Deoxyadenosine kinase (dAK) forms a heterodimer with either deoxyguanosine kinase (dGK) or deoxycytidine kinase (dCK), and is heterotropically activated 3–5 times by dGuo or dCyd. Expressed alone, dAK is inactive and exhibits no response to dGuo or dCyd; activity and heterotropic response are fully restored upon reassociation with dGK or dCK. However, turnover of independently expressed dGK or dCK is nearly maximal, being further activated only 50–100% upon reassociation with dAK. In neither case is the heterotropic activation due to ligand-induced heterodimer formation.

A proline/alanine substitution within a dAK segment homologous to loop G2 of Ras proteins yielded a heterodimer with dAK permanently cis-activated 2-fold, with a corresponding reduction in heterotropic activation by dGuo. A chimeric dAK, with 25% of its C terminus substituted by the homologous sequence from dGK, was inactive alone, and its characteristics were unchanged in the reconstituted heterodimer. Superimposing the Pro/Ala substitution on this chimera also reduced heterotropic activation by half. Cross-linking the dimer by 1,5-difluoro-2,4-dinitrobenzene was inhibited by ATP, dATP, dGTP, and dAdo, suggesting the proximity of the active site(s) to the interface. These data suggest that dAK depends on dGK or dCK in a manner resembling the reliance of Ras upon GTPase-activating protein.

Subunit interaction is the basis for a wide spectrum of regulatory mechanisms controlling and coordinating chemical events in vivo (1, 2), and the stereochemical nature of these allosteric interactions in several protein systems has been elucidated in great detail over the past 60 years (3). Lactobacillus acidophilus R-26, lacking the ribonucleotide reductase of the deoxynucleotide de novo pathway, possesses all four deoxynucleoside kinases by which it generates building blocks of DNA through salvage pathways (4). While thymidine kinase is readily separated from the other three kinase activities (4), dAdo kinase (dAK) exists as a heterodimer with either dGuo kinase (dGK) or dCyd kinase (dCK) subunit (5–8). The turnover of the dAK active site of either heterodimer is only one-seventh to one-tenth of that of dGK or dCK, but is heterotropically activated 3–5-fold to its full activity potential by dGuo or dCyd, respectively, in contrast with the minimal effect (20%, at most) of dAdo on dGK or dCK, which are almost fully active (7, 9). Therefore, the combined output of these two heterotropically-regulated dimers provides the nearly equal quantities of dAMP, dCMP, and dGMP needed for DNA precursor synthesis (7). While dGK and dCK are kinetically and structurally parallel in a relaxed conformation (6, 7, 10), dAK is in a constrained conformational state which is relaxed only through subunit-subunit interaction in the presence of heterotropic activators. The constrained state of dAK has been inferred from experiments involving chemical modification (5), limited proteolysis, activation by chaotropic salts, and affinity labeling (10). The largely unidirectional heterotropic activation of dAK by dGuo or dCyd has not been correlated in detail with the unique conformational states of dAK, and a detailed structural mechanism of this heterotropic activation is yet to be described.

The tandem dak/dgk structural genes of L. acidophilus R-26 have been cloned; the DNA insert consists of a common endogenous promoter, the dak gene preceded by its Shine-Dalgarno sequence, a 21-base pair spacer containing the Shine-Dalgarno sequence for dgk expression, the dgk gene, and a transcription termination loop (11). The derived amino acid sequences of dAK and dGK are more than 60% identical. The remaining sequence divergences must therefore account for any differences in conformation and specificity of the two polypeptides. The sequences of dGK and dCK, on the other hand, are identical except at their extreme N termini, and these polypeptides are now believed to be alternative processing products of the dgk gene (12). Hereafter, as a first approximation, the conformations of dGK and dCK will be considered to be essentially identical within their respective heterodimers.

To examine the largely unidirectional interactions leading to optimal conformations for both subunits of a heterodimer, unmodified dAK and dGK (or dCK) were expressed separately in vivo, enabling us to study the impact of heterodimer reconstitution in vitro on the catalytic competence of these two subunits. Short-range chemical cross-linking was carried out to explore further the interface of the dAK/dGK heterodimer.

dAK and dGK each contain a segment homologous in varying degrees to the G2 loop of p21<sup>TM</sup> protein, which, in Ras, is responsible for GTP binding and interaction with GTPase-activating protein (GAP) (13). The proline residue within this loop is of particular interest, since it is believed to stiffen the polyacrylamide gel electrophoresis; pAK, porcine adenylate kinase; es, a subunit of the GTP-binding regulatory protein that stimulates adenylate cyclase.

* This work was supported in part by National Institutes of Health Grant GM49635. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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* S. Ikeda, unpublished observations.
Fig. 1. Construction of the independent chimeric dak gene. The dak/dgk tandem genes cloned in the plasmid pBlueScript(+) KS were, in Step 1, restricted with StyI endonuclease, which does not cleave the vector. In Step 2, the larger restriction fragment was, after separation from the smaller StyI insert, ligated to yield an independent chimeric dak gene loop (14). Knowing that dAK is in a constrained state, a site-directed proline/alanine substitution was made within this loop in an attempt to relax its conformation.

An obvious structural difference between dGK and dAK, and a possible basis for their differing behaviors, is the extra nine amino acid residues at the C terminus of dGK. Therefore, a chimeric dAK was constructed and expressed independently, in which nearly 25% of its C terminus was replaced by the homologous sequence from dGK, either with or without the additional proline/alanine substitution.

EXPERIMENTAL PROCEDURES

Enzyme Assays—One unit of kinase activity is defined as 1 nmol of deoxynucleoside 5’-monophosphate formed per minute, assayed in 20 μM deoxynucleoside (Sigma) and 10 mM ATP (Sigma)-Mg2+ at 20 °C; [3H]dAdo, [3H]dGuo, or [3H]dCyd (Moravek Biochemicals) was included to allow the product detection using the anion exchange disk method (15). Specific activity is expressed as units per mg of protein. Substrate concentrations were varied for steady-state kinetics analysis as indicated in each case, but each reaction mixture contained a constant amount of the radioactive deoxynucleoside.

Construction of the Independent Chimeric dAK—As shown in Fig. 1, the phagemid pBlueScript(+) KS (Stratagene) construct (11) containing the cloned tandem dak/dgk genes was cleaved at two StyI restriction sites with endonuclease from Life Technologies Inc., and the residual phagemid was religated following isolation and purification on Qiagen columns.

Independent Subcloning of the dak, dgk, and dck Genes—Plasmids with the separated dgk or engineered dak genes have recently been prepared and the kinetic properties of their products described (12). Construction of a dak clone was achieved by deleting the dgk gene from the original pBlueScript clone containing the tandem dak/dgk genes. This mutagenesis was carried out with the Mutagen-Gene® phagemid in vitro mutagenesis kit (version 2) from Bio-Rad, and the primer was synthesized at the Ohio State University Biotechnology Center. The primer, with its 5’ terminus complementary to the DNA sequence immediately downstream from dgk’s stop codon and its 3’ terminus complementary to the DNA sequence between dak’s stop codon and dgk’s Shine-Dalgarno sequence, was annealed to the single-stranded dak/dgk template. Upon obtaining the putative mutant colonies, the desired deletion was confirmed by sequencing the plasmid by the dideoxy method, using the Sequenase version II kit from U. S. Biochemical Corp. The independently subcloned dGK, dCK, and dAK proteins were expressed in Escherichia coli XL1-Blue strain (Stratagene) grown overnight in LB medium (components from Difco) containing ampicillin (100 μg/ml). Ammonium sulfate fractions (70% saturation) were prepared from all cell extracts. Further purification of specific preparations was carried out as described.

Site-directed P155A and S156T Substitutions of dAK in the dAK/dGK Heterodimer—In general, materials and methods for the mutagenesis were the same as described above. However, in order to specifically mutagenize the dak gene without altering the homologous sequence in dgk, the independent chimeric dak gene served as the template. Upon obtaining the desired mutants, a mutant dak/dgk construct was assembled by rescaling the StyI fragment from normal dak/dgk into the StyI site of the mutant chimeric dak construct (i.e. the tandem gene sequence is restored, except for the mutation within dak, reversing the steps shown in Fig. 1). DNA sequencing and assays for the dGK activity were carried out to identify colonies with the StyI insert.

Estimation of the Approximate Dissociation Constant (Kd) of dAK/dGK—The inactive dAK was partially purified as an ammonium sulfate fraction (~70% saturation), while dCK was further purified by gel filtration chromatography. The latter involves applying ammonium sulfate fraction (50 mg total protein in 8 ml) to a Sephacryl S-200 HR (Pharmacia) gel permeation column (2.5 cm × 148 cm, 726-ml volume) equilibrated with elution buffer containing 15 mM potassium phosphate (pH 8.0) and 5% glycerol. About 75% of the dAK activity was recovered with the dCK protein peak (about 80% pure, as estimated from SDS-PAGE), with a protein concentration of about 0.08 mg/ml, as determined by the Bradford method (16) using dye reagent from Bio-Rad. The molar concentration of dAK was then calculated from its subunit molecular weight. Various amounts of dCK were mixed with a fixed amount of dAK, incubated on ice for 10 min, and then diluted and incubated at 20 °C for further 5 min. Aliquots (10 μl each) were then taken from each diluted mixture for assay (at 20 °C) of the dAK activity (total volume of 40 μl) in the presence of 400 μM dCyd, which both activated the dAK subunit and blocked the secondary dAK activity of the heterodimeric dCK protein. The progress of the dAK and dCK heterodimerization was monitored by this measurement of the dAK activity, since the total catalytic turnover of dAdo within the 30-min assay period is linearly correlated with the amount of heterodimeric dAK/dCCK in equilibrium. The data were then fitted to Equation 1, which assumes that dCK used during titration existed as monomers before associating with dAK,

\[
AC = (K_{\text{d}} + A_r + A_r)^2 - [(K_{\text{d}} + A_r + A_r)^2 - (A_r + C_T)^{1/2}]
\]

Correspondingly, K_{dA} is the dissociation constant of dAK/dCCK, C_T is the total dCK concentration added each time, A_r is the total dAK concentration, and AC is the concentration of heterodimeric dAK/dCCK in equilibrium.

Enzyme Assays—The dGK or dCK active sites have secondary dAK activities (but with much lower rates), and dAK active sites have secondary dCK activities (but with much higher rates), regardless of whether the dGK or dCK is expressed independently of the other. The activities were collected. Those active fractions were then pooled and further purified on a blue-Sepharose column (2.5 cm × 2.5 cm), as

Results are based on the secondary slopes of plots of 1/V versus 1/[dAdo]; S. Ikeda, personal communication.
Ras G2-like Sequence Mediates Heterotropic Activation of dAK

**RESULTS**

Independently Expressed dAK Is Inactive Until Associated with dGK—With the primer 5'-CCA TAT TGG ACC GCA GTT TTG CT C GTT AAC TAG TTT AAA TTC CCT-3', the dgk structural gene was completely looped out from the original (11) DNA construct employing the Mutα-Gene® phagemid in *vitro* mutagenesis kit from Bio-Rad. RNA sequencing confirmed that the dgk gene was deleted, leaving only the dak structural gene immediately followed by the transcription terminator of the original tandem genes. Consequently, the dak gene in this construct should be transcribed and translated just as it is in the tandem dak/dgk genes, but without the dgk gene product. The dAK protein thus expressed was enzymatically inactive until the heterodimeric enzyme form was reconstituted in *vitro* with independently expressed dGK or dCK peptides (expressed in *E. coli* as a heterodimer in *vitro*, the specific dAK activity in a comparable ammonium sulfate fraction is usually ~3 units/mg).

The dAK activity of reconstituted dAK/dGK or dAK/dCK heterodimer could be further activated 3–5-fold heterotropically by dGuo or dCyd, respectively (Table I), just as has been technically by dGuo or dCyd, respectively (Table I), just as has been independently expressed dGK or dCK (data not shown) and dCK (Fig. 2, A and B) was already almost fully active, being activated only an additional 50–100% upon heterodimer reconstitution in *vitro*. This activation is purely a *V* max effect with no significant changes in the *K* m for either the deoxynucleoside substrate or for ATP-Mg 2+. This relatively modest dependence of the dGK or dCK activity upon the dAK subunit is paralleled by the small heterotropic activation (5–20%) of dGK (Fig. 2, C and D) or dCK (data not shown) in the heterodimer by dAdo. The heterotropic activation is also a *V* max effect with no changes of *K* m values for either the deoxynucleoside substrate (7, 9) or ATP-Mg 2+, within experimental error (Fig. 2, C and D). These experimental results can also be predicted theoretically: since dGuo binding to dGK does not change the affinity of dAK for dAdo in the presence of saturating ATP (7), according to the linked-function analysis (1) (Fig. 3), dAdo binding to dGK should not change dGK's affinity for dGuo, either. In this analysis, the assumption was made that if *K* m is not changed during heterotropic activation, neither is *K* a, and vice versa.

**Heterotropic Activation Is Not Caused by Induced Heterodimer Formation**—With the finding that dAK expressed without dGK is enzymatically inactive until the heterodimer is formed, the question naturally arises as: does the deoxynucleoside substrate strength the inter-subunit affinity of the heterodimer, hence driving the equilibrium further toward heterodimer formation and activating dAK and dGK? The independent cloning of dAK, dGK, and dCK provided the means to answer this question. During the *in vitro* heterodimer reconstitution, the amount of the dAK protein was fixed while the dGK or dCK amounts were varied so that, in effect, dAK was being titrated by the formation of heterodimer. However,
the extent of heterotropic activation of dAK by dGuo or dCyd was unaltered regardless of the progress of titration (Fig. 4), leading to the conclusion that heterotropic activation is not due to the formation of additional heterodimeric dAK. Therefore, we may exclude the possibility of a ligand-induced change of the heterodimer association constant as the mechanism of the heterotropic activation. In fact, it is clear from Fig. 4 that dGK (or dCK) had the same affinity for dAK both in the presence and absence of dGuo (or dCyd). Therefore, dAK was being titrated. Because dAK is inactive unless in the heterodimer, measuring the dAK activity from the dAK subunit revealed the progress of the titration. When dAK was being titrated nearly to completion by dGK or dCK, the heterotropic activation factor (2- or 4-fold, respectively) was unaffected, which proves that the heterotropic activation is not the result of ligand-induced heterodimer formation.

**FIG. 2.** The activity of dGK or dCK was only partially dependent on the dAK protein. The references to dGK and dCK are regarded as interchangeable in terms of their conformation. The $V_{\text{max}}$ of dGK was activated 50–100% upon forming the heterodimer with dAK. A fixed amount of the dCK protein, either alone or in the heterodimeric form with dAK, was assayed for the dCK activity at different concentrations of dCyd, and fixed 10 mM ATP and 12 mM MgCl$_2$ (A) or at different concentrations of ATP-Mg$^{2+}$ and fixed 20 mM dCyd (B). The $V_{\text{max}}$ of dGK in the dAK/dGK heterodimer was heterotropically activated only about 5–20% by dAdo. Either with or without dAdo (400 μM), the heterodimeric dAK/dGK was assayed for the dGK activity with varying concentrations of dGuo, 10 mM ATP, and 12 mM MgCl$_2$ (C), or with varying concentrations of ATP-Mg$^{2+}$ and 20 mM dGuo (D).

**FIG. 3.** Linked function analysis. The square stands for the dAK subunit, and the circle for dGK. A denotes bound dAdo, and $G$ bound dGuo. $K_1$ represents dGK’s affinity for dGuo when dAdo does not bind to dAK. $K_2$ stands for dAK’s affinity for dAdo when dGK is bound with dGuo. $K_3$ represents dAK’s affinity for dAdo when dGK is free. $K_4$ stands for the affinity of dGK for dGuo when dAK is bound with dAdo. The equation $K_1 \times K_2 = K_3 \times K_4$ applies. Since dGuo binding to dGK does not change dAK’s affinity for dAdo, $K_2$ equals $K_3$. Therefore, $K_1$ must be equal to $K_4$. It means that dAdo binding to dAK should not affect dGuo binding to dGK in the heterodimeric dAK/dGK.

**FIG. 4.** Heterotropic activation is not due to the ligand-induced heterodimer formation. The dAK activity from dAK in the reconstituted heterodimer was measured. The amount of dGK subunit (A) or dCK subunit (B) was varied while the dAK amount was fixed; therefore, dAK was being titrated. Because dAK is inactive unless in the heterodimer, measuring the dAK activity from the dAK subunit revealed the progress of the titration. When dAK was being titrated nearly to completion by dGK or dCK, the heterotropic activation factor (2- or 4-fold, respectively) was unaffected, which proves that the heterotropic activation is not the result of ligand-induced heterodimer formation.

**Estimation of the Heterodimer Equilibrium Dissociation Constant**—The dissociation constant of dAK/dGK was estimated, based on the following observations: (i) dAK is inactive by itself (Table I), (ii) the secondary dAK activity of heterodimeric dCK should be completely blocked by saturating dCyd (as discussed earlier), and (iii) the affinity between dAK and dGK (or dCK) is unaltered by dGuo (or dCyd) (Fig. 4). Furthermore, since dAK is active only in the heterodimeric form, the equilibrium concentration of dAK/dGK should be...
Fig. 5. Estimation of the dissociation constant ($K_d$) for the heterodimer. Purified dCK protein was used to titrate dAK, in the presence of saturating dCyd (400 μM) to block the secondary dAK activity from heterodimeric dCK. Hence, the measured dAK activity was solely from heterotropically activated dAK of the formed heterodimer and served to monitor the progress of the titration. The data were fitted by SigmaPlot™ with Equation 1 or 2.

directly proportional to the amount of dAdo converted to dAMP during the 30-min assay period. Therefore, the heterodimer formation was monitored directly by measuring the dAK activity of the “revived” and heterotropically activated dAK subunit. The data obtained (Fig. 5) were then fitted into Equation 1, yielding a $K_d$ of $4 \times 10^{-8}$ M. An identical $K_d$ value was also obtained through Equation 2, when an assumption was made that the dCK proteins used for the titration were in the homodimeric form before associating with dAK. Since the heterodimer formation was monitored by measuring the dAK activity from the dAK subunit in the heterodimer, the $K_d$ value should be regarded as having been obtained under current assay conditions: 100 mM Tris-HCl, 5% glycerol at 20 °C. The actual $K_d$ value under the host’s physiological conditions (37 °C) is expected to be somewhat different, especially since it has been observed that salt (e.g. NaCl) can affect the association between dAK and dGK (data not shown). However, that estimated $K_d$ should be reasonably close to the true value in vivo, because it has been determined that nearly 100% of the dCK molecules used for the titration are active. In the chemical cross-linking reactions (described in a later section), where both dAK and dGK were present at concentrations of about $2 \times 10^{-6}$ M, nearly 100% of dAK and dGK should be in the heterodimeric form according to the magnitude of this $K_d$.

The Structural Elements Distinguishing dAK from dGK Must be Located between Residues 19 and 171—Since the longer dGK protein has nine additional amino acid residues at the very C terminus that have no counterparts in the highly homologous dAK protein, the different behaviors of these two proteins might be explained in terms of this structural difference. Therefore, a chimeric dAK was constructed, containing residues 1–171 of dAK and residues 172–224 from dGK. However, this replacement of over 20% of the dAK polypeptide apparently did not change the unique conformational state of dAK. The chimeric dAK was still inactive until reconstituted into the heterodimer, and the extent of heterotropic activation of this revived chimera remained essentially the same as in the native heterodimer (data not shown). Apart from the first three amino acid residues at the very N terminus which distinguish the specificities of dGK and dCK (12), wild type dAK and dGK share an identical N-terminal amino acid sequence up to residue 18 (11). Therefore, the unique structural determinants of dAK which differentiate its properties from those of dCK and dGK must reside between residues 19 and 171.

### Table II

| dAK source            | Relative dAK activity | Heterotropic activation factor |
|-----------------------|-----------------------|-------------------------------|
| dAK/dGK               | 1.0                   | 4.3                           |
| dAK(P155A)/dGK        | 2.0                   | 4.3                           |
| dAK(S156T)/dGK        | 1.0                   | 4.2                           |

* dGuo concentration was 400 μM.

* dAK specific activity was 3 units/mg.
GTPase activity of Ras proteins and GAP, GAP directly activates the GTPase superfamily. In the interaction with GAP, the catalytic efficiency, but to greatly different extents. Reflecting the role of dGK in affecting the conformation and activity of dAK, the dAK and dGK polypeptides were independently expressed. The fact that dAK, by itself, has no discernable dAK activity therefore parallels the situation with Ras of the GTPase superfamily. In the interaction between Ras proteins and GAP, GAP directly activates GTPase activity of Ras ~100,000-fold (21–23), possibly by supplying one or more critical positive charges important for efficient catalysis (24–26). Furthermore, in G proteins of the GTPase superfamily, when both the Gα GTP-binding core domain (R-2) and a GAP-like Gα insert domain (G-2) are separately expressed as recombinant proteins, G-2 stimulates the GTPase activity of R-2 under conditions where neither alone hydrolyzes GTP (23). In another GTPase family, which includes proteins involved in protein translocation across membranes, a G2 loop-containing FtsY again has no measurable GTPase activity alone unless combined with Ffh-4.5S ribonucleoprotein (27).

Analysis of the amino acid sequences derived from the tandem dakh/dgk genes reveals that dAK and dGK both contain a segment (residues 153–161 at site iv) which is homologous, to varying degrees, with the G2 sequence of Ras proteins (Fig. 7) of the GTPase superfamily (11). While within each GTPase family the amino acid sequence corresponding to the G2 region is highly conserved, some of the residues vary between families of the superfamily. But an invariant element in all the GTPase families is a threonine residue within each putative G2 sequence (13, 28). It is interesting to note that this site iv of dAK, with its G2-like sequence, is only 6 residues away from the arginine-rich site iii (residues 140–146). While absent from Ras, this arginine-rich region is highly conserved among all the other deoxynucleoside kinases sequenced so far, as well as in other kinases such as porcine adenylylase kinase (pAK) (29, 30).

Since the Arg-138 residue of pAK (or, the Arg-156 of E. coli adenylate kinase) was shown to interact with the transferred phosphoryl group at the transition state (31, 32), it is reasonable to assume that an arginine residue in site iii of dAK or dGK has a similar function (11). In equivalent manner, this stabilizing function is also carried out by an arginine residue within the G2 sequence among the α-subunits (e.g. as (33)) of G proteins (13, 28, 34, 35). However, this catalytically important Arg is missing from the corresponding G2 sequence of Ras (13, 36), and is thought to be provided for Ras in trans by Arg-903 of its GAP (25). While required for activity, this Arg is not necessary for the association of GAF with Ras (26). The association with GAP, and the binding of GTP-Mg<sup>2+</sup>, as well, is a function of the Ras G2 sequence (13, 37). In G1, the N-terminal Ras sequence resembles the conserved P-loop of many nucleotide-binding proteins and recent model studies on Ras-catalyzed hydrolysis of GTP suggest that Gly-13 and Lys-16 also contribute significantly to transition state stabilization. Again, this GTPase superfamily’s G1 sequence is also conserved in dAK and dGK as site v (see Fig. 7).

The interaction between dAK and dGK (or dCK) subunits is viewed as occurring in two stages. The first stage occurs immediately after protein synthesis as the subunits associate into heterodimers, each subunit reciprocally affecting the other’s catalytic efficiency, but to greatly different extents. Reflecting in their contrasting conformations (10), dGK (or dCK) turnover is elevated 1.5-2-fold, while that of dAK is raised from practi-
Ras G2-like Sequence Mediates Heterotropic Activation of dAK

![Comparison of conserved sequences at sites i and iv of dAK and dGK (11) with the G1 and G2 consensus sequences of Ras-like proteins.](image)

The P-loops of both dAK or dGK (site i) and of Ras (G1 Sequence) are very near the N termini of each polypeptide, 0 to about one-seventh of its partner's activity. In a second fine-tuning stage, dAK is brought into its fully active conformation heterotropically; the binding of dGuo (or dCyd) at the dGK (or dCK) active site presumably causes a conformational change which is transmitted to the neighboring dAK subunit. This is an example of the unusual V-type activation, the $K_m$ values for substrates of dAK being unchanged (7, 9). In the opposite direction, dAdo produces only a very minor (5–20%) effect on the turnover of its partner. It is also important to note that the heterotropic activation is not caused by additional heterodimer formation.

Efforts were therefore initiated to probe the structural elements determining the contrasting conformations and activities of dAK and dGK, which occur despite highly homologous primary structures overall. Upon substituting about 25% of dAK's C terminus with dGK's homologous sequence, the character of dAK remains unchanged. The chimeric dAK is still inactive until re-associated with dGK, and subsequent heterotropic activation is of normal magnitude. This result clearly indicates that elements contributing to dAK's unique conformational properties (i.e. dAK's total dependence on dGK for its activity and the large heterotropic activation of dAK by dGuo or dGTP), as well as its specificity, are further upstream.

Just several residues upstream of the site selected for chimeric replacement, dAK has a Ras G2-like loop, but which differs slightly from that of dGK. Within this loop, among other differences, dAK has a serine residue while dGK has a threonine at position 156. However, the lack of an effect of the S156T mutation on dAK indicates that this particular amino acid replacement is not sufficient to convert dAK to dGK in any apparent way. However, since the conformation of the equivalent Ras loop changes during Ras-GAP interaction, alteration of its conformation in dAK may be expected to affect subunit interaction and substrate turnover if dAK-dGK interaction parallels that of Ras-GAP. Accordingly, a P155A substitution on dAK of the dAK/dGK heterodimer resulted in a dAK, permanently cis-activated half-way toward its maximal turnover potential, correspondingly reducing the heterotropic activation effect of dGuo. Presumably, this mutation increases loop flexibility, altering dAK's interaction with dGK in a way that partially mimics the impact on dAK when dGuo binds to dGK. This effect of the mutation on dAK is not surprising in the light of mutagenic studies on other allosteric proteins such as dimeric glutathione reductase from E. coli (38) and tetrameric pyruvate kinase of yeast (39), where point mutations of subunit interface regions are capable of altering protein conformation, intersubunit communication, and hence, allosteric behavior and catalysis. Nevertheless, the chimeric dAK, even with the addition of the proline/alanine mutation, is still inactive by itself, indicating that the alteration of the loop conformation in this way only partially mimics the process occurring during the second stage of subunit-subunit interaction.

Chemical cross-linking with a 3-Å linker offers an opportunity to study the interface of the heterodimeric dAK/dGK. By analogy with Ras-GAP, the dGK subunit contacts the dAK subunit at dAK's active site which catalyzes the transfer of the phosphoryl group. This is evidenced by the fact that ATP, dADP, and dADP all partially block the cross-linking reaction. Therefore, it is likely that both elements of dAK's active site are at the interface. dGTP can also reduce the cross-linking efficiency, suggesting that the ATP triphosphate-binding site of dGK, which dGTP is believed to partially overlap, is also close to the interface, and opposite the dAdo site. It is unfortunate that extensive apparently random monovalent addition of this reagent makes peptide mapping analysis impractical. The cross-linking event, on the other hand, is specific and useful, even if it does not afford analysis of the specific residues involved.

In conclusion, all the data up to this point are consistent with the hypothesis that dGK acts, in some way, like a "GAP" for dAK. It is obvious that the exact functional mechanisms in which the G2-sequence participates are different between dAK (as a kinase) and Ras (as a GTPase), reflecting nature's versatility in utilizing a similar structural element in different three-dimensional environments. While the heterotropic activation of dAK $V_{max}$ does not fall into the category of complex signal transduction in the conventional sense, but it does reflect the ability of protein/enzyme to regulate the downstream events in response to substrate conditions, as seen in many other systems (2). Finally, it is worth noticing that the Ras G2-like sequence conserved in the heterodimeric dAK/dGK and dAK/dCK expands the repertoire of this loop's functions to prokaryotes, and may provide a useful model for further study. In a parallel case, many of the energetic and conformational features required for chemomechanical energy transducing assemblies have also been observed in the less-complex allosteric enzymes, leading to the suggestion that structural and energetic data obtained by study of allosteric proteins can help to understand the more complicated mechanisms occurring in such eukaryotic macromolecular assemblies (40).

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