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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

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Abstract

Deoxycytidine kinase (dCK) uses either ATP or UTP as phosphoryl donors 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 situated distant to 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 enzyme adopting the open conformation. Glu74 stabilizes the open conformation by directly interacting with the indole side chain of Trp58, a residue that is in close proximity to 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 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 form. 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

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SUPPORTING INFORMATION AVAILABLE

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

(5). The enzyme undergoes a main-chain rearrangement at the nucleotide base-sensing loop to accommodate UTP relative to its conformation with ATP (Fig. S1) (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 solved 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 since co-crystallization with the triphosphates would result in the reaction taking place, and only poor crystals were obtained with ATP or UTP non-hydrolysable analogs). 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, since in the closed conformation glutamic acid 53 (Glu53) directly interacts (distance ~ 2.5 Å) with the nucleoside's 5'-hydroxyl group (Fig. S1). The function of Glu53 is to activate the 5'-hydroxyl group to attack the gamma 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 over 4 Å, thereby disabling 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 beta sheet core surrounded by several alpha helices (Fig. 1A). In dCK and dGK, but not in TK2, an insert region that consists of 12–15 residues connects helices 2 and 3 (Fig. S2). The function of this insert was a mystery, but recently it was shown that dCK undergoes post-translational phosphorylation on serine 74 (Ser74) (7), which is a part of the insert region (residues no. 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 increased rate with the pyrimidine dC, it displays decreased rate with the purines dA and dG. Since dCK is constitutively expressed (6), (albeit at very dissimilar levels in different tissues (<http://biogps.gnf.org>)), phosphorylation of Ser74 could be a mechanism to regulate the specificity of the kinase to favor dC.

How this mutation (and in analogy, the phosphorylation of Ser74) achieves the observed effects on the catalytic activity of dCK was unclear since this residue is far from the active site (Fig. 1B). To gain insight into the mechanism of dCK activity modulation by phosphorylation of Ser74 we sought to solve 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 where this region forms an artificial conformation due to 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 in 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-bromo-deoxycytidine (5Br-dC) were purchased from ChemGenes Corporation, 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 (C₄S-dCK), in which four of six cysteine residues are mutated to serines (C9S/C45S/C59S/C146S), is more amenable for crystallization but otherwise behaves like wild type (WT) dCK (9,10). The three S74E containing structures reported here were solved in the background of this C₄S-dCK variant. When we refer to WT dCK in this report, we mean the C₄S-dCK variant. To allow the binding of thymidine, additional two mutations were carried out – R104M and D133A, as previously reported. All constructs were expressed in C41 (DE3) *Escherichia coli* using a pET14b vector, grown in 2YT media, and induced with 0.1 mM IPTG over 4 h at 37° C. Cells were harvested and the pellet lysed by sonication. Lysates were cleared by centrifugation at 30,000 × g for 1 hour 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 until use at –80 °C.

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 performed in triplicate at 37 °C in a buffer containing 100 mM Tris, pH 7.5, 100 mM KCl, 10 mM MgCl₂ and 1 mM ATP. The enzyme concentration was 0.33 μM. To determine the K_m and k_{cat} values the nucleoside concentration was varied with all other components being fixed. Data were fit to the Michaelis-Menten equation using SigmaPlot. Due to the moderate absorption coefficient for NADH (the signal of our assay), this assay is not suitable when measuring substrate concentration lower than ~5 μmolar.

Crystallization, X-ray data collection and refinement

Crystals of dCK complexed with nucleoside and/or nucleotide were grown using the vapor diffusion method from hanging drops. The specific conditions for the complexes were: for the S74E-DM-dCK variant in complex with L-dT + 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 + 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 0.9–1.5M trisodium citrate dihydrate and 100 mM Tris, pH 7.5. Crystals were cryoprotected with mineral oil. In case of S74E-dCK variant complexed with UDP alone, diffraction data were collected from a single frozen crystal using a RAXIS-IV⁺⁺ detector mount on a Rigaku RH-200 rotating anode x-ray generator. For other two complexes (S74E-dCK complexed with 5Br dC + UDP and S74E-DM-dCK complexed with L-dT + UDP) diffraction data were collected at the Advanced Photon Source using SERCAT beamline BM-22. Data were processed with XDS (12). The structure was solved by molecular replacement using the program MOLREP (13) and the dCK structure 1P5Z as search model. Refinement was carried out with REFMAC (14). All three data sets are perfectly twinned, and refinement was carried out with the Twin

option active in REFMAC. Data collection and refinement statistics are presented 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 solved the structures of S74E-containing dCK in three conditions: (i) only with UDP, (ii) with 5-bromo-deoxycytidine (5Br-dC) and UDP, and (iii) with L-deoxythymidine (L-dT) and UDP. Data collection and refinement statistics are presented in Table 1. The overlay of a dCK monomer solved in six different conditions (three previously reported and three reported here) showcases the conformational plasticity of the enzyme (Fig. 1C). The nature of the nucleotide (UDP versus 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 (Fig. 1C, bottom right zoom and Fig. S1). This overlay of the structures also shows that despite the different base-sensing loop conformation, the nucleotides UDP or ADP bind at the same position (Fig. 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 show a lower k_{cat} value with a concomitant lower K_m value in comparison to those measured with ATP (2,4). The possible communication mechanism 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 example, cladribine (2-chlorodeoxyadenosine, 2CDA), which is a dA analog used in cancer chemotherapy, is different to dA only by the presence of a chlorine atom at the adenine base 2-position. Yet, wild type (WT) dCK's conformation is open when solved in the presence of dA (2) (Fig. 1C, in brown), and closed in the presence of cladribine (1) (Fig. 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 complex with dA and UDP was up to this new work the only dCK structure that displayed the open conformation. We speculated that the open conformation is not unique to the 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 between the enzyme, a nucleoside, and a nucleotide. We were also successful in solving the structure of dCK only in the presence of a nucleoside (i.e. in the absence of a nucleotide) (4). In contrast, up to this point we could not obtain the structure of WT dCK in the absence of a nucleoside (i.e. solely with a nucleotide). The closest we got were non-diffraction 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 S74E-dCK in complex with UDP shows the open enzyme conformation (Fig. 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 plus UDP did not form good crystals since it was interconverting between the open and closed states

In addition to this nucleoside-free structure, we solved two structures of S74E-containing dCK in the presence of nucleosides: The S74E complex with 5Br-dC and UDP (Fig. 1C, pink), and the complex with L-dT and UDP (Fig. 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-containing complexes and the WT dCK in complex with dA and UDP), the nucleoside is positioned differently relative to the position adopted in structures with the closed conformation (Fig. 1C, left zoom inset, and Fig. S1). 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 by the shorter aspartic acid reduces k_{cat} by over 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 indicate that we have generated conditions that favor the open over the closed conformation. Since 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.

The structural differences between the open and closed states

To analyze in more detail the differences between the open and closed conformation we compare two structures: a double mutant (DM) variant of dCK (R104M+D133A) that permits binding of thymidine (16,17), in 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 (Fig. 2). Ideally we would compare two structures where the sole difference is the presence or absence of the S74E mutation. However, we could not obtain crystals of DM-dCK in complex with L-dT and UDP, nor 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 whereas the other UDP. Both contain the DM mutations, which permit the binding of nucleosides with a thymine base to dCK.

The right zoom inset of Fig. 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 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 zoom inset of Fig. 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 further distance in the case of the S74E-DM + L-dT + UDP complex (open state).

The closed state has the side chain of Trp58 in close proximity to the base of the nucleoside. In contrast, in the open state, Trp58 swings away from the nucleoside (Fig. 2, bottom left zoom). Thus, the open state is characterized by a more open nucleoside binding pocket (Fig. S1). We observe the side chain of Glu74 to be within 3.1 – 3.5 Å to 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 by 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 + UDP, to the three S74E dCK mutant structures presented here shows an identical open conformation. This point is shown in Fig. 3, that for simplicity presents only the overlay of the S74E variant in complex with 5-bromo-deoxycytidine (5Br-dC) + UDP (pink) on the WT dCK in complex with dA + 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 the phosphorylated form of dCK can attain the same open state, and that the interaction between Glu74 and the tryptophan's indole ring is not essential to achieve the open state.

In all cases of the open state, whether elucidated using WT dCK or the S74E dCK variant, the nucleoside base adopts the same position, which is different to 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 of 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.

How different are the open and closed states? As a representative of the open state we selected the S74E dCK complex with 5Br-dC and UDP (Fig. 4, pink), and that of the closed state we selected WT dCK with cladribine and UDP (Fig. 4, yellow). We chose the latter complex for this analysis since we wanted a closed state that has UDP and not ADP at the phosphoryl donor site, in order to minimize the differences between the complexes. Fig. 4 reveals a nearly perfect overlay of the open and closed enzyme states, with differences limited to the nucleoside binding site region. Not surprising, since 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 (Fig. 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 cladribine + UDP structure, residues 61 to 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 (Fig. S1). Specifically for the 5Br-dC + UDP structure, we could model the insert region except for residues between amino acid 63 to 70. Importantly, residue 74 has now acquired unambiguous electron density, and is at an interaction distance to the indole nitrogen of Trp58 (Fig. 4, zoom).

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 (Fig. 5). This interaction is broken as the tryptophan side chain rotates in the open enzyme conformation. In its place is the direct 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 W58F-S74E dCK variant, and characterized it kinetically. This mutant, which cannot form this interaction, retains phosphorylation activity, and is kinetically more similar to WT dCK than S74E-dCK (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 above shows that abolishing an interaction that stabilizes the open state, by substituting Trp58 with a phenylalanine, induces a more WT-like kinetic character to S74E-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 takes place only in the closed state conformation. We exchanged Ser103 by an alanine in the background of the S74E variant. This protein has lost most of its ability to phosphorylate the purines dA and dG, but is very active with the 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 in the case with purines versus that with pyrimidines. This is consistent with the fact that the WT dCK complex with dA + UDP adopts the open state, yet the complex dC + UDP adopts the closed state, i.e. the purine-bound enzyme favors the open state, 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 to modulate the activity of this enzyme (7,19). The structural and kinetic study presented here of the Ser74-phosphomimic, the S74E-dCK 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) examining 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 due to the increased distance of the nucleoside to Glu53? We propose that the open state is required for the binding of the substrate nucleoside and the later release of the monophosphorylated product. Fig. 6 presents surface representations of the closed (DM-dCK + L-dT + ADP, blue) and the open (S74E-DM-dCK + L-dT + UDP, green) structures. The nucleotide ADP or UDP is visible on the surface of dCK in both the closed (Fig. 6a) and open (Fig. 6b) states. This means that binding and release of the nucleotide is compatible with either state. In contrast, the nucleoside L-dT is totally hidden within dCK in the closed state (Fig. 6c), yet is solvent-exposed in the open state (Fig. 6d). This suggests that the nucleoside cannot dissociate from, or bind to, the closed state, and that nucleoside binding/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 of 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 was detectable 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 Fig. 7.

We propose that in the absence of substrates, dCK is in equilibrium between an open state (designated by E^O) and a closed state (E^C). In the case of WT dCK, E^C is favored over E^O . This would rationalize the pre-steady state kinetic data that shows that nucleoside binds much less favorably than nucleotide to the apo enzyme (22). Since the nucleoside binding site in the closed state is not solvent accessible (Fig. 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 are added, but not when ATP or ADP are added to the apo enzyme (2). The transition E^C to E^O results in the change of Trp58 position, 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 states, 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 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. Since 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 S74E dCK 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 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 since only the closed state can catalyze phosphoryl transfer. Therefore, the S74E dCK variant is characterized by increased K_m values for the nucleosides. Interestingly, while both UTP and phosphorylation of Ser74 acts 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 double mutant R104M/D133A). This analysis (Table S1) shows that the presence of S74E invariably increases the K_m value of the nucleoside, but also the k_{cat} values which 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 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 S74E-dCK variant compared to 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 S74E dCK variant, dCK transitions from an open nucleoside binding site to a closed nucleoside binding site. The former is compatible with the binding/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 by 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 understanding of phosphoryl transfer catalyzed by dCK by adding an additional snapshot (the open state) to the structurally characterized dCK conformations. Together with previous snapshots of this enzyme (closed state), we increase our appreciation of the dynamic behavior required by dCK to catalyze phosphoryl transfer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

dCK	deoxycytidine kinase
S74E dCK	serine-to-glutamic acid mutant of dCK
DM	R104M+D133A variant of dCK
WT	wild type

5Br-dC	5-bromo-deoxycytidine
L-dT	L-deoxythymidine

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Fig. 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-bromo-deoxycytidine (5Br-dC) at the nucleoside acceptor site. One monomer is shown in pink, the other in magenta. The nucleosides/nucleotides are depicted in cyan. The N- and C-termini are labeled “N” and “C”, respectively. B. A view 90° rotated relative to that shown in panel A. The location of Insert, a 15 residue long region that connects two helices, is marked. A jagged black line shows the 6–8 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: WT dCK in complex with dA and UDP (brown; PDB ID 2ZI6); WT dCK in complex with cladribine and UDP (yellow; PDB ID 2ZIA); dCK with the double mutation (DM) of R104M-D133A in complex with L-dT and ADP (blue; PDB ID 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 + DM mutations in complex with L-dT and UDP (green). Right bottom zoom: 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. Right upper inset: the nucleotides adopt a nearly identical position despite the conformational differences of the base-sensing loop. Left zoom: in contrast, the nucleoside in the closed state bind at a different position relative to nucleosides binding in the open state.

Fig. 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). Center: the structures overlay perfectly except for the base-sensing loop and the nucleoside-binding region near the insert. Right zoom showcases the differences in the base-sensing loop (red arrows). Left top zoom showcases the different positions of L-dT between the open and closed conformation. 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 far to allow activation by Glu53. The position of the double mutation R104M-D133A present in both structures is indicated. Left bottom zoom: a view with a 90° rotation relative to the top zoom. Trp58 undergoes a dramatic conformational change upon transitioning from the closed to open state. The open state (green) is stabilized via an interaction between the side chain of E74 to 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.

Fig. 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 S74E dCK variant in complex with 5Br-dC and UDP (pink). The structures overlay well throughout, including the base-sensing loop, since both had bound UDP, and the nucleoside binding site since both adopt the open state. Right panels: two 90°-rotated views of the nucleosides that demonstrate the similar binding position of dA and 5Br-dC.

Fig. 4.

The open state is 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 S74E-dCK variant in complex with 5Br-dC and UDP (pink; open state). Since both complexes were solved 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 to 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 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 versus 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 is 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.

Fig. 5.

Different interactions stabilize the open versus 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 towards the base of the nucleoside L-dT, and the nitrogen of the indole group is at a 2.7 Å distance to 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 at a 3.1 Å distance to 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).

Fig. 6.

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

WT dCK S74E dCK **Fig. 7.**

Proposed reaction scheme for WT and S74E dCK. 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.

Table 1

Data collection and refinement statistics

	S74E + UDP	S74E + 5Br-dC + UDP	DM-S74E + L-dT + UDP
<i>Data Collection statistics</i>			
PDB code	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 – 2.00	30.0 – 1.90
Reflections			
Observed	46265	273588	330797
Unique	18394	37632	44080
Completeness (%) ^a	95.2 (99.1)	99.5 (97.8)	99.7 (98.3)
<i>R</i> _{sym} (%)	5.7 (55.4)	6.1 (41.9)	6.7 (74.0)
<i>I</i> / σI	11.6 (1.6)	17.6 (3.9)	19.1 (2.9)
Space group	P4 ₁	P4 ₁	P4 ₁
Unit cell			
a = b (Å)	68.43	69.14	68.76
c (Å)	120.37	118.90	120.60
Molecules per a.u.	2	2	2
<i>Refinement statistics</i>			
<i>R</i> _{work} (%)	20.1	19.7	19.5
<i>R</i> _{free} (%)	23.6	24.7	24.4
Number 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
R.m.s. deviation			
Bond length (Å)	0.011	0.013	0.013
Bond angles (°)	1.387	1.576	1.538
Average B-factors (Å ²) / 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 is in parenthesis

Table 2

Steady state kinetic data for WT and mutant dCK

Nucleoside	<u>WT dCK^a</u>			<u>S74E dCK^a</u>			<u>W58F + S74E dCK</u>			<u>S103A + S74E dCK</u>		
	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	-	-	-
dG	0.73	245.8	2.9	0.36	601.8	0.6	0.66	514.1	1.28	-	-	-

^a data from McSorley et al, 2008^b k_{cat} units are sec^{-1} , K_m units are in μM .^c $\times 10^3 \text{ sec}^{-1}/\text{M}$

-activity undetectable or extremely weak