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Short communication

Mutations within the Putative Active Site of Heterodimeric Deoxyguanosine Kinase Block the Allosteric Activation of the Deoxyadenosine Kinase Subunit

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Replacement of the Asp-84 residue of the deoxyguanosine kinase subunit of the tandem deoxyadenosine kinase/ deoxyguanosine kinase (dAK/dGK) from *Lactobacillus acidophilus* R-26 by Ala, Asn, or Glu produced increased K_m values for deoxyguanosine on dGK. However, it did not seem to affect the binding of Mg-ATP. The Asp-84 dGK replacements had no apparent effect on the binding of deoxyadenosine by dAK. However, the mutant dGKs were no longer inhibited by dGTP, normally a potent distal end-product inhibitor of dGK. Moreover, the allosteric activation of dAK activity by dGTP or dGuo was lost in the modified heterodimeric dAK/dGK enzyme. Therefore, it seems very likely that Asp-84 participates in dGuo binding at the active site of the dGK subunit of dAK/dGK from *Lactobacillus acidophilus* R-26.

Keywords: Deoxyguanosine kinase, Deoxyadenosine kinase, *Lactobacillus acidophilus,* Site-directed mutation

Introduction

Because of their important role in chemotherapy, deoxynucleoside kinases have long been the subject of intensive study (Arnér and Eriksson, 1995; Ives and Ikeda, 1998). In spite of this, the active sites of this enzyme are still not well understood. A useful model is found in *Lactobacillus acidophilus* R-26, where all four of these kinases are expressed. In contrast with most other organisms, which supply most of their need for DNA precursors by means of ribonucleotide reductases, these cells are solely dependent upon salvage pathways that lead to deoxynucleoside triphosphates. These tandem pathways are initiated and controlled by three deoxynucleoside kinase activities that are situated on two heterodimeric proteins, deoxyadenosine kinase/deoxyguanosine kinase (dAK/dGK) and deoxyadenosine kinase/deoxycytidine kinase (dAK/dCK), along with a separate and apparently typical bacterial thymidine kinase (Ives and Ikeda, 1998). Regulation of the paired enzymes is achieved by a combination of end-product inhibition of each activity-its homologous deoxynucleoside triphosphate, and by allosteric activation of the dAK subunits only by the nucleoside substrate or nucleoside triphosphate end-product of the adjacent dCK or dGK subunit. The deoxynucleoside triphosphates are understood to behave as bisubstrate analogs, overlapping both the deoxynucleoside site and polyphosphate-binding domain of the ATP-Mg site (Ikeda *et al.*, 1986; Ikeda and Ives, 1994).

Tandem genes for dAK and dGK were cloned from *L. acidophilus* R-26. They can be expressed in *E. coli* using a common upstream promoter and independent Shine/Dalgarno sequences (Ma *et al.*, 1995). However, no distinct gene for dCK was found in the lactobacillus donor. Therefore, we infer that the specificity for deoxycytidine (dCyd) arises upon the N-terminal editing of the nascent dGK peptide by this organism since the mature dCK and dGK peptides appear otherwise to be identical (Ma *et al.,* 1996). A dimeric protein with physical, chemical, and regulatory properties that are identical to those of native dAK/dCK was expressed in *E.coli* upon deletion of codons 2 and 3 from the *dgk* portion of the tandem genes.

Site-directed mutagenesis was used to identify the function of amino acids in various enzymes (Huang *et al.*, 1999; Kim *et al.*, 2001; Lee and Cho, 2001). Efforts to identify the active sites of the cloned lactobacillus enzyme began with sequence comparisons with herpes-viral thymidine kinase (TK). These revealed several conserved regions, including glycine- and arginine-rich ATP-binding motifs and a DRS (Asp-Arg-Ser) motif that is like the putative TK deoxynucleoside site (Balasubramaniam *et al.*, 1990; Gentry, 1992; Ma *et al.*, 1995). A conserved *ras* G2-like motif that was also present has been implicated in the heterotropic activation of dAK

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(Guo and Ives, 1997). Earlier work in our laboratories found that the replacement of amino acids in the *Lactobacillus* DRS motif modified the enzyme activities of both dGK and dAK. The replacement of Asp-78 for both subunits with Glu, Ala, or Asn virtually inactivated both activities. The conservative replacement of Arg-79 with Lys, either on both subunits in tandem (R79K) or on the dGK subunit only (R79K : dGK), yielded active but kinetically modified enzymes (Hong *et al*., 1995). A crystallographic study of herpesviral TK clearly implicated this motif in nucleoside binding. In the present work, we attempted to extend our knowledge of the deoxynucleoside domain in the *Lactobacillus acidophilus* R-26 dAK/dGK model system. A possible functional residue that is located near the DRS motif is Asp-84, which was probed by replacing it with Ala, Glu, and Asn.

Materials and Methods

Materials The 2'-deoxyadenosine (dAdo) and 2'-deoxyguanosine (dGuo) were obtained from Sigma; 2'-[2,8-^{3}H]deoxyadenosine and 2'-[8-^{3}H]deoxyguanosine were from Moravek Biochemicals, Inc. Deoxyadenosine 5'-[α-^{35}S]thiophosphate was purchased from the Amersham Corp. *Escherichia coli* XL1-Blue was from Stratagene. The Sequenase version II kit was obtained from U.S. Biochemical Corp. All other materials, including protocols and enzymes for site-directed mutagenesis, were from the Muta-Gene Phagemid *in vitro* Mutagenesis Version II kit, purchased from Bio-Rad.

Enzyme assays Deoxynucleoside kinase activities were assayed using the method of Ives (Ives, 1984) with minor modifications as follow. In the standard assay, the final concentrations of reagents in the reaction mixture (in a 1.5 ml Eppendorf tube) were: ATP, 10 mM; $MgCl_2$, 12 mM; Tris/HCl, pH 8.0, 0.1 M; [^{3}H]dGuo, 0.2 mM (0.5 µCi/assay), respectively. The reaction was started by adding 20 µl of enzyme that was diluted in a 15 mM potassium phosphate buffer, pH 8.0, containing 20% (v/v) glycerol to the warmed reaction mixture tube (final volume, 40 µl). After 30 min of incubation at 20ºC, 0.1 ml of formic acid was added to stop the reaction. Aliquots (20 µl) of the mixture were spotted on Whatman DE-81 anion exchange papers for the measurements of radioactivity.

Site-directed mutagenesis The materials and protocols of the Muta-Gene phagemid *in vitro* site-directed mutagenesis kit (version II) from Bio-Rad, based on a method described by Kunkel, was used (Kunkel, 1985). Reverse primers used for the site-directed mutagenesis were: for the D84A mutant, (5'-GAAAAGAGCAGC TTCGTAG); the D84N mutant, (5'-GAAAAGAGCATTTTCGTA G); and the D84E mutant, (5'-GAAAAGAGCCTCTTCGTAG). Pure oligomers were air-dried, dissolved in 1 ml of a 10 mM Tris buffer, pH 8.0, and stored at −20ºC until use. The 5' end of each mutagenic oligomer was phosphorylated with polynucleotide kinase to improve the frequencies of mutagenesis. After the polymerization/ligation reaction was continued for 90 min at 37ºC, the reaction was stopped by adding a Tris-EDTA buffer. Transformants were grown on LB plates that contained ampicillin. Each instance of mutagenesis was confirmed by DNA sequencing using the dideoxynucleotide termination method (Sanger *et al.*, 1977).

Extraction of enzyme *E. coli* XL1-Blue cells that contained mutants of the recombinant pBluescript KS(+) clone GTM-K48 were cultured in a LB liquid medium that contained 100 µg/ml ampicillin for expression of the enzyme (Ma *et al.*, 1995). The cell pellets were washed in an ice-cold cell suspension buffer (0.1 M Tris-HCl, pH 8.0, 25 mM EDTA, 20% glycerol) and used immediately or stored at −20ºC for later use. The washed cell pellets were suspended in a cell suspension buffer, broken by ultrasonication, and centrifuged at 13,500 × *g* for 1 h at 4ºC.

Results and Discussion

In order to increase our understanding of the active site(s) of deoxynucleoside kinases, particularly elements participating in the binding of the nucleoside substrate, we have begun probing residues that are located in a short region that are conserved in both the heterodimeric dAK and dGK subunits (adjacent to the DRS motif for which there is already substantial evidence of active-site involvement) (Hong *et al*., 1995; Brown *et al*., 1995). Asp-84 in dGK of *Lactobacillus acidophilus* is one such conserved residue (Fig. 1). It was also conserved in human dGK (Johansson and Karlsson, 1996), human dCK (Johansson and Karlsson, 1996), dNK of *Lactococcus lactis* (Bolotin *et al*., 2001), dGK/dAK of *Streptococcus pyogenes* (Ferreti *et al*., 2001), dGK/dAK of *Mycoplasma pneumonia* (Dandekar *et al*., 2000), dGK of *Ureaplasma gentitalium* (Glass *et al*., 2000), dGK/dAK of *Deinococcus radiodurans* (White *et al*., 1999), and dGK/dAK *of Borrelia burgdorfri* (Fraser *et al*., 1997). However, no

78	**D R** S I Y E **D** A L F F	88	*L.acidophilus* dGK (Ma *et al*., 1995)
78	**D R** S I Y E **D** A L F F	88	*L.acidophilus* dAK (Ma *et al*., 1995)
141	E **R** S V Y S **D** R Y I F	151	human dGK (Johansson and Karlsson, 1996)
127	E **R** S V Y S **D** R Y I F	137	human dCK (Johansson and Karlsson, 1996)
78	**D R** S I F E **D** E – – L F	86	*Streptococcus pyogenes* dGK/dAK (Ferreti *et al*., 2001)
100	**D R** S I F E **D** W – L F	109	*Mycoplasma pneumonia* dGK/dAK (Dandekar *et al*., 2000)
104	**D R** S I F E **D** W – L F	113	*Ureaplasma urealyticus* dGK (Glass *et al*., 2000)
78	**D R** T V F E **D** A N I F	88	*Deinococcus radiodurans* dGK/dAK (White *et al*., 1999)
83	**D R** S I Y G **D** C V F A	93	*Borrelia burgdoferi* dGK/dAK (Fraser *et al*., 1997)

Fig. 1. Comparison of amino acid sequences of dAK/dGK from *Lactobacillus acidophilus,* human dGK and dCK, dGK/dAK from *Streptococcus pyogenes*, dGK/dAK from *Mycoplasma pneumonia*, dGK from *Ureaplasma urealyticus*, dGK/dAK from *Deinococcus radiodurans,* and dGK/dAK from *Borrelia burgdoferi* in regions near the DRS motif. Identical amino acid residues are indicated by boldface.

Table 1. Effect of D84 replacements on kinetic parameters

Mutants	K_m (μM)	V_{max} (nmole/mg/min)	V_{max}/K_m (nmole/mg/min/M)
UMCE	8.7	410	47
D84A	360	5.3	0.015
D84N	340	19	0.056
D84E	550	170	0.31

UMCE: Unmodified cloned enzyme (apparently identical to wild-type). The expression level of the enzymes was estimated to be 3.0% of total protein. The V_{max} values were calculated on the basis of this estimation.

Table 2. Effect of deoxynucleoside triphosphate on activities of various mutant enzymes

dNTP (0.1 mM)		Relative activity (%)			
		UMCE	D84A	D84N	D84E
dGK	dATP	78	63	65	86
	dGTP	14	90	98	106
dAK	dATP	50	25	42	40
	dGTP	340	109	140	100

Activities are relative to the uninhibited form of the same enzyme genotype.
The dGuo or dAdo concentration was 20 μM.

previous research has been performed to identify the role of the amino; therefore, it was selected for the present study. A common strategy that is used here is the charge-conservative replacement, charge-neutralization, and replacement with the smallest L-amino acid, alanine. Mutations at or near the substrate binding or catalytic site are likely to affect the kinetic properties of the enzyme. These parameters were therefore screened with enzyme that was extracted from the various mutants. Among the mutant enzymes, the replacement of Asp-84 with Ala (D84A) resulted in the most drastic decrease in the dGK efficiency ratio (V_{max}/K_m) (Table 1). The K_m values for dGuo increased dramatically for all three of the mutant enzymes. The D84A, D84N, and D84E mutants had comparable K_m values of 360 μM, 340 μM, and 550 μM, respectively. This was nearly two orders of magnitude larger than in the unmodified control. Correspondingly, the dGK V_{max} values also decreased in all mutants. Interestingly, the replacement of Asp-84 with Glu, which conserves the negative charge, only reduced the maximum velocity slightly over one-half; whereas the non-conservative replacements with asparagine or alanine reduced the turnover by more than 95%. Thus, the variations in the efficiency ratios are due directly to the variations in the maximum velocities. This suggests that the extra methylene group in glutamic acid might provide a steric hindrance for the D84E mutant to bind dGuo, thereby increasing the K_m value. The conservation of the negative charge might be essential for catalysis of the

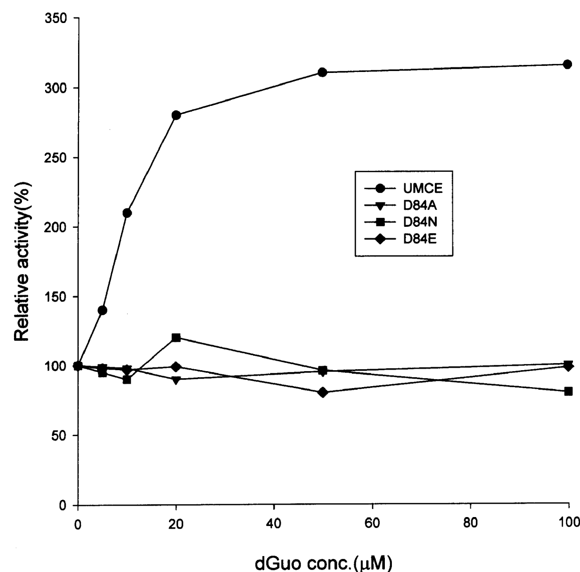

Fig. 2. Effect of dGuo concentration on dAK activities of various mutant enzymes. The dAdo and Mg-ATP concentrations were 20 μM and 10 mM, respectively. The half-maximal heterotropic activation of dAK was achieved by dGuo concentration that corresponded to the K_m value for dGuo (8.7 μM).

enzyme since the D84E mutant exhibited the high V_{max} value compared to those of D84A and D84N . The K_m values for Mg-ATP were also determined for both UMCE and the D84E mutant with dGuo as the co-substrate. The K_m values of Mg-ATP for UMCE and D84E were 6.9 mM and 7.2 mM, respectively (data not shown). Therefore, this mutation at Asp-84 seems to have little effect on the Mg-ATP binding site.

The large increases in the dGuo K_m that was observed upon modification of Asp-84 in dGK should also affect the binding of its regulatory ligand, dGTP; if as an earlier work has suggested, the nucleoside portion of dGTP occupies the dGuo binding site (Ikeda *et al.*, 1986; Ikeda and Ives, 1984). As can be inferred from Table 2, the normally strong inhibition of the unmodified dGK by dGTP is reduced or even eliminated by D84 replacements. Conversely, whereas UMCE is stimulated three-fold by 0.1 mM dGTP, these modifications also prevent dGTP from allosterically activating dAK. Both of these effects are consistent with the understanding that dGTP behaves as a multisubstrate analog that bridges the deoxynucleoside and ATP-phosphate domains of the active site. This weakens the binding of dGuo and also prevents the dGuo portion of dGTP from binding strongly to the dGK subunit. Fig. 2 that exhibits the heterotropic activation of dAK by dGuo was also abolished in the D84 replacement, while the dAK activity of UMCE was normally heterotropically activated about 3.0-fold by dGuo (at 100 μM). The *cis*-inhibition of dAK by dATP is largely unaffected by these alterations of D84 on dGK.

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