).

active site. Since the binding of 8-azido-Ade to the dGuo kinase active site is more than 10 times weaker than its binding to the dAdo kinase active site, any stimulation of dAdo kinase by 8-azido-Ade reversibly bound to dGuo kinase active site seems to be cancelled out by its inhibitory effect on dAdo kinase. The degree of dAdo kinase stimulation produced by the labeling of dGuo kinase with 8-azido-Ade has been found to be at most 2-fold and is not so high as the activation generated reversibly by dGuo or dGTP (5−7-fold). This is probably due to the incomplete fitting of 8-azido-Ade derivative to the subsite for dGuo at the active site of dGuo kinase.

Following the same experimental protocol used with nonradioactive affinity ligand, the dGuo kinase was selectively labeled with 0.1 mM [8-$^{14}$C]-8-azido-Ade in the presence of 10 mM ATP and 0.1 mM dAdo. After irradiation for 3 min, aliquots were taken for assays, and the remainder of the enzyme solution was denatured and applied to SDS−PAGE (pH 6.6). Assays (not shown) confirmed the almost total loss of dGuo kinase and the stimulation of dAdo kinase activity by nearly 200%. As shown in Figure 8, only the faster-moving (F) subunit band exhibited radioactivity when exposed to X-ray film, confirming that the labeling with [8-$^{14}$C]-8-azido-Ade took place at the dGuo kinase subunit, for this fast-moving (F) band had been assigned to the dGuo kinase subunit by using limited proteolysis in the presence of dNTP in our previous work (Ikeda et al., 1994).

These results directly indicate that the activation of dAdo kinase is caused by the binding of the substrate (analog) to the active site of the counterpart dGuo kinase subunit. At the same time, affinity labeling of dGuo kinase subunit with 8-azido-Ade produced a new species of kinase heterodimer in terms of enzyme conformation, in which the specific activity of dAdo kinase is nearly two times higher than the original activity without the addition of any other activator (Figure 7j). The enzyme conformation of this permanently activated dAdo kinase may be close to that induced in dAdo kinase by the reversible binding of dGuo or dGTP to the dGuo kinase subunit (Figure 2f and g). Analysis of the kinase heterodimer molecules including this modified species by physical methods may provide in the future some useful information on the relationship between enzyme activity and its conformation.

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