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Post-Translational Phosphorylation of Serine 74 of Human Deoxycytidine Kinase Favors the Enzyme Adopting the Open Conformation Making It Competent for Nucleoside Binding and Release

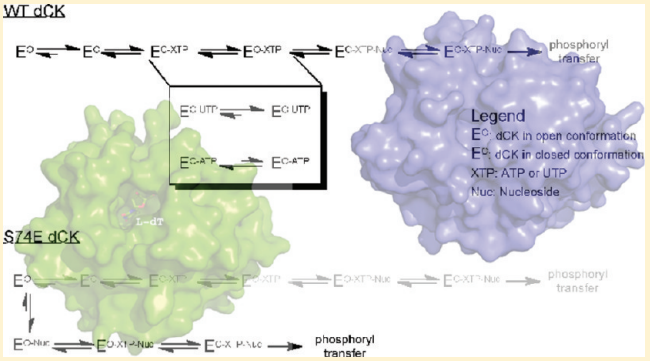
Saugata Hazra,[†] Andrzej Szewczak,[‡] Stephan Ort,[‡] Manfred Konrad,[‡] and Arnon Lavie*[,†]

[†]Department of Biochemistry and Molecular Genetics, University of Illinois, 900 South Ashland Avenue, Chicago, Illinois 60607, United States

[‡]Max Planck Institute for Biophysical Chemistry, Am Fassberg 11, D-37077 Göttingen, Germany

Supporting Information

ABSTRACT: Deoxycytidine kinase (dCK) uses either ATP or UTP as a phosphoryl donor to catalyze the phosphorylation of nucleoside acceptors. The kinetic properties of human dCK are modulated in vivo by phosphorylation of serine 74. This residue is a part of the insert region and is distant from the active site. Replacing the serine with a glutamic acid (S74E variant) can mimic phosphorylation of Ser74. To understand how phosphorylation affects the catalytic properties of dCK, we examined the S74E variant of dCK both structurally and kinetically. We observe that the presence of a glutamic acid at position 74 favors the adoption by the enzyme of the open conformation. Glu74 stabilizes the open conformation by directly interacting with the indole side chain of Trp58, a residue that is in the proximity of the base of the nucleoside substrate. The open dCK conformation is competent for the binding of nucleoside but not for phosphoryl transfer. In contrast, the closed conformation is competent for phosphoryl transfer but not for product release. Thus, dCK must make the transition between the open and closed states during the catalytic cycle. We propose a reaction scheme for dCK that incorporates the transition between the open and closed states, and this serves to rationalize the observed kinetic differences between wild-type dCK and the S74E variant.



Human deoxycytidine kinase (dCK) is a nucleoside kinase responsible for the phosphorylation of deoxycytidine (dC), deoxyadenosine (dA), and deoxyguanosine (dG) to their monophosphate forms. As such, dCK plays a pivotal role in the salvage pathway of deoxyribonucleosides for their ultimate conversion to triphosphorylated forms suitable for incorporation into DNA. The enzyme catalyzes phosphoryl group transfer by simultaneously binding the nucleoside phosphoryl group acceptor at one binding site and the nucleotide phosphoryl group donor at a second binding site. Our previous work[1−3] has revealed that dCK can adopt several distinct conformations, which are a function of the nature of the nucleoside and nucleotide bound.[2,4] Both ATP and UTP can serve as phosphoryl donors.[5] The enzyme undergoes a main chain rearrangement at the nucleotide base-sensing loop to accommodate UTP relative to its conformation with ATP (Figure S1 of the Supporting Information).[4] Moreover, the nature of the nucleotide at the donor site can determine the conformation of the enzyme at the acceptor site. For example, the conformation of dCK's nucleoside binding site determined in the presence of dA is dependent on the nature of the nucleotide: a closed conformation in the presence of ADP but an open conformation in the presence of UDP[2] (note that structures in complex with nucleotides were determined using the diphosphates because cocrystallization with the triphosphates would result in the reaction taking place, and only poor crystals were obtained with ATP or UTP nonhydrolyzable analogues). The designation "open" or "closed" relates to the compactness of the nucleoside binding site. The closed state has been interpreted as the catalytically active state, because in the closed conformation glutamic acid 53 (Glu53) directly interacts (distance of ∼2.5 Å) with the nucleoside's 5′-hydroxyl group (Figure S1 of the Supporting Information). The function of Glu53 is to activate the 5′-hydroxyl group to attack the γ-phosphate of the nucleotide donor UTP or ATP. In contrast, in the open state conformation, the distance between the side chain of Glu53 and the nucleoside 5′-hydroxyl group increases to more than 4 Å, thereby disabling

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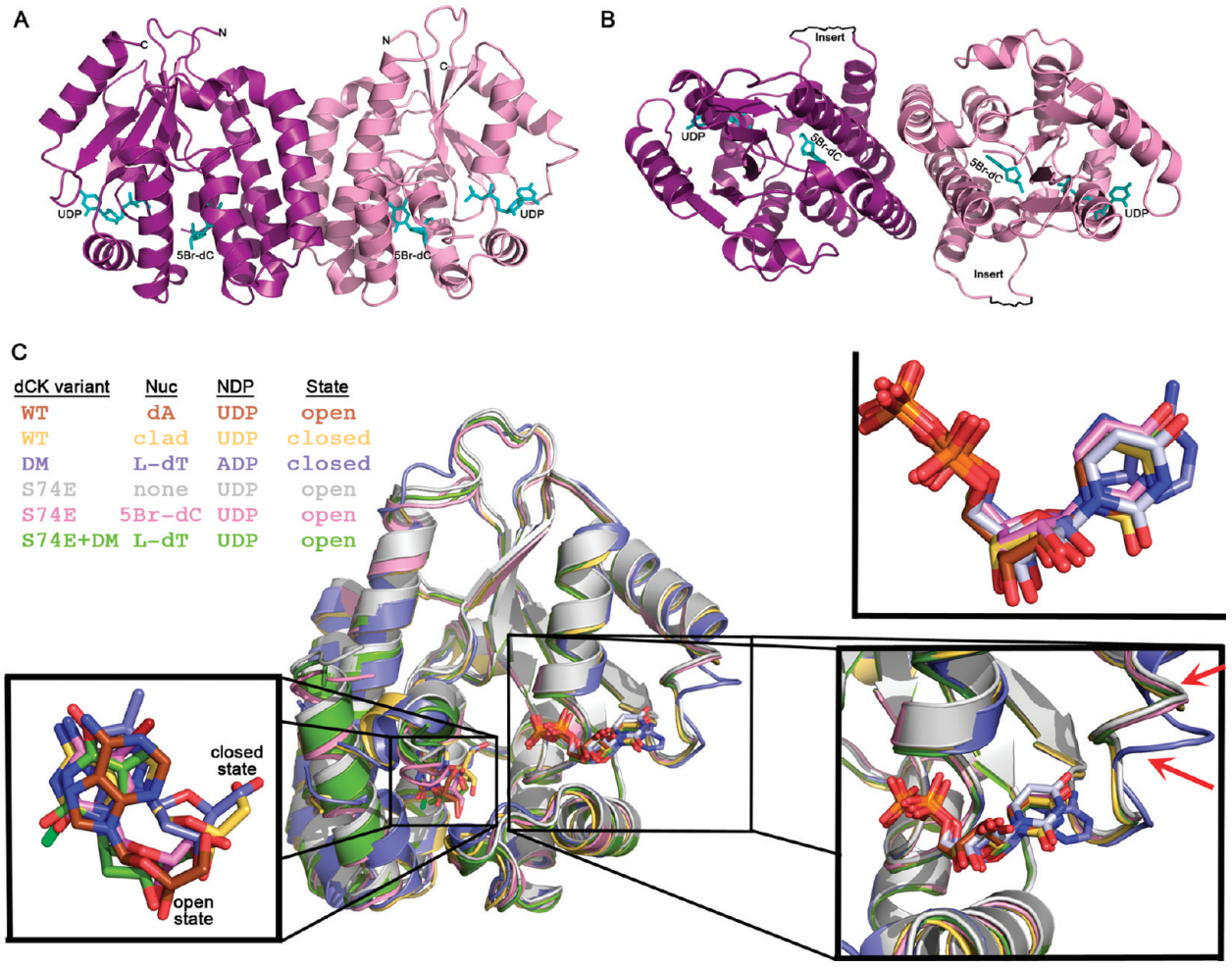

Figure 1. Conformational plasticity in human dCK at the nucleotide donor and nucleoside acceptor binding sites. (A) Ribbon diagram of a dCK dimer at a view perpendicular to the three dimer interface helices. Shown is the S74E dCK variant in complex with UDP at the phosphoryl donor site and 5-bromodeoxycytidine (5Br-dC) at the nucleoside acceptor site. One monomer is colored pink and the other magenta. The nucleosides and nucleotides are colored cyan. The N- and C-termini are labeled N and C, respectively. (B) View rotated 90° relative to panel A. The location of the insert, a 15-residue region that connects two helices, is marked. A jagged black line shows the six to eight insert residues that could not be seen in the electron density. (C) Ribbon diagram of an overlay of a dCK monomer from six different structures: wild-type (WT) dCK in complex with dA and UDP (brown, PDB entry 2ZI6), WT dCK in complex with cladribine and UDP (yellow, PDB entry 2ZIA), dCK with the R104M/D133A double mutation (DM) in complex with L-dT and ADP (blue, PDB entry 3EXK), dCK with the S74E mutation and UDP (gray), dCK with the S74E mutation in complex with UDP and 5Br-dC (pink), and dCK with the S74E and DM mutations in complex with L-dT and UDP (green). In the bottom right close-up, the conformation at the nucleotide binding site is dependent on the nature of the nucleotide, UDP or ADP, at this site. Red arrows indicate the main chain differences of the base-sensing loop between the UDP- and ADP-containing complexes. In the top right inset, the nucleotides adopt a nearly identical position despite the conformational differences in the base-sensing loop. In the left close-up, in contrast, the nucleoside in the closed state binds at a different position relative to nucleosides binding in the open state.

the ability of Glu53 to activate the nucleoside and making this state an inactive conformation of the enzyme.

The enzyme dCK belongs to a family of nucleoside kinases that includes human deoxyguanosine kinase (dGK) and human thymidine kinase 2 (TK2).[6] The fold of these homodimeric kinases consists of a β-sheet core surrounded by several α-helices (Figure 1A). In dCK and dGK, but not in TK2, an insert region that consists of 12−15 residues connects helices 2 and 3 (Figure S2 of the Supporting Information). The function of this insert was a mystery, but recently it was shown that dCK undergoes post-translational phosphorylation at serine 74 (Ser74),[7] which is a part of the insert region (residues 63−77), and that this modification influences the enzyme's activity. To study the function of this post-translational modification, we mimicked the Ser74 phosphorylated state by mutating the residue to a glutamic acid. Analysis of the Ser74-to-glutamic acid (S74E) mutant revealed a change in the kinetic properties of dCK that encompassed both the k_{cat} and K_m values.[8] Some of these effects were dramatic, such as a 10-fold increase in the rate of dC phosphorylation. Interestingly, while the S74E variant has an increased rate with the pyrimidine dC, it displays a decreased rate with the purines dA and dG. Because dCK is constitutively expressed[6] [albeit at very dissimilar levels in different tissues (http://biogps.gnf.org)], phosphorylation of Ser74 could be a mechanism for regulating the specificity of the kinase to favor dC.

How this mutation (and, in analogy, the phosphorylation of Ser74) has the observed effects on the catalytic activity of dCK was unclear because this residue is far from the active site

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(Figure 1B). To gain insight into the mechanism of dCK activity modulation by phosphorylation of Ser74, we sought to determine the structure of the S74E dCK variant in complex with its substrates. The challenge was that in all prior crystal structures of dCK the insert region lacked electron density and could not be modeled (except for structures in which this region forms an artificial conformation because of crystal contacts). Indeed, the insert region remained disordered when we determined the structure of the S74E dCK variant in complex with dC and ADP (data not shown). Thus, we sought crystallization conditions that would stabilize the conformation of the insert region. Previous structural results with dCK have shown that the specific nature of the nucleoside and/or nucleotide can dramatically affect the dCK conformation. Therefore, we tested alternative combinations of nucleosides and nucleotides and were successful in discovering three conditions under which the electron density for and around E74 was of high quality. In the work reported here, we present the analysis of these three crystal structures of dCK containing the S74E mutation. The structures suggest a mechanism for how phosphorylation of Ser74 can affect the catalytic properties of dCK.

EXPERIMENTAL PROCEDURES

Materials. General laboratory reagents were purchased from Fisher (Pittsburgh, PA) and Sigma-Aldrich (St. Louis, MO). L-Deoxythymidine (L-dT) and 5-bromodeoxycytidine (5Br-dC) were purchased from ChemGenes Corp. (Wilmington, MA). All other nucleosides and nucleotides were obtained from Sigma.

Protein Expression and Purification. Our previous work has shown that a variant of dCK (C_4S-dCK), in which four of six cysteine residues are mutated to serine (C9S/C45S/C59S/C146S), is more amenable to crystallization but otherwise behaves like wild-type (WT) dCK.[9,10] The three S74E mutation-containing structures reported here were determined in the background of this C_4S-dCK variant. When we refer to WT dCK in this report, we mean the C_4S-dCK variant. To allow the binding of thymidine, two additional mutations were introduced, R104M and D133A, as previously reported. All constructs were expressed in C41(DE3) *Escherichia coli* using a pET14b vector, grown in 2YT medium, and induced with 0.1 mM IPTG over 4 h at 37 °C. Cells were harvested, and the pellet was lysed by sonication. Lysates were cleared by centrifugation at 30000*g* for 1 h at 4 °C and subjected to purification with HisTrap HP (GE Healthcare) following the supplier's protocol. After elution with 250 mM imidazole, the protein was further purified by gel filtration using an S-200 column. Protein fractions were pooled, concentrated, aliquoted, frozen in liquid nitrogen, and stored at −80 °C until they were used.

Kinetic Assay. The activities of WT dCK and mutants were determined using an NADH-dependent enzyme-coupled assay and a Carry UV spectrophotometer.[3,11] All measurements were taken in triplicate at 37 °C in a buffer containing 100 mM Tris (pH 7.5), 100 mM KCl, 10 mM $MgCl_2$, and 1 mM ATP. The enzyme concentration was 0.33 μM. To determine the K_m and k_{cat} values, we varied the nucleoside concentration and kept all the other components fixed. Data were fit to the Michaelis−Menten equation using SigmaPlot. Because of the moderate absorption coefficient for NADH (the signal of our assay), this assay is not suitable for measuring a substrate concentration lower than ~3 μM.

Crystallization, X-ray Data Collection, and Refinement. Crystals of dCK in complexes with nucleosides and/or nucleotides were grown using the vapor diffusion method from hanging drops. The specific conditions for the complexes were as follows. For the dCK S74E/DM variant in complex with L-dT and UDP, 1 μL of protein at 9 mg/mL, 5 mM L-dT. and 5 mM UDP were mixed with 1 μL of reservoir buffer at room temperature; for the dCK S74E variant in complex with 5Br-dC and UDP or UDP alone, 1 μL of protein at 12 mg/mL (15 mg/mL for UDP only), 5 mM 5Br-dC, and 5 mM UDP were mixed with 1 μL of reservoir buffer at 12 °C. The reservoir solution contained 0.9−1.5 M trisodium citrate dihydrate and 100 mM Tris (pH 7.5). Crystals were cryoprotected with mineral oil. In the case of the dCK S74E variant in a complex with UDP alone, diffraction data were collected from a single frozen crystal using a RAXIS-IV^{++} detector mounted on a Rigaku RH-200 rotating anode X-ray generator. For two other complexes (dCK S74E in a complex with 5Br-dC and UDP and dCK S74E/DM in a complex with L-dT and UDP), diffraction data were collected at the Advanced Photon Source using SERCAT beamline BM-22. Data were processed with XDS.[12] The structure was determined by molecular replacement using MOLREP[13] and the dCK structure (PDB entry 1P5Z) as a search model. Refinement was conducted with REFMAC.[14] All three data sets are perfectly twinned, and refinement was conducted with the Twin option active in REFMAC. Data collection and refinement statistics are listed in Table 1.

RESULTS AND DISCUSSION

The goal of this work is to elucidate how phosphorylation of Ser74, a residue far from the active site, influences the catalytic activity of dCK. We surmised that the presence of a negative charge at that position affects the enzyme's conformation, and hence its activity. To test this hypothesis, we crystallized and determined the structures of S74E mutation-containing dCK under three conditions: (i) with only UDP, (ii) with 5-bromodeoxycytidine (5Br-dC) and UDP, and (iii) with L-deoxythymidine (L-dT) and UDP. Data collection and refinement statistics are listed in Table 1. The overlay of dCK monomer structures determined under six different conditions (three previously reported and three reported here) showcases the conformational plasticity of the enzyme (Figure 1C). The nature of the nucleotide (UDP vs ADP) influences the conformation of the base-sensing loop; all complexes with UDP have the base-sensing loop adopt one conformation and those with ADP another (Figure 1C, bottom right close-up, and Figure S1 of the Supporting Information). This overlay of the structures also shows that despite the different base-sensing loop conformation, the nucleotide UDP or ADP binds at the same position (Figure 1C, top right). Note that the kinetic parameters for a particular nucleoside acceptor are very different when the donor is ATP versus UTP; for most nucleosides, the kinetic parameters determined with UTP are a lower k_{cat} value with a concomitant lower K_m value in comparison to those measured with ATP.[2,4] The possible mechanism of communication between the nucleotide base-sensing loop and the nucleoside binding site has been the topic of previous reports.[1,2] Here we focus on conformational plasticity at the nucleoside binding site.

The nature of the nucleoside can determine if the enzyme adopts the closed (and active) or open (inactive) conformation. Interestingly, very small changes in the structure of the nucleoside suffice to select the open or closed conformation. For

Table 1. Data Collection and Refinement Statistics[a]

	S74E and UDP	S74E, 5Br-dC, and UDP	S74E/DM, L-dT, and UDP
Data Collection			
PDB entry	3QEJ	3QEN	3QEO
X-ray source	rotating anode	SER-CAT BM	SER-CAT BM
wavelength (Å)	1.5418 (Cu anode)	1.0	1.0
temperature (K)	100	100	100
resolution range (Å)	30.0−2.50	30 0.0−2.00	30.0−1.90
no. of reflections			
observed	46265	273588	330797
unique	18394	37632	44080
completeness (%)	95.2 (99.1)	99.5 (97.8)	99.7 (98.3)
R_{sym} (%)	5.7 (55.4)	6.1 (41.9)	6.7 (74.0)
$I/\sigma I$	11.6 (1.6)	17.6 (3.9)	19.1 (2.9)
space group	$P4_1$	$P4_1$	$P4_1$
unit cell dimensions			
$a = b$ (Å)	68.43	69.14	68.76
c (Å)	120.37	118.90	120.60
no. of molecules per asymmetric unit	2	2	2
Refinement			
R_{work} (%)	20.1	19.7	19.5
R_{free} (%)	23.6	24.7	24.4
no. of atoms			
protein	1946, 1945	1956, 1945	1940, 1939
UDP	25 × 2	25 × 2	25 × 2
nucleoside	0	17 × 2	17 × 2
water	90	138	156
root-mean-square deviation			
bond lengths (Å)	0.011	0.013	0.013
bond angles (deg)	1.387	1.576	1.538
average B factor (Å^2) per chain			
protein	56.3, 56.3	41.6, 40.2	34.3, 34.4
UDP	65.9, 71.1	43.0, 35.8	36.3, 34.1
nucleoside	−	44.3, 45.5	51.1, 66.1
water molecules	47.5	41.6	34.3
Ramachandran plot regions			
most favored (%)	90.0	89.5	91.4
additionally allowed (%)	9.1	9.5	7.9
generously allowed (%)	0.7	0.9	0.5
disallowed (%)	0.2	0.0	0.2

[a] Values for the highest-resolution shell are in parentheses.

example, cladribine [2-chlorodeoxyadenosine (2CDA)], which is a dA analogue used in cancer chemotherapy, is different from dA only in the presence of a chlorine atom at the adenine base 2-position, yet WT dCK's conformation is open when determined in the presence of dA[2] (Figure 1C, brown) and closed in the presence of cladribine[1] (Figure 1C, yellow), where in both cases UDP occupied the donor site. Thus, the sole difference between these two structures is a single atom located at the nucleoside's base, and this in turn determined which enzyme conformation was most stabilized under the crystallization conditions. This structure of WT dCK in a complex with dA and UDP was until now the only dCK structure that displayed the open conformation. We speculated that the open conformation is not unique to nucleoside dA and that the rate of transition between the open (catalytically incompetent) and closed (catalytically competent) conformations could explain the different kinetic rates observed for dCK substrates. In the three structures of dCK containing the S74E mutation reported here, we were able to observe this open conformation.

Structures of dCK Containing the S74E Mutation Adopt the Open State. The majority of the dCK crystal structures have been determined as a ternary complex of the enzyme, a nucleoside, and a nucleotide. We were also successful in determining the structure of dCK only in the presence of a nucleoside (i.e., in the absence of a nucleotide).[4] In contrast, until now, we could not determine the structure of WT dCK in the absence of a nucleoside (i.e., solely with a nucleotide). The closest we got were nondiffraction quality crystals of WT dCK in the presence of

UDP, yet with the S74E mutation, we obtained good crystals in the presence of UDP. The structure of dCK S74E in a complex with UDP shows the open enzyme conformation (Figure 1C, gray). This suggests that the presence of the S74E mutation was sufficient to stabilize the enzyme in a single conformation that is compatible with crystallization, specifically the open state conformation. It also suggests that WT dCK and UDP did not form good crystals because it was interconverting between the open and closed states.

In addition to this nucleoside-free structure, we determined two structures of S74E mutation-containing dCK in the presence of nucleosides: the complex with 5Br-dC and UDP (Figure 1C, pink) and the complex with L-dT and UDP (Figure 1C, green). Both of these also adopt the open state. Significantly, in all structures of dCK with nucleosides that adopt the open conformation (these two S74E mutation-containing complexes and WT dCK in complex with dA and UDP), the nucleoside is positioned differently relative to the position adopted in structures with the closed conformation (Figure 1C, left close-up, and Figure S1 of the Supporting Information). Invariably, the open conformation positions the nucleoside 5′-hydroxyl group at a distance too large for a direct interaction with Glu53, thereby eliminating the ability of this carboxylic acid to activate the nucleoside. Showcasing the importance of a direct interaction between the side chain of Glu53 and the nucleoside 5′-hydroxyl group are mutagenesis experiments performed on the analogous *Drosophila melanogaster* deoxynucleoside kinase (dNK). In these experiments, it was shown that replacing the glutamic acid with the shorter aspartic acid reduces the k_{cat} by more than 4 orders of magnitude while negligibly changing the K_m values.[15]

Note that all of the structures were determined with nucleosides that can be phosphorylated by dCK, a fact that implies that the enzyme does have the ability to assume the closed and catalytically competent conformation with these substrates. The fact that we observe the open conformation indicates that we have generated conditions that favor the open conformation over the closed conformation. Because the crystallization conditions used for the open and closed structures are identical, these cannot be the deciding parameters that determine whether the open or closed conformation is adopted. Rather, it is the nature of the dCK variant, WT versus S74E, and the nature of the nucleoside that determine which conformation is preferred.

Structural Differences between the Open and Closed States. To analyze in more detail the differences between the open and closed conformations, we compare two structures: a double mutant (DM) variant of dCK (R104M/D133A) that permits binding of thymidine,[16,17] in a complex with L-dT and ADP, and the other variant that in addition to the DM mutations contains the S74E substitution, also with L-dT, but this time with UDP (Figure 2). Ideally, we would compare two structures

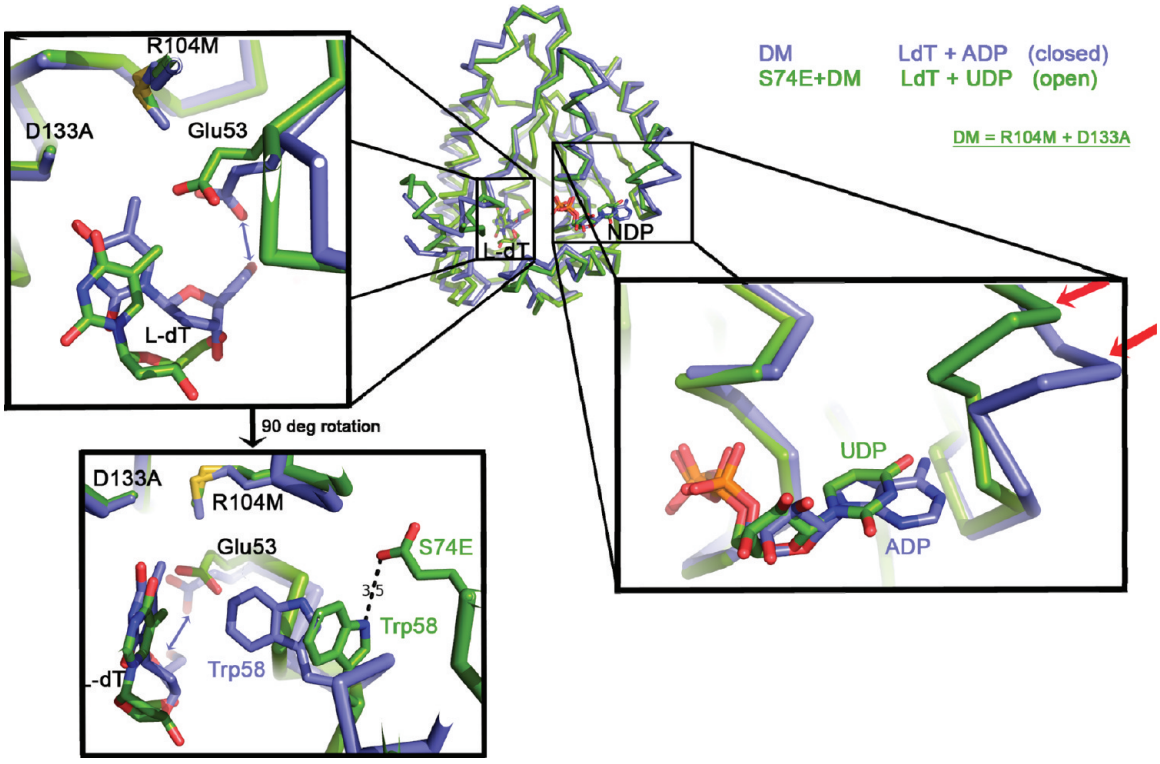

Figure 2. Mutation of Ser74 to a glutamic acid residue, done to mimic its phosphorylation, induces the open dCK conformation in the complex with L-dT. To allow for the binding of thymidine, a double mutant (DM) of dCK was generated. The dCK DM protein in complex with L-dT and ADP (blue) adopts the closed conformation, whereas the dCK DM/S74E version in complex with L-dT and UDP adopts the open conformation (green). In the center, the structures overlay perfectly except for the base-sensing loop and the nucleoside-binding region near the insert. The right close-up showcases the differences in the base-sensing loop (red arrows). The top left close-up showcases the different positions of L-dT between the open and closed conformations. Glu53 makes an interaction with the L-dT 5′-hydroxyl group only in the closed state (blue double arrow). In contrast, in the open state, L-dT is too distant to allow activation by Glu53. The position of the R104M/D133A double mutation present in both structures is indicated. The bottom left close-up is a view with a 90° rotation relative to the top close-up. Trp58 undergoes a dramatic conformational change upon making the transition from the closed to open state. The open state (green) is stabilized via an interaction between the side chain of E74 and the Trp58 nitrogen of the indole ring. E74 is a part of the insert region that could be resolved in the open state. This region lacks electron density in the closed state.

in which the sole difference is the presence or absence of the S74E mutation. However, we could not obtain crystals of dCK DM in complex with L-dT and UDP or of the S74E/DM variant with L-dT and ADP. Hence, in this structural comparison, in addition to the presence or absence of the S74E mutation, one complex has ADP and the other UDP. Both contain the DM mutations, which permit the binding of nucleosides with a thymine base to dCK.

The right close-up of Figure 2 serves to show the already discussed and expected differences of the nucleotide base-sensing loop due to the different nature of the nucleotide (UDP or ADP). Red arrows highlight the differences in the main chain conformation that is determined by the nucleotide present. However, we are interested in the conformation at the nucleoside binding site. Our previous work has shown that both ADP and UDP are consistent with the enzyme adopting a closed conformation at the nucleoside binding site, but with UDP, we also observed the open conformation when dA was present as the nucleoside. As the top left close-up of Figure 2 shows, L-dT is bound in a different position between the two complexes: within interaction distance via its 5′-hydroxyl to the side chain of Glu53 (blue double arrow) in the case of the DM−L-dT−ADP complex (closed state) but at a greater distance in the case of the S74E/DM−L-dT−UDP complex (open state).

The closed state has the side chain of Trp58 in the proximity of the base of the nucleoside. In contrast, in the open state, Trp58 swings away from the nucleoside (Figure 2, bottom left close-up). Thus, the open state is characterized by a more open nucleoside binding pocket (Figure S1 of the Supporting Information). We observe the side chain of Glu74 to be within 3.1−3.5 Å of the indole nitrogen atom of the tryptophan residue (distance varies between the two monomers in the asymmetric unit). This suggests that Glu74 stabilizes the open state via this interaction with Trp58. However, the open state does not require the substitution of Ser74 with a glutamic acid: in the dA−UDP structure,[2] the open state is adopted despite the residue at position 74 being a serine. In fact, comparing the structure of WT dCK in complex with dA and UDP to the three S74E dCK mutant structures presented here shows an identical open conformation. This point is shown in Figure 3, which for the sake of simplicity presents only the overlay of the S74E variant in complex with 5-bromodeoxycytidine (5Br-dC) and UDP (pink) on the WT dCK in complex with dA and UDP (brown). The enzyme residues for these structures overlay perfectly, as do the UDPs, and 5-Br-dC with dA. The conclusion is that the unphosphorylated and phosphorylated forms of dCK can attain the same open state, and that the interaction between Glu74 and the tryptophan's indole ring is not essential for the achievement of the open state.

In all cases of the open state, whether elucidated using WT dCK or the dCK S74E variant, the nucleoside base adopts the same position, which is different from that adopted in the closed state. Additionally, the side chain of Trp58 is rotated whenever the open state is achieved. We[2] and others[18] have reported a change in the tryptophan fluorescence signal upon substrate binding. We can now attribute this signal change to the change in the conformation of Trp58. While not essential, the presence of a glutamic acid instead of a serine at position 74 seems to increase the propensity of the open state. Presumably, phosphorylation of Ser74 would result in the same open state-favoring effect, where the interaction would be between the phosphate group and the indole of Trp58.

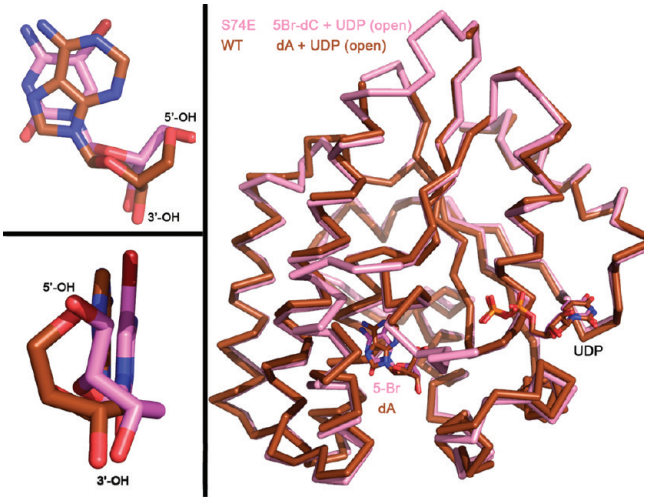

Figure 3. dCK can adopt the open state also in the absence of the S74E mutation. Shown is an overlay of WT dCK in complex with dA and UDP (brown) with the dCK S74E variant in complex with 5Br-dC and UDP (pink). The structures overlay well throughout, including the base-sensing loop, because both had bound UDP, and the nucleoside binding site because both adopt the open state. The left panels show two 90°-rotated views of the nucleosides that demonstrate the similar binding position of dA and 5Br-dC.

How different are the open and closed states? As a representative of the open state, we selected the dCK S74E complex with 5Br-dC and UDP (Figure 4, pink), and for the closed state, we selected WT dCK with cladribine and UDP (Figure 4, yellow). We chose the latter complex for this analysis because we wanted a closed state that has UDP and not ADP at the phosphoryl donor site, to minimize the differences between the complexes. Figure 4 reveals a nearly perfect overlay of the open and closed enzyme states, with differences limited to the nucleoside binding site region. Not surprisingly, because both complexes contained UDP, the nucleotide base-sensing loop is at the identical conformation. As noted above, the hallmarks of the open state are the rotated conformation of the side chain of Trp58 and the more distant binding position of the nucleoside relative to the side chain of Glu53 (Figure 4). In addition to these differences, we observe differences in the insert region. In structures of the closed state, we could not resolve the insert region. Specifically for the structure with cladribine and UDP, residues spanning amino acids 61−78 could not be modeled. The lack of electron density for the insert region is an invariant observation made in all structures of the closed state of dCK. In contrast, in structures of the open state, we are able to trace a large fraction of the insert region. The length and relative angles of helices 2 and 3, which span the insert region, are altered upon formation of the open state, as the insert adopts a defined conformation (Figure S1 of the Supporting Information). Specifically, for the structure with 5Br-dC and UDP, we could model the insert region except for residues between amino acids 63 and 70. Importantly, residue 74 has now acquired unambiguous electron density and is within interaction distance of the indole nitrogen of Trp58 (Figure 4, close-up).

The Open State Is Stabilized by Direct Interactions with Glu74. In the closed enzyme state, the Trp58 nitrogen of the indole ring interacts with the side chain of Ser103 (Figure 5). This interaction is broken as the tryptophan side chain rotates in the open enzyme conformation. In its place is the direct

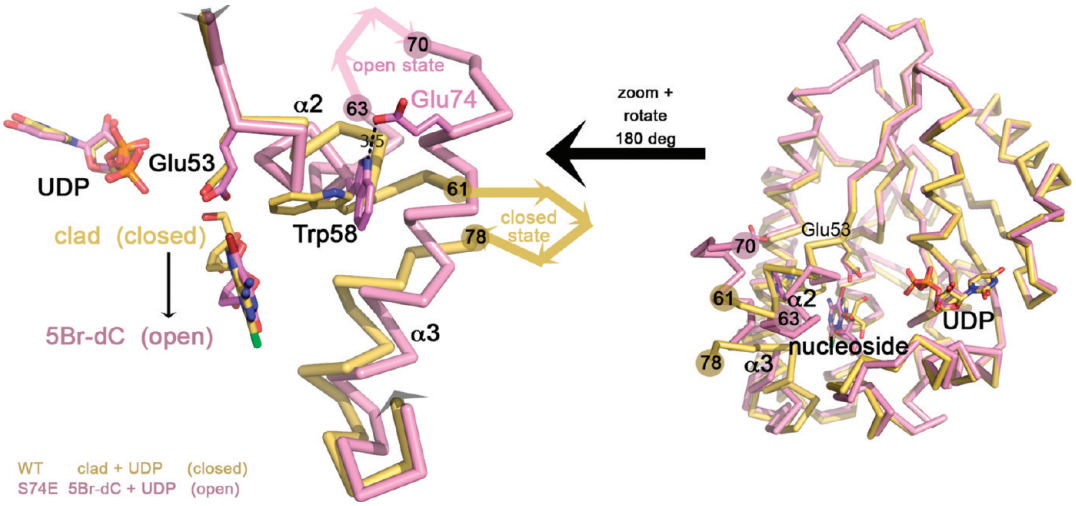

Figure 4. Open state characterized by a different angle of the helices spanning the insert region and a different conformation of Trp58. Shown is an overlay of WT dCK in complex with cladribine and UDP (yellow, closed state) and the dCK S74E variant in complex with 5Br-dC and UDP (pink, open state). Because the structures of both complexes were determined in the presence of UDP, the conformation of the base-sensing loop is identical, as is the position of UDP. The cladribine complex is in the closed state, in which we could not model the residues spanning amino acids 61−78 (encompassing the insert region). However, the S74E mutation induced the open state, and now a large portion of the insert could be modeled (a break limited to residues between positions 63 and 70). The open state conformation is stabilized by an interaction between Glu74 and Trp58 (indicated by a dashed line). Note the different position of the nucleosides, cladribine vs 5Br-dC, as indicated by the black arrow. Cladribine, which is in the closed state, can interact with the side chain of Glu53, whereas 5Br-dC in the open state it too distant to maintain this interaction. Glu53 serves to activate the 5′-hydroxyl group to attach the phosphoryl donor. Hence, the closed state is a catalytically competent conformation, whereas the open state is not.

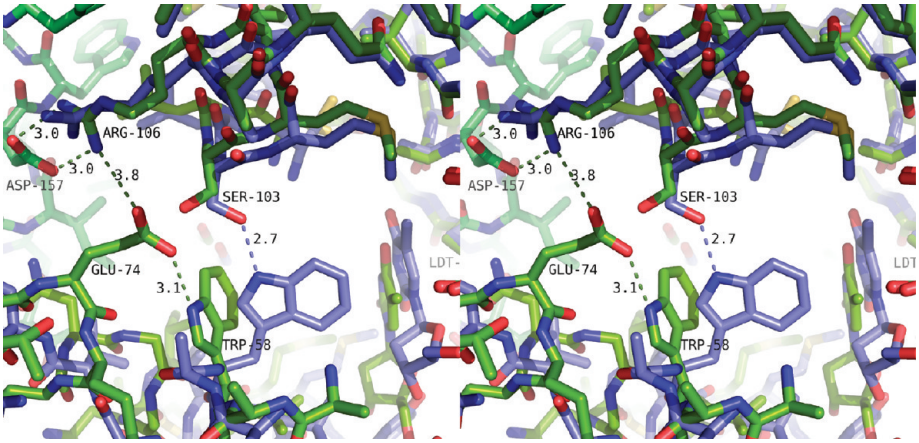

Figure 5. Different interactions stabilize the open vs closed states. Shown is a stereoview of the overlay of the insert region near residue 74 from the dCK DM variant structure in complex with L-dT and ADP (blue) and the dCK DM/D74E variant in complex with L-dT and UDP (green). In the closed state, Trp58 points toward the base of the nucleoside L-dT, and the nitrogen of the indole group is 2.7 Å from the side chain of Ser103. The insert region is not visible in electron density maps of dCK in the closed state. Upon transition to the open state, Trp58 rotates, with a concomitant adjustment of the Ser103 side chain. Additionally, the insert region, which includes residue 74, displays clear electron density. Glu74 is 3.1 Å from the Trp58 indole nitrogen atom and 3.8 Å from Arg106. The side chain of Arg106 makes a dimer interface interaction through a salt bridge with Asp157 that originates from the other monomer (light green).

interaction with the side chain of Glu74. An analogous interaction would be possible in the case of the Ser74-phosphorylated enzyme. To probe the role of the Trp58−Glu74 interaction, we generated the dCK W58F/S74E variant and characterized it kinetically. This mutant, which cannot form this interaction, retains phosphorylation activity and is kinetically more similar to WT dCK than dCK S74E (Table 2). This suggests that the observed Trp58−Glu74 interaction is indeed important in modifying the activity of dCK, and that the substitution of Trp58 with a residue that cannot recapitulate this interaction has generated an enzyme with near-WT character despite the presence of Glu74.

The data given above show that abolishing an interaction that stabilizes the open state, by substituting Trp58 with a phenylalanine, induces a more WT-like kinetic character in S74E mutation-containing dCK. The alternative to destabilizing the open state by eliminating the Trp58−insert interaction is to destabilize the closed state. This could potentially be accomplished by eliminating the Trp58−Ser103 interaction that occurs only in the closed state conformation. We exchanged Ser103 with

Table 2. Steady State Kinetic Data for WT and Mutant dCK

	WT dCK[a]			dCK S74E[a]			dCK W58F/S74E			dCK S103A/S74E		
nucleoside	k_{cat}[b]	K_m[b]	k_{cat}/K_m[c]	k_{cat}	K_m	k_{cat}/K_m[c]	k_{cat}	K_m	k_{cat}/K_m[c]	k_{cat}	K_m	k_{cat}/K_m[c]
dC	0.04	<3	>13.3	0.45	8.9	50.6	0.14	10.6	13.2	0.27	17.06	15.8
dA	0.77	116.9	6.6	0.69	517.9	1.3	0.95	233.1	4.07	—[d]	—[d]	—[d]
dG	0.73	245.8	2.9	0.36	601.8	0.6	0.66	514.1	1.28	—[d]	—[d]	—[d]

[a] From ref 8. [b] k_{cat} in units of s^{-1} and K_m in units of μM. [c] At $\times 10^3$ s^{-1}/M. [d] Activity undetectable or extremely weak.

an alanine in the background of the S74E variant. This protein has lost most of its ability to phosphorylate purines dA and dG but is very active with pyrimidine dC (Table 2). One interpretation of these results is that the energy barrier for the formation of the closed (and catalytically competent state) is higher with purines than with pyrimidines. This is consistent with the fact that the WT dCK complex with dA and UDP adopts the open state, yet the complex with dC and UDP adopts the closed state; i.e., the purine-bound enzyme favors the open state and the pyrimidine-bound enzyme the closed state. Hence, by eliminating an interaction that stabilizes the closed state, we have increased the barrier to the formation of catalytically competent state in the case of purine substrates, but not enough in the case of pyrimidine substrates such as dC.

Understanding the dCK Mechanism in Terms of the Open and Closed States. The discovery that dCK undergoes post-translational phosphorylation of the insert Ser74 residue exposed a mechanism for modulating the activity of this enzyme.[7,19] The structural and kinetic study presented here of the Ser74 phosphomimic, the dCK S74E variant, reveals how this mechanism functions, namely, by increasing the propensity of dCK to adopt the open state. This conclusion is supported by the results of Keszler et al.,[20] who examined the activation of dCK in lymphocytes that revealed that dCK activation is accompanied by a conformational change to a more open state.

Why is the open state important for the function of dCK, though it is a catalytically incompetent conformation because of the increased distance between the nucleoside and Glu53? We propose that the open state is required for the binding of the substrate nucleoside and the later release of the monophosphorylated product. Figure 6 presents surface representations of the closed (dCK DM—L-dT—ADP, blue) and open (dCK S74E/DM—L-dT—UDP, green) structures. The nucleotide ADP or UDP is visible on the surface of dCK in both the closed (Figure 6a) and open (Figure 6b) states. This means that binding and release of the nucleotide are compatible with either state. In contrast, the nucleoside L-dT is totally hidden within dCK in the closed state (Figure 6c) yet is solvent-exposed in the open state (Figure 6d). This suggests that the nucleoside cannot dissociate from, or bind to, the closed state, and that nucleoside binding and/or release must occur via the dCK open conformation. Thus, a consequence of Ser74 phosphorylation is the modulation of the transition from the nucleoside-binding competent conformation (open) to the catalytically competent conformation (closed).

How does this new understanding of the dynamic nature of dCK, which originates from structural studies, fit with kinetic data for this enzyme? Steady state analysis by the Shewach group has revealed a dCK mechanism that is dependent on the nature of the phosphoryl donor.[21] With ATP, a random bi-bi mechanism was proposed, in contrast to an ordered reaction sequence with UTP, in which UTP binds before the nucleoside. Subsequent pre-steady state studies by the Eriksson group also indicated that UTP would bind prior to the nucleoside.[22] Critically, UTP binding resulted in a dCK conformational change that could be detected by a change in tryptophan fluorescence. In our hands, titration of dCK with ATP (or ADP) resulted in no intrinsic fluorescence change, yet with UTP (or UDP), we detected a change in tryptophan fluorescence.[2] The sum of these data supports a mechanism for dCK as shown in Figure 7.

We propose that in the absence of substrates, dCK is in equilibrium between an open state (designated E^O) and a closed state (E^C). In the case of WT dCK, E^C is favored over E^O. This would explain the pre-steady state kinetic data that show that nucleoside binds much less favorably to the apoenzyme than nucleotide.[22] Because the nucleoside binding site in the closed state is not solvent accessible (Figure 6c), E^C will preferentially bind nucleotide. ATP or UTP can bind to either the open or closed states, but the binding of UTP will favor a transition from E^C to E^O. In support of this, our titration experiments showed a tryptophan signal when UTP or UDP is added, but not when ATP or ADP is added to the apoenzyme.[2] The transition from E^C to E^O results in the change in the position of Trp58, so this is the likely source for the change in fluorescence observed by others and us. While both enzyme—nucleotide binary complexes can adopt either the open or closed state, UTP prefers the open state more than ATP. In addition to these kinetic and fluorescence data, our structural results also support this conclusion. First, the fact that all of the open structures to date have been determined with UDP implies that this nucleotide induces the enzyme to favor the open state to a greater degree than ADP does. Second, the structure of WT dCK in complex with the nucleoside dA and the nucleotide ADP is closed yet with UDP is open.

It is in this open state that nucleosides can bind. However, the ternary complex that forms first (E^O—nucleotide—nucleoside) is not catalytically competent, as suggested by the structures that reveal a long distance between the 5′-hydroxyl group of the nucleoside and the side chain of Glu53. Hence, prior to the chemical step, the enzyme must undergo the transition to the closed state. This mechanistic proposal for WT dCK can also explain the dependence of the nucleoside K_m value on the nature of the nucleotide, where the K_m is lower in the presence of UTP compared to that measured in the presence of ATP. Because ATP is not as efficient as UTP in converting E^C to E^O, with ATP a smaller fraction of the enzyme is in a state that is compatible with nucleoside binding, and this ultimately results in an increased K_m value.

The situation is different when Ser74 is phosphorylated, as indicated by our results with the dCK S74E variant. We propose that the stabilization of the open Trp58 conformation by the phosphorylated insert (mimicked by S74E) results in the open state being the preferred apo state, instead of the closed state in

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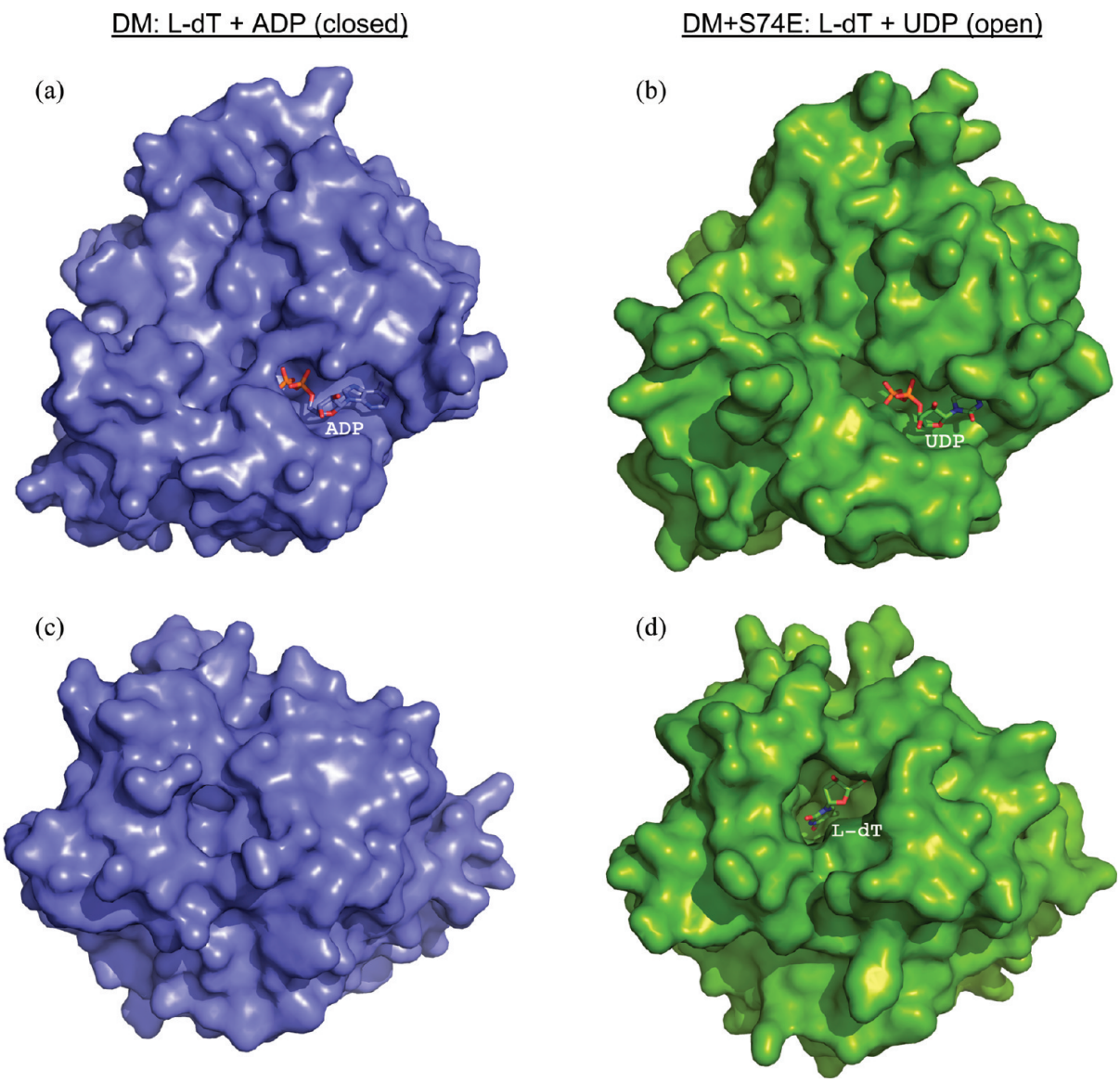

Figure 6. The open state, but not the closed state, is compatible with the binding of nucleoside to dCK. Shown are surface representations of the dCK DM−L-dT−ADP complex structure (blue) as an example of the closed state and the dCK DM/S74E−L-dT−UDP complex structure (green) as an example of the open state. Identical views of the closed (a) and open (b) nucleotide binding site show ADP or UDP at the surface of the protein. (c) View of the nucleoside binding site in the closed state indicating that L-dT is not accessible to the surface of the protein. (d) In contrast, the same orientation of the open state complex reveals the solvent accessibility of L-dT. Hence, nucleosides require the open state to bind to or be released from dCK.

unphosphorylated dCK. Hence, nucleoside can bind directly to apo-dCK. However, this stabilization of the open state, while conducive for nucleoside binding, becomes a barrier for the reaction because only the closed state can catalyze phosphoryl transfer. Therefore, the dCK S74E variant is characterized by increased K_m values for the nucleosides. Interestingly, while both UTP and phosphorylation of Ser74 act to select the open state, in the former case the effect reduces the nucleoside K_m value and in the latter it increases the nucleoside K_m value. We have analyzed the kinetic behavior of dCK with and without the S74E mutation with numerous nucleoside substrates (mostly in the background of the R104M/D133A double mutant). This analysis (Table S1 of the Supporting Information) shows that the presence of S74E invariably increases the K_m value of the nucleoside, but also the k_{cat} values that may be determined by product release being the rate-limiting step for the dCK reaction. Both effects can be rationalized by a conformational change in dCK between the open and closed states. The higher K_m for nucleosides is due to an increased energy barrier to the formation of the catalytically competent closed state. The increased k_{cat} is due to the S74E mutation as a result of the more rapid transition to the open state after phosphoryl transfer has occurred, allowing for the release of the nucleoside monophosphate product (which is not possible from the closed state).

The fact that all of the open structures to date have been determined with UDP implies that this nucleotide induces the enzyme to favor the open state to a greater degree than ADP does. Consistent with this interpretation are the considerable differences in K_m values for nucleoside substrates when measured in the presence of ATP versus UTP. By influencing the

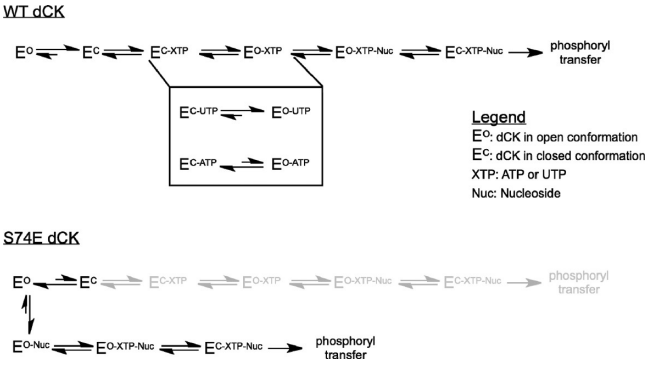

Figure 7. Proposed reaction scheme for WT dCK and dCK S74E. WT dCK is in equilibrium between the open conformation (E^O) and the closed conformation (E^C), but with a preference for the closed state. Nucleotide (ATP or UTP) can bind to this enzyme state, but nucleoside binding is dependent on dCK adopting the open state. Prior to phosphoryl transfer, the closed ternary complex must form. In contrast, the S74E mutation opens an alternative pathway by preferentially stabilizing the open state.

equilibrium between the open and closed states, the nucleotides affect the ability of the enzyme to productively bind the nucleoside substrates, and hence their K_m values. Phosphorylation of Ser74, or the mutation of Ser74 to a glutamic acid, would serve to favor the open state, and indeed, the K_m values for all three physiological substrates of dCK (dC, dA, and dG) are increased in the dCK S74E variant compared to that of WT dCK.

CONCLUSION

The activity of human dCK is modulated by several factors that include the nature of the nucleotide phosphoryl donor, the nucleoside phosphoryl acceptor, and the state of phosphorylation of residue Ser74 located in the insert region. This work reveals, by examining the phosphomimic dCK S74E variant, dCK undergoes a transition from an open nucleoside binding site to a closed nucleoside binding site. The former is compatible with the binding and release of the substrate at the nucleoside binding site, but not with phosphoryl transfer. The latter is compatible with the phosphoryl transfer reaction, but not with substrate binding. Substituting Ser74 with a glutamic acid acts to preferentially stabilize the open conformation. Phosphorylation of Ser74, as found in the cellular environment, is expected to have a very similar effect. We propose that the rate of conformational transition between the open and closed states is modulated by the properties of the residue at position 74. This rate acts to determine to a large extent the rate of nucleoside phosphorylation by dCK. Our work improves our understanding of phosphoryl transfer catalyzed by dCK by adding an additional snapshot (the open state) to the structurally characterized dCK conformations. With this and previous snapshots of this enzyme (closed state), we increase our level of appreciation of the dynamic behavior required by dCK to catalyze phosphoryl transfer.

ASSOCIATED CONTENT

Supporting Information. Detailed kinetic analysis of WT, DM, and DM/S74E (Table S1), an overlay of the structure of DM/S74E in complex with L-dT and UDP (open conformation) with the structure of DM in complex with L-dT and ADP (closed conformation) (Figure S1), and a structure-based sequence alignment of dCK with homologous nucleoside kinases (Figure S2). This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Department of Biochemistry and Molecular Genetics, University of Illinois, MBRB, room 1108, 900 S. Ashland Ave., Chicago, IL 60607. Telephone: (312) 355-5029. Fax: (312) 355-4535. E-mail: Lavie@uic.edu.

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ABBREVIATIONS

dCK, deoxycytidine kinase; dCK S74E, serine-to-glutamic acid mutant of dCK; dCK DM, R104M/D133A variant of dCK; WT, wild type; 5Br-dC, 5-bromodeoxycytidine; L-dT, L-deoxythymidine; PDB, Protein Data Bank.

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