The "Catalytic" Triad of Isocitrate Dehydrogenase Kinase/Phosphatase from *E. coli* and Its Relationship with That Found in Eukaryotic Protein Kinases

Christelle Oudot,[‡] Jean-Claude Cortay,[§] Christophe Blanchet,[‡] David C. Laporte,[||] Attilio Di Pietro,[‡] Alain J. Cozzone,[‡] and Jean-Michel Jault*,[‡]

Institut de Biologie et Chimie des Protéines, Université Claude Bernard Lyon I, UMR 5086 du CNRS, Lyon, France, Immuno-Virologie Moléculaire et Cellulaire, Université Lyon I, UMR 5537 du CNRS, Lyon, France, and Department of Biochemistry, University of Minnesota, Minneapolis, Minnesota

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ABSTRACT: The isocitrate dehydrogenase kinase/phosphatase (IDHK/P) of *E. coli* is a bifunctional enzyme responsible for the reversible phosphorylation of isocitrate dehydrogenase (IDH) on a seryl residue. As such, it belongs to the serine/threonine protein kinase family. However, only a very limited homology with the well-characterized eukaryotic members of that family was identified so far in its primary structure. In this report, a new region of amino acids including three putative residues involved in the kinase activity of IDHK/P was identified by sequence comparison with eukaryotic protein kinases. In IDHK/P, these residues are Asp-371, Asn-377, and Asp-403. Their counterpart eukaryotic residues have been shown to be involved in either catalysis (former residue) or magnesium binding (the two latter residues). Site-directed mutagenesis was performed on these three IDHK/P residues, and also on the Glu-439 residue equivalent to that of the Ala-Pro-Glu motif found in the eukaryotic protein kinases. Mutations of Asp-371 into either Ala, Glu, or Gln residues drastically lowered the yield and the quality of the purification. Nevertheless, the recovered mutant enzymes were barely able to phosphorylate IDH either in vitro or after expression in an *aceK* $^-$ mutant strain. In contrast, mutation of either Asn-377, Asp-403, or Glu-439 into an Ala residue altered neither the yield of purification nor the maximal phosphorylating capacity of the enzyme. However, when IDH was phosphorylated in the presence of increasing concentrations of magnesium ions, the two former mutants displayed a much lower affinity for this cation, with a K_m value of 0.6 or 0.8 mM, respectively, as compared to 0.1 mM for the wild-type enzyme. On the other hand, the Glu439Ala mutant has an affinity for magnesium essentially unaffected. Therefore, and in contrast to the current opinion, our results suggest that the catalytic mechanism of IDHK/P exhibits some similarities with that found in the eukaryotic members of the protein kinase family.

Protein phosphorylation is known to play a crucial role in the metabolism and signal transduction of living organisms. In bacteria, three types of protein phosphorylation systems have been identified which are mechanistically unrelated (*2*). The first mechanism is the well-characterized phospho-enolpyruvate-dependent phosphotransferase system, which permits the selective entry of sugar by phosphoryl transfer to protein histidyl and cysteinyl residues (*3, 4*). The second system concerns the ATP-dependent sensor kinase that responds to environmental stimuli by autophosphorylation onto a histidyl residue, followed by transfer of the phosphate group to an aspartyl residue belonging to a response regulator (*5, 6*). The third system involves an ATP-dependent kinase that modulates the activity of its target protein(s) by phosphorylation on either seryl, threonyl, or tyrosyl residues (*7*).

Despite the sudden awareness in the late 70's that the latter mechanism of phosphorylation occurred in many bacterial species, purification of the involved protein kinases was tedious (*8*), and only a few examples have been reported so far (*9, 10*). One of the first members characterized in this group phosphorylates the Krebs-cycle enzyme isocitrate dehydrogenase (IDH)[1] at a single seryl residue (Ser-113), thereby leading to inactivation of the enzyme (*11, 12*). This regulatory mechanism enables the bacteria to bypass the Krebs cycle via the glyoxylate shunt, when bacteria are grown on acetate or ethanol as the sole carbon source (*13, 14*). In fact, the reversible phosphorylation of IDH is catalyzed by an unusual single bifunctional enzyme responsible for both the phosphorylation and dephosphorylation activities, which is therefore called isocitrate dehydrogenase kinase/phosphatase (IDHK/P) (*15*). This enzyme also possesses a high ATPase activity, even in the absence of its natural substrate IDH (*16*), and it has been proposed that

* To whom correspondence should be addressed at the Institut de Biologie et Chimie des Protéines, UMR 5086 du CNRS, 7 Passage du Vercors, 69367 Lyon Cedex 07, France. Phone: 33-4-72-72-26-29; Fax: 33-4-72-72-26-05; E-mail: jm.jault@ibcp.fr.

[‡] Institut de Biologie et Chimie des Protéines, Université Claude Bernard Lyon I.

[§] Immuno-Virologie Moléculaire et Cellulaire, Université Lyon I.

[||] Department of Biochemistry, University of Minnesota.

[1] Abbreviations: DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; IDH, isocitrate dehydrogenase; IDHK/P, isocitrate dehydrogenase kinase/phosphatase; ePKs, eukaryotic protein kinases; PKA, cAMP-dependent protein kinase; SDS−PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

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both kinase, phosphatase and ATPase activities occur at a single catalytic site (*17*).

In contrast, protein phosphorylation in eukaryotes mainly involves ATP-dependent serine/threonine/tyrosine protein kinases (ePKs), and all members of this well-defined superfamily exhibit extensive sequence similarity (*18*). Consistently, crystal structures of several eukaryotic proteine kinases have revealed that their catalytic cores share a similar fold (*19*). The overall structure consists of two domains, with an *N*-terminal lobe providing some residues for ATP binding, and a *C*-terminal lobe containing a catalytic loop and the protein substrate binding site; the catalysis occurs at the cleft between the two lobes (*20, 21*). Several residues highly conserved among these ePKs are scattered throughout the catalytic core (*22*): in the *N*-terminal lobe, these are essentially the glycine-rich motif (50-GlyXGlyXXGly-55[2]), involved both in nucleotide binding and in catalysis (*23, 24*), and a lysine residue (Lys-72) found invariably and known to interact with the α- and β-phosphates of bound MgATP (*1*). In the *C*-terminal lobe, the most conserved residues are an aspartate residue (Asp-166), presumed to act as a catalytic base (*25, 26*), and two other residues, one asparagine (Asn-171) and one aspartate (Asp-184), each involved in chelating one of the two ATP-liganded Mg^{2+} ions in PKA (*27, 28*). This cluster of three residues forms a "catalytic" triad which is invariably found in all members of the ePK family. Recently, genome sequencing programs have allowed identification of several putative bacterial members belonging to this family, since they display most, if not all, of the conserved residues mentioned above (*29, 30*). In contrast, *E. coli* IDHK/P has long been considered as an orphan in the kinase world (*5, 31*). Indeed, its structural relationship with ePKs was restricted only to a stretch of about 25 amino acids, encompassing a glycine rich-like motif (315-APGIRG-320) and a critical lysine residue (Lys-336) proposed to be equivalent to Lys-72 of the cAPK (*17, 32*).

The recently determined structure of a bacterial aminoglycoside kinase revealed a three-dimensional fold strikingly similar to that of ePKs, despite a virtually complete lack of sequence homology but allowing, nonetheless, the strategic location of conserved key residues (*33*). This prompted us to reexamine the sequence of IDHK/P: a faint, but significant, homology with ePKs was detected, possibly including a "catalytic triad" of IDHK/P. These newly identified putative critical residues of IDHK/P were put to the test by site-directed mutagenesis, and the kinetic parameters of the corresponding mutants are reported here. The results suggest that the kinase activity of IDHK/P is relevant to the catalytic mechanism occurring in ePKs. From a general viewpoint, this would imply an evolutionary link between ePKs and *E. coli* IDHK/P.

EXPERIMENTAL PROCEDURES

Materials. Unless specified, all the reagents were from Sigma, and the enzymes used for molecular biology techniques were from Promega.

Bacterial Strains. The strains used in this study were the following: *E. coli* strains JM109 (*end*A1, *gyr*A96, *hsd*R17, *mcr*B$^+$, *rec*A1, *rel*A1, *sup*E44, *thi*-1, Δ(*lac-pro*AB), F′-[*tra*D36, *pro*AB, *lac*I^{q}ZΔM15]) (*17*) and BMH 71-18 (*sup*E, *thi*-1, Δ(*lac-pro*AB), F′[*proAB*$^+$, *lac*I^{q}ZΔ*M*15], *mut*S:: Tn10). IL2 was an *E. coli* mutant strain (ΔaceK and lacIq) obtained as previously described (*32*).

Purification of IDHK/P. Recombinant wild-type IDHK/P, fused with 6×His at its *N*-terminal end, was prepared from *E. coli* JM109 cells harboring the overproducing plasmid pJCD4, as previously described (*34*). Mutant IDHK/Ps were purified under the same conditions as the wild-type enzyme.

DNA Manipulation. Routine DNA manipulations and site-directed mutagenesis were performed as previously described (*35*). The oligonucleotide primers used for the respective changes (underlined) were the following:

Asp371Ala, 5′-GGCCGAATGGCG<u>GCT</u>ACCCAGGA-GTTTGAAA-3′

Asp371Gln, 5′-GGCCGAATGGCG<u>CAG</u>ACCCAGGA-GTTTGAAA-3′

Asp371Glu, 5′-GGCCGAATGGCG<u>GAA</u>ACCCAGGA-GTTTGAAA-3′

Asn377Ala, 5′-CAGGAGTTTGAA<u>GCT</u>TTTGTGCTG-GAG-3′

Asp403Ala, 5′-GAAAAAATCACC<u>GCT</u>CTCGGCGA-ACAA-3′

Glu439Ala, 5′-CGCGACGCCATT<u>GCT</u>GAATACGGT-AAC-3′

The mutations introduced were confirmed by DNA sequencing using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical) according to (*36*).

Purification of IDH and in Vitro Phosphorylation. The recombinant IDH, fused with 6×His at its *N*-terminal end, was prepared as previously reported (*37*). Phosphorylation of purified IDH was routinely performed at 37 °C using a 20-μL assay mixture containing 7 μCi of [γ-^{32}P]ATP (3000 Ci/mmol), 15 μM ATP, 25 mM Mops-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$, 100 mM NaCl, 10 μg of IDH, and 1 μg of IDHK/P. The reaction mixture was incubated for the time specified in the figure legends. When the apparent K_m for ligands was determined, the incubation period was only 30 s. This time interval was chosen to maintain the initial rate of phosphorylation throughout the experiments, as checked on separate kinetic experiments. When the phosphorylation activities were measured in the presence of increasing concentrations of magnesium, EDTA was omitted from the reaction mixture, and the ATP concentration was set at 50 μM. The phosphorylation assays were stopped by adding the gel-electrophoresis loading buffer (31.2 mM Tris-HCl, pH 6.8, 75 mM β-mercaptoethanol, 0.5% sodium dodecyl sulfate, 2.5% glycerol, and 0.005% bromophenol blue) and heating immediately for 5 min at 100 °C. Samples were then submitted to 10% polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS−PAGE), and the dried gels were autoradiographed at −80 °C on a Kodak MS film. Quantification of the phosphorylation spots obtained by autoradiography was performed with a Personal Densitometer SI (Molecular

[2] The numbering of mouse cAMP-dependent protein kinase (PKA) was taken as a reference for the eukaryotic protein kinases throughout the manuscript [cf. (*1*)].

Dynamics), and curve fitting of the data was performed by using the MacCurvefit 1.0.8 software.

Phosphorylation of Purified IDH by a Crude Cellular Extract. The host strain IL2 harboring either plasmid pJCD4 [a pQE30 derivative encoding the wild-type IDHK/P (*34*)] or plasmid pCOD (the same as pJCD4 but encoding an Asp371Ala mutant of IDHK/P) was grown in 2×TY medium at 37 °C. When the $OD_{600\ nm}$ reached a value of 0.7−0.8, isopropyl β-thiogalactopyranoside (2 mM) was added and the incubation was continued for 3 h. Cells were harvested by low-speed centrifugation (14 000 rpm) and disrupted by sonication. A 5-mL fraction of the crude cellular extract was then submitted again to low-speed centrifugation, and a 8-μL aliquot of the recovered supernatant, containing about 10 μg of protein, was used to phosphorylate 5 μg of purified recombinant IDH.

RESULTS

Partial Sequence Alignment of E. coli IDHK/P and Members of ePKs. When the full-length sequence of IDHK/P was compared to various ePKs sequences, the only similarity detected by the clustal W program was that described previously (*17, 32*), dealing with a glycine-rich-like motif of IDHK/P (315-APGIRG-320) and a lysine residue (Lys-336) proposed to be equivalent to Lys-72 of the cAPK (*17, 32*). In most ePKs members, the invariant Lys residue (Lys-72) is distant from the putative catalytic base (Asp-166) by about 100 residues (*22*). However, these two critical residues lie on different subdomains of ePKs [subdomains II and VI based on Hanks's nomenclature for Lys-72 and Asp-166, respectively (*18, 22*)] and, as such, might be separated by a variable length in the primary sequence, depending on whether insertion or deletion occurred during evolution. For instance, an insertion of 58 residues is found between the 2 equivalent residues of an antibiotic kinase, which otherwise adopts a tertiary fold very similar to that of ePKs (*33*). In such a case, global alignment programs may overlook some local similarity between sequences (*38*), especially if the overall sequence identity is low. Therefore, we used truncated sequences of IDHK/P, free from the previously detected homologous region, and we compared them with the complete sequences of different ePKs. A partial sequence alignment was thus obtained by the clustal W program, between residues Ala-343 and Arg-411 of IDHK/P, which is shown in Figure 1 (residues 1−81 in the alignment). The right side of Figure 1 (residues 81−140) was aligned manually by using the MPSA program, which allows a better fitting of the alignment by computer-generated gaps (*39*). The most noticeable difference between these sequences concerns one additional residue between Asp-371 and Asn-377 in IDHK/P. However, the recent crystal structure of an atypical ePK, the actin-fragmin kinase, has shown that, despite an insertion of nine residues between the Asp and Asn residues, these two residues occupy a similar spatial position as the equivalent residues of PKA (*40*). The AspPheGly (Asp-184) and AlaProGlu (Glu-208) motifs which are highly conserved in all ePKs [in 87% and 70% of the 390 sequences registered in the Protein Kinase Resource (*41*), respectively] are, however, sometimes replaced by AspLeuGly (in 5.6% of the sequences) and AlaIleGlu (in 2%) as reported here for IDHK/P. In the only other IDHK/P sequence found in the Swiss-Prot database (*42*), from *Salmonella typhimurium*, the residues highlighted in this partial alignment are totally conserved (*43*).

The availability of the crystallographic coordinates from several ePKs allowed us to estimate, by using the dssp program, their secondary structure for the sequence stretches used in Figure 1. This was done for 14 different kinases found in the PDB (1KOB chain A, 1TKI chain A, 1PHK, 1ERK, 1P38, 1HCK, 1BI8 chain A, 1A6O, 3LCK, 1AGW chain A, 1IR3 chain A, 2CPK chain E, 1CKI chain A, and 1CSN). The results obtained, shown for only five of them in Figure 1, indicated that the juxtaposition of secondary structures is conserved among all the ePKs. The secondary structures of the 14 kinases were then compared with those predicted by different methods including PHD (*44*), SOPMA (*45*), DSC (*46*), GOR IV (*47*), Predator (*48*), and SIMPA 96 (*49*). The best prediction was obtained by the SOPMA algorithm (67.7%), followed by SIMPA 96 (66.9%), PHD (66.1%), Predator (65%), DSC (59.3%), and GOR IV (46.2%). When only the serine/threonine subfamily of ePKs was considered, the level of prediction was slightly increased for each method except Predator (63.9%), SOPMA still giving the best prediction (71.6%), as compared to SIMPA 96 (68.5%) and PHD (66.9%). Therefore, SOPMA was chosen for the secondary structure prediction of all sequence stretches shown in Figure 1.

The comparison of the consensual secondary elements found by dssp and SOPMA predictions gave the following structural information: a long α-helix (residues 2−26) is predicted for all sequences including IDHK/P, but KYK1. Asp-166 is located in a coil for 13 sequences, including IDHK/P, out of a total of 18. Asn-171 is at the end of a short 3-10 helix and precedes a β-strand. This residue is predicted 5 times in an α-helix (including IDHK/P) but also 10 times in a turn, and the following β-strand (at least two residues) is rather well predicted for all sequences but INSR and 7LESS. Asp-184 is in a coil preceded by a β-strand. This is not predicted for either IDHK/P, or FGFR, FRK, SME1, and KYK1. Glu-208 is located in an α-helix which is only predicted 8 times (including IDHK/P) out of 18 sequences. The last long α-helix (residues 125−140) is more or less well-predicted for most sequences but is not detected in IDHK/P.

Mutation of the Predicted "Catalytic" Triad of IDHK/P. The partial alignment of Figure 1 suggested that Asp-371, Asn-377, Asp-403, and Glu-439 residues of IDHK/P might play a role similar to that of their counterpart residues in ePKs (Asp-166, Asn-171, Asp-184, and Glu-208, respectively). In ePKs, the former residue has been proposed to be the catalytic base responsible for hydrolysis of the γ-phosphate of ATP (*20, 26, 50*) whereas Asn-171 and Asp-184 interact with ATP-chelated magnesium ion(s), present as either a single (*51*−*53*) or two different ions (*26, 27, 50*) in the different crystallized enzymes. Because crystallographic data show that Glu-208 lies away from the nucleotide binding site (*25*), this residue is unlikely to play a direct role in catalysis. Nevertheless, its substitution by an alanine residue in yeast PKA seems to greatly reduce the affinity for a pseudo-substrate peptide (*54*).

To investigate the functional role of Asp-371, Asn-377, Asp-403, and Glu-439 residues in the phosphorylating activity of IDHK/P, site-directed mutagenesis experiments were carried out on these residues. Asp-371 was replaced

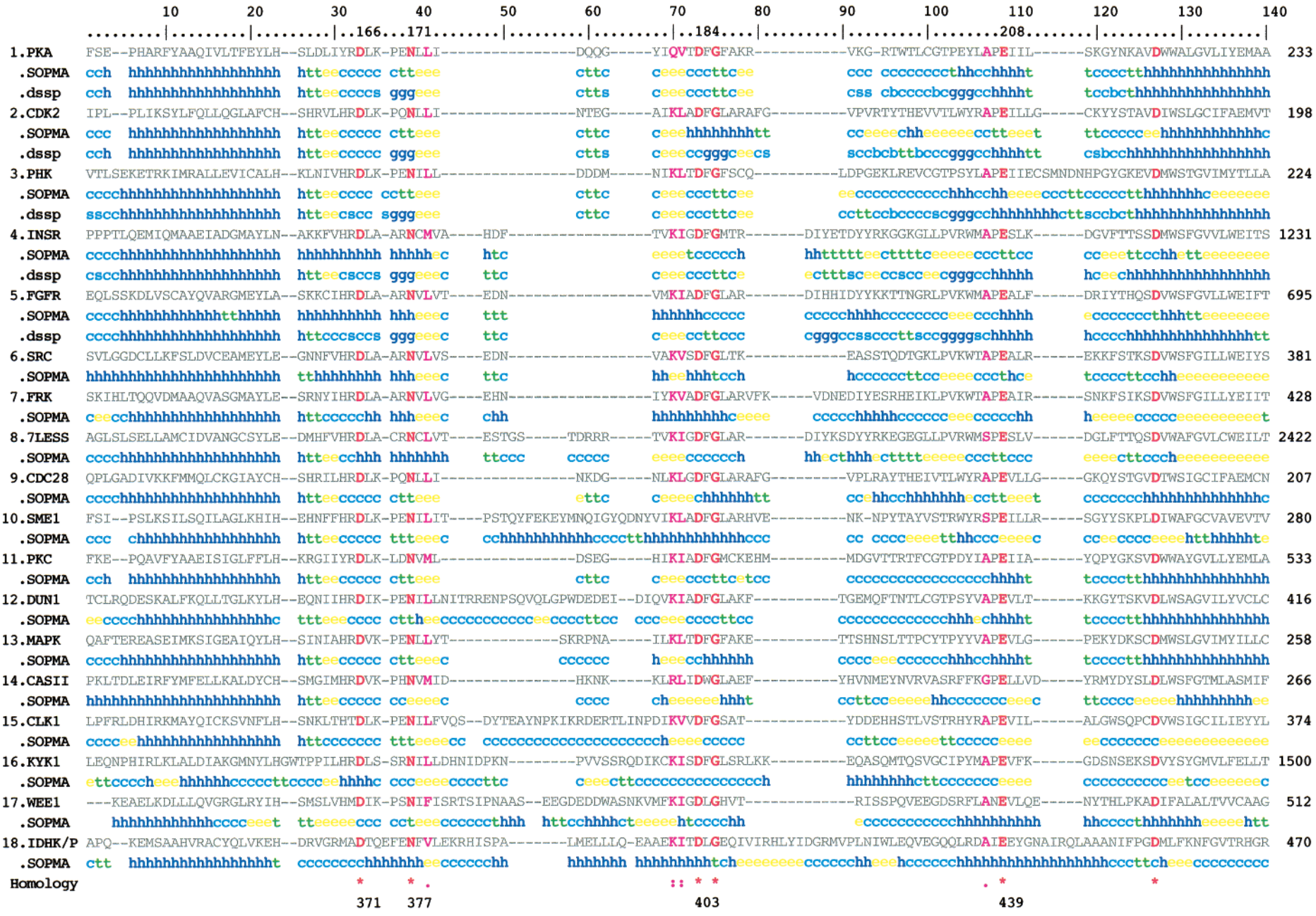
10 20 30 40 50 60 70 80 90 100 110 120 130 140
1.PKA FSE--PHARFYAAQIVLTFEYLH--SLDLIYRDLK-PENLLI----------DQQG-----YIQVTDFGFAKR----------VKG-RTWTLCGTPEYLAPEIIL------SKGYNKAVDWWALGVLIYEMAA 233
.SOMPA cch hhhhhhhhhhhhhhhhh htteecccccttceee cttc ceeecccttcee ccc ccccccccthhcchhhht tcccctthhhhhhhhhhhhhh
.dssp cch hhhhhhhhhhhhhhhhh htteecccccs gggeee ctts ceeecccttcee css cbccccbcgggcchhhht tccbcthhhhhhhhhhhhhhh
2.CDK2 IPL--PLIKSYLFQLLQGLAFCH--SHRVLHRDLK-PQNLLI----------NTEG-----AIKLADFGLARAFG---------VPVRTYTHEVVTLWYRAPEILLG-----CKYYSTAVDIWSLGCIFAEMVT 198
.SOMPA ccc hhhhhhhhhhhhhhhhh htteecccccc cttceee cttc ceeehhhhhhhtt cceeeechheeeeeecctteeet ttccccceehhhhhhhhhhhc
.dssp cch hhhhhhhhhhhhhhhhh htteecccccc gggeee ctts ceeeccgggceecs sccbcbttbcccgggcchhhhtt csbcchhhhhhhhhhhhhhhh
3.PHK VTLSEKETRKIMRALLEVICALH--KLNIVHRDLK-PENILL----------DDDM-----NIKLTDFGFSCQ----------LDPGEKLREVCGTPSYLAPEIIECSMNDNHPGYGKEVDMWSTGVIMYTLLA 224
.SOMPA ccchhhhhhhhhhhhhhhhhh htteecccc ccttceee cttc ceeecccttcee eecccccccccchhhcchheeeecccttccccctthhhhhhhceeeeeee
.dssp sscchhhhhhhhhhhhhhhhh htteecscc sgggeee cttc ceeecccttcee ccttccbccccscgggcchhhhhhhhcttsccbcthhhhhhhhhhhhhhh
4.INSR PPPTLQEMIQMAAEIADGMAYLN--AKKFVHRDLA-ARNCMVA----HDF----------TVKIGDFGMTR-------DIYETDYYRKGGKGLLPVRWMAPESLK------DGVFTTSSDMWSFGVVLWEITS 1231
.SOMPA ccchhhhhhhhhhhhhhhhhh hhhhhhhhh hhhhhec htc eeeettccccch hhtttttecttttceeeeeccttcc cceeettcchhetteeeeeeee
.dssp csschhhhhhhhhhhhhhhhhh htteecsccs gggeeec ttc ceeecccttce ecttttscceecscccceecgggcchhhhh hcceechhhhhhhhhhhhhhh
5.FGFR EQLSSKDLVSCAYQVARGMEYLA--SKKCIHRDLA-ARNVLVT----EDN----------VMKIADFGLAR-------DIHHIDYYKKTTNGRLPVKWMAPEALF------DRIYTHQSDVWSFGVLLWEIFT 695
.SOMPA ccchhhhhhhhhhhhttthhhhh hhhhhhhhh hhheeec ttt hhhhhcccccc ccccchhhhccccccccceeecchhhh eccccccchhhttceeeeeeee
.dssp ccchhhhhhhhhhhhhhhhhh httcccsccs gggeeec ttc ceeecctttccc cgggccsscccttsccggggschhhhh hcccchhhhhhhhhhhhhhtt
6.SRC SVLGGDCLLKFSLDVCEAMEYLE--GNNFVHRDLA-ARNVLVS----EDN----------VAKVSDFGLTK----------EASSTQDTGKLPVKWTAPEALR------EKKFSTKSDVWSFGILLWEIYS 381
.SOMPA hhhhhhhhhhhhhhhhhhhhh tthhhhhhhh hhheeec ttc hheehhhtcch hccccccttccceeeecccthce tcccctttcchheeeeeeeeee
7.FRK SKIHLTQQVDMAAQVASGMAYLE--SRNYIHRDLA-ARNVLVG----EHN----------IYKVADFGLARVFK-----VDNEDIYESRHEIKLPVKWTAPEAIR------SNKFSIKSDVWSFGILLYEIIT 428
.SOMPA ceecchhhhhhhhhhhhhhhhh httccccchh hhheeec chh hhhhhhhhhceeee cccccchhhhcccccceeecccchh heeeeecccccceeeeeeeeet
8.7LESS AGLSLSELLAMCIDVANGCSYLE--DMHFVHRDLA-CRNCLVT----ESTGS-----TDRRR-----TVKIGDFGLAR-------DIYKSDYYRKEGEGLLPVRWMSPESLV------DGLFTTQSDVWAFGVLCWEILT 2422
.SOMPA ccchhhhhhhhhhhhhhhhhh htteecchhh hhhhhhh ttccc ccccc eeeecccccch hheecthhhecttttceeeeccttccc eeeecttcccheeeeeeeeeee
9.CDC28 QPLGADIVKKFMMQLCKGIAYCH--SHRILHRDLK-PQNLLI----------NKDG-----NLKLGDFGLARAFG---------VPLRAYTHEIVTLWYRAPEVLLG-----GKQYSTGVDTWSIGCIFAEMCN 207
.SOMPA ccchhhhhhhhhhhhhhhhhh htteecccccc cttceee ettc cceeeechhhhhhtt ccehhcchhhhhhhecctteeet ccccccchhhhhhhhhhhhhc
10.SME1 FSI--PSLKSILSQILAGLKHIH--EHNFFHRDLK-PENILIT----PSTQYFEKEYMNQIGYQDNYVIKLADFGLARHVE---------NK-NPYTAYVSTRWYRSPEILLR-----SGYYSKPLDIWAFGCVAVEVTV 280
.SOMPA ccc chhhhhhhhhhhhhhhh htteecccccc ttteeec cchhhhhhhhhhccccttthhhhhhhhhhhccc cc ccccceeeettchhcccceeeec ccecccceeeehtthhhhhte
11.PKC FKE--PQAVFYAAEISIGLFFLH--KRGIIYRDLK-LDNVML----------DSEG-----HIKIADFGMCKEHM---------MDGVTTRTFCGTPDYIAPEIIA------YQPYGKSVDWWAYGVLLYEMLA 533
.SOMPA cch hhhhhhhhhhhhhhhhh htteecccccc cttceee cttc ceeecccttcetcc ccccccccccccccccchhhht tcccctthhhhhhhhhhhhhh
12.DUN1 TCLRQDESKALFKQLLTGLKYLH--EQNIIHRDIK-PENILLNITRRENPSQVQLGPWDEDEI--DIQVKIADFGLAKF----------TGEMQFTNTLCGTPSYVAPEVLT------KKGYTSKVDLWSAGVILYVCLC 416
.SOMPA eecccchhhhhhhhhhhhhhhc ttteeecccc ctthecccccccccceecccttcc ccceeecccttcc ccccccccccccchhhechhhht tcccctthhhhhhhhhhhhhh
13.MAPK QAFTEREASEIMKSIGEAIQYLH--SINIAHRDVK-PENLLYT----------SKRPNA-----ILKLTDFGFAKE----------TTSHNSLTTPCYTPYYVAPEVLG------PEKYDKSCDMWSLGVIMYILLC 258
.SOMPA ccchhhhhhhhhhhhhhhhhh htteecccccc cttceeec ccccc heeecchhhhhh cccceeecccccchhhcchhhht tcccctthhhhhhhhhhhhhh
14.CASII PKLTDLEIRFYMFELLKALDYCH--SMGIMHRDVK-PHNVMID----------HKNK-----KLRLIDWGLAEF----------YHVNMEYNVRVASRFFKGPELLVD-----YRMYDYSLDLWSFGTMLASMIF 266
.SOMPA hhhhhhhhhhhhhhhhhhhhh htteecccccc cceeeec cccc cheeeeeehhht ccttcceeeeehhcccccccceeec ttcccceeeeehhhhhhhhee
15.CLK1 LPFRLDHIRKMAYQICKSVNFLH--SNKLTHTDLK-PENILFVQS--DYTEAYNPKIKRDERTLINPDIKVVDFGSAT----------YDDEHHSTLVSTRHYRAPEVIL------ALGWSQPCDVWSIGCILIEYYL 374
.SOMPA ccccceehhhhhhhhhhhhhhhh httcccccccc ttteeeecc cccccccccccccccccccccheeeecccccc ccttcceeeeettccccceeee eecccccccceeeeeeeeeeee
16.KYK1 LEQNPHIRLKLALDIAKGMNYLHGWTPPILHRDLS-SRNILLDHNIDPKN--------PVVSSRQDIKCKISDFGLSRLKK---------EQASQMTQSVGCIPYMAPEVFK------GDSNSEKSDVYSYGMVLFELLT 1500
.SOMPA ettccccheeehhhhhhccccctttccccceehhhhc ccceeeecccttc ceeecttcccccccccccccccch hhhhhhhhhcttcccccceeee cccccccccceetcceeeeeec
17.WEE1 ---KEAELKDLLLQVGRGLRYIH--SMSLVHMDIK-PSNIFISRTSIPNAAS--EEGDEDDWASNKVMFKIGDLGHVT----------RISSPQVEEGDSRFLANEVLQE-----NYTHLPKADIFALALTVVCAAG 512
.SOMPA hhhhhhhhhhhhccccceeet tteeeeeccc cctecccccccthhh httcchhhhctecceehtcccchh ecccccccccchhhhhhhhhh hhcccctthhhhhhheeeeehtt
18.IDHK/P APQ--KEMSAAHVRACYQLVKEH--DRVGRMADTQEFENFVLEKRHISPA--------LMELLLQ-EAAEKITDLGEQIVIRHLYIDGRMVPLNIWLEQVEGQQLRDAIEEYGNAIRQLAAANIFPGDMLFKNFGVTRHGR 470
.SOMPA ctt hhhhhhhhhhhhhhhhht ccccccccchhhhhhheecccccchh hhhhhhh hhhhhhhhhtchecceeeeeeccccccchheeehccccccchhhhhhhhhhhhhhhhhhhhccccttchecccccccccc
Homology * * . :: * * . * *
371 377 403 439

FIGURE 1: Partial sequence alignment of several protein kinases. PKA, cAMP-dependent protein kinase (Swiss-Prot accession number: P05132, PDB code: 2CPK chain E); CDK2, cell division protein kinase 2 (P24941, 1FIN chain C); PHK, phosphorylase kinase (P00518, 1PHK); INSR, insulin receptor (P06213, 1IR3 chain A); FGFR, basic fibroblast growth factor receptor 1 (P11362, 1AGW chain A fragment 4); SRC, tyrosine−protein kinase CSK (P32577); FRK, tyrosine−protein kinase FRK (P42685); 7LESS, sevenless protein (P13368); CDC28, cell division control protein 28 (P00546); SME, meiosis induction protein kinase IME2/SME1 (P32581); PKC, protein kinase C (P04409); DUN, DNA damage response protein kinase DUN1 (P39009); MAPK, MAP kinase-activated protein kinase 2 (P49137); CASII, casein kinase II (P15790); CLK1, protein kinase CLK1 (P49759); KYK, nonreceptor tyrosine kinase spore lysis A (P18160); WEE1, WEE1-like protein kinase (P30291); IDHK/P (P11071). The alignment was first generated by using Clustal W (*75*), and manual adjustment was then performed by using the MPSA program (*39*). For primary structure, red residues indicated by a star are fully conserved. Pink residues indicated by either one or two dots are weakly or strongly homologous, respectively, as defined in (*75*). Numbers below the sequence of IDHK/P correspond to the residues mutated here, and the corresponding numbers in PKA are also indicated above its sequence. On the right side is indicated the exact numbering of the last residue for each sequence. For secondary structure, predictions were made by using SOPMA (*45*) and compared, when available, with the crystallographic structures obtained from the PDB files (see codes above) which were analyzed by the dssp program (*76*). The symbols used and the color codings are as follows: h, α-helix (dark blue); g, 3-10 helix (dark blue); e, β-strand (yellow); t, turn (green); c, coil (light blue); s, bend (light blue); and b, β-bridge (light blue).

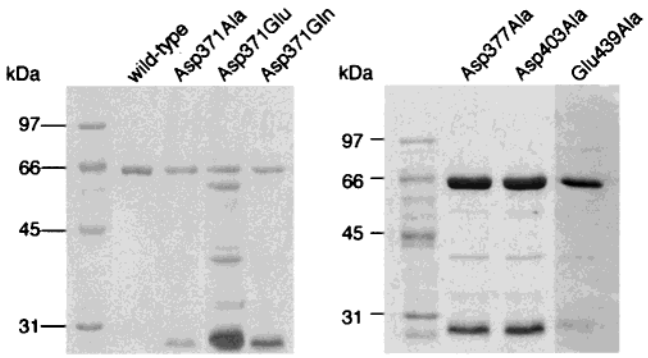

FIGURE 2: SDS−PAGE of IDHK/P from wild-type or mutant strains. Wild-type and mutant proteins were purified by one-step affinity chromatography on a nickel-agarose column and analyzed by SDS−PAGE. All samples were mixed with an electrophoresis loading buffer and heated for 5 min at 100 °C prior to electrophoresis. After electrophoresis, the gel was stained with R250 Coomassie blue. The proteins, wild-type or mutants, loaded onto the gel are indicated on the figure.

by either Ala, Gln, or Glu residues whereas Asn-377, Asp-403, and Glu-439 residues were replaced by an Ala residue. Each mutant was expressed and purified by the same procedure as that described for the wild-type enzyme. However, the amounts of protein recovered from the Asp-371 mutants were much lower as compared to the wild-type: about a few hundred micrograms instead of a few milligrams starting from 4 L of culture medium. This low yield of purification led to a substantial contamination of the Asp371Ala and the Asp371Gln mutant preparations (Figure 2, left panel) by a 25-kDa protein which was identified, by sequencing, as the WHP protein. This histidine-rich protein has been described as a common contaminant of proteins purified by nickel-agarose chromatography (*55*), and is also detected with the wild-type IDHK/P when high amounts of enzyme are loaded on the gel. The Asp371Glu mutant was even more contaminated by the WHP protein after purification, and two additional bands at about 60 and 40 kDa were detected on the gel after electrophoresis. An additional anion-exchange chromatography was performed for the Asp371Gln mutant, allowing elimination of the WHP protein, but considerably lowering the amount of purified protein recovered (data not shown).

In contrast, the purification yield and the electrophoretic pattern of the other three mutants, Asn377Ala, Asp403Ala, and Glu439Ala, were quite similar to that obtained for the wild-type enzyme (Figure 2, right panel).

Is the Asp-371 Residue Critical for IDHK/P Phosphorylating Activity? Despite the presence of some contaminant(s) in the Asp-371 mutant preparations, the phosphorylating capacity of each mutant was compared to that of the wild-type by adding a similar amount of wild-type or mutant IDHK/P to the phosphorylation mixture, as estimated from the electrophoretic pattern (see in Figure 2 the band intensities at about 60 kDa in the respective lanes). Figure 3 shows that the phosphorylating capacity, measured during a 30-min incubation period, was greatly impaired for each IDHK/P mutant, the Asp371Ala mutant retaining the highest residual activity among them (lane 2). When this experiment was repeated with the Asp371Gln mutant further purified by anion-exchange chromatography, identical results were obtained (data not shown), indicating that the presence of

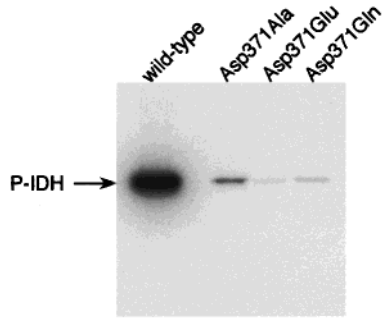

FIGURE 3: Phosphorylating activity of wild-type and Asp371 mutants of IDHK/P. Phosphorylation of purified IDH was performed in the presence of purified IDHK/P from either wild-type, Asp371Ala mutant, Asp371Glu mutant, or Asp371Gln mutant for 30 min at 37 °C, as described under Experimental Procedures. After SDS−PAGE, the gel was submitted to autoradiography. The position of phosphorylated IDH is shown on the figure.

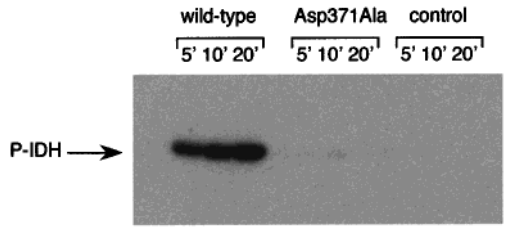

FIGURE 4: Phosphorylation of purified IDH by a cellular extract containing either the wild-type IDHK/P or the Asp371Ala mutant. The host strain IL2 either nontransformed (control) or harboring the plasmid pJCD4 (expressing the wild-type IDHK/P) or the plasmid pCOD (expressing the Asp371Ala-mutated IDHK/P) was used. After induction by isopropyl β-thiogalactopyranoside, cellular extracts from each culture were prepared and used to phosphorylate purified IDH, as described under Experimental Procedures. After the time specified, samples were withdrawn, mixed with electrophoresis loading buffer, and heated for 5 min at 100 °C prior to SDS−PAGE. The gel was then submitted to autoradiography; the position of the phosphorylated recombinant IDH is shown on the left side.

contaminant(s) did not alter the phosphorylation measurements. The incubation period used in this assay was deliberately long so as to detect whether the mutants were capable of any phosphorylating activity. Indeed, a kinetic experiment performed with the wild-type enzyme showed that an initial rate of phosphorylation was maintained during about 2 min, and then the phosphorylating activity reached a saturating level. The phosphorylating activity of the Asp371Ala mutant was barely detectable when the incubation lasted less than 2 min, and, therefore, we could only estimate that its activity represents much less than a few percent of that of the wild-type enzyme (data not shown). A possible explanation for the lack of activity of the Asp-371 mutants is that the corresponding proteins were less stable than the wild-type enzyme, and therefore became partially denatured during the purification procedure. To investigate this possiblity, the *E. coli* IL2 strain devoided of IDHK/P was used (*32*). It was complemented with either the wild-type IDHK/P or the Asp371Ala mutant, and phosphorylation experiments were performed with each crude cellular extract. Figure 4 shows that the wild-type complemented strain was capable of phosphorylating exogenous IDH to a significant and sustained level, whereas the phosphorylating activity of the strain complemented with the Asp371Ala-mutated IDHK/P was hardly detectable. No phosphorylation occurred when a control was performed by using directly the IL2 strain. Assuming a similar level of expression of the wild-type IDHK/P and the Asp371Ala mutant for the IL2 strain transformation, this would suggest that the Asp371Ala mutant is intrinsically much less active and/or less stable than the wild-type.

The Asn-377 and Asp-403 Residues of IDHK/P Are Involved in Magnesium Binding. Phosphorylation experiments were also performed on the other three mutants, Asn377Ala, Asp403Ala, and Glu439Ala. First, their affinity for ATP was compared to that of the wild-type enzyme. As shown in Figure 5A, the same amount of enzyme (IDH and IDHK/P) was used in each set of experiments, and the phosphorylation of IDH gradually increased when the ATP concentration was raised (Figure 5B). From the phosphorylation intensities reported in Figure 5C, the apparent fitted K_m for ATP was calculated to be 6.9 ± 1.5 μM for the wild-type IDHK/P and was nearly identical for the Asp403Ala (8.7 ± 1.9 μM) and the Glu439Ala mutants (9.8 ± 4 μM). In contrast, the apparent fitted K_m for ATP was about 2-fold higher for the Asn377Ala mutant (14.7 ± 3 μM, see Table 1). Since these three mutants did not show a marked decrease in affinity, this implies that their overall structure was unaffected by the newly introduced mutation.

In another set of experiments, the affinity for magnesium during the phosphorylating activity was assessed (Figure 6). It was found that, at saturating concentrations of IDH and ATP, the wild-type IDHK/P had an apparent fitted K_m for magnesium of about 0.1 ± 0.02 mM and it was essentially unaffected by the mutation Glu439Ala (0.2 ± 0.05 mM). In contrast, it was much higher for either Asn377Ala or Glu403Ala mutants, with respective values of 0.6 ± 0.2 and 0.8 ± 0.3 mM (Table 1). Therefore, these results suggest that both Asn-377 and Asp-403 of IDHK/P are somehow involved in the binding of magnesium ion(s).

DISCUSSION

The results reported in this paper shed new light on the catalytic mechanism of the *E. coli* isocitrate dehydrogenase kinase/phosphatase, especially on the residues involved in its kinase activity. Up to now, the structure of IDHK/P was supposed to be unique and unrelated to that of ePKs (*5, 31, 56*), except for a short stretch of amino acid residues encompassing a glycine-rich loop and a lysine residue (*17, 32*). In ePKs, these residues belong to subdomains I and II, respectively, as defined by Hanks et al. (*18, 22*), and interact with the polyphosphate moiety of the bound nucleotide. Inactivation of different members of the ePKs family by the ATP analogue 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine invariably labeled the conserved lysine residue of subdomain II (*57*−*60*). Surprisingly, we found that the equivalent lysine residue of IDHK/P, Lys-336, could not be labeled by the same ATP analogue, suggesting that this residue was less reactive and/or less accessible than in ePKs (*35*). In the primary structure of IDHK/P, Lys-336 is much closer to the "catalytic triad" (about 35 residues) than in the ePKs (about 100 residues). In the tertiary structure of ePKs, these two elements belong to two different lobes of the protein, and the nucleotide is nestled in the cleft between them (*19*−*21*). It is therefore conceivable that the shorter length in the IDHK/P sequence between these two elements might impose

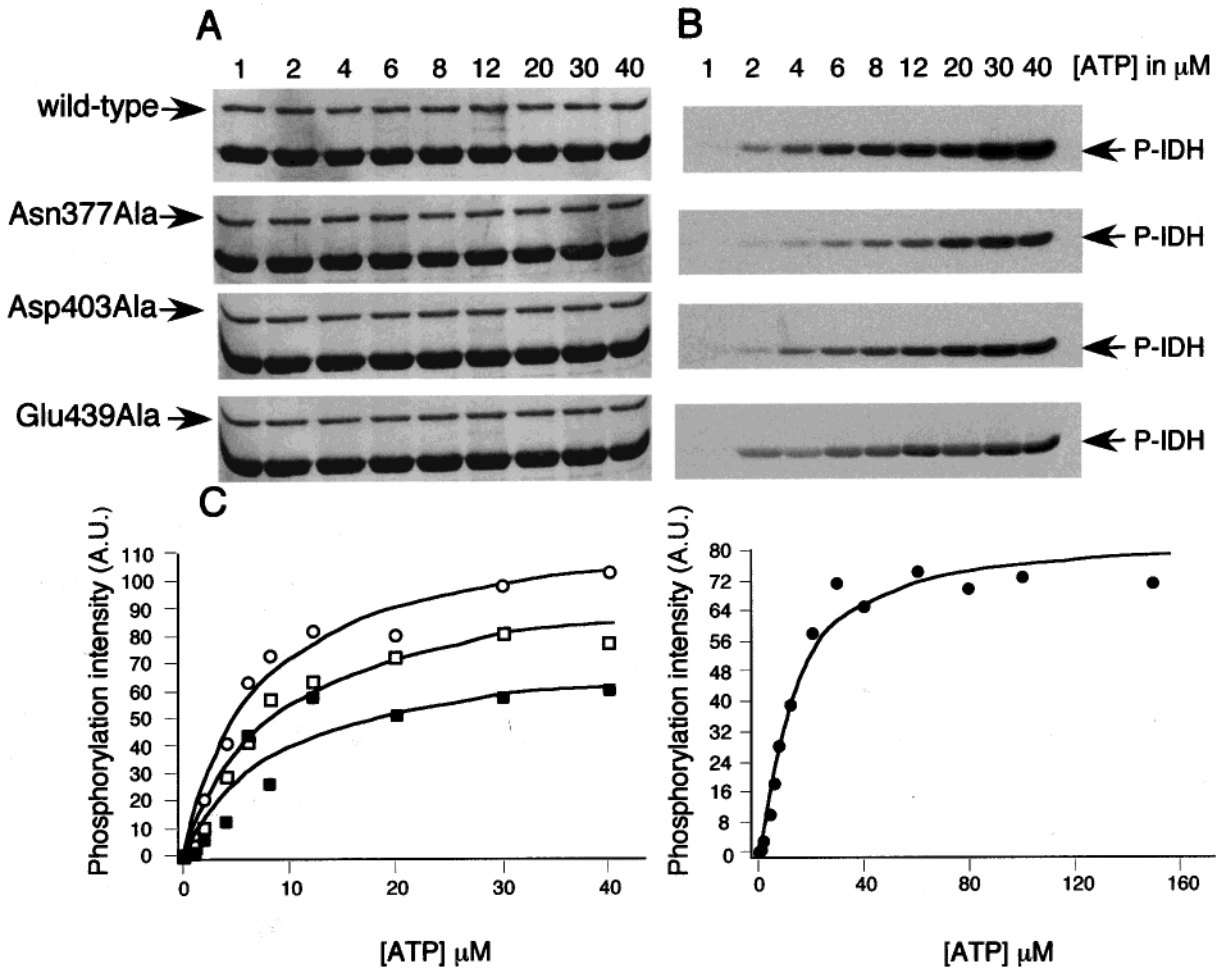

FIGURE 5: Kinase activity of wild-type and mutant IDHK/P, in the presence of increasing concentrations of ATP. Purified recombinant IDH was incubated for 30 s at 37 °C in a phosphorylation mixture containing 5 mM $MgCl_2$ and either wild-type IDHK/P, Asn377Ala, or Asp403Ala mutants, in the presence of increasing concentrations of ATP as indicated. For the Asn377Ala mutant, four additional ATP concentrations were tested (60, 80, 100, and 150 μM), but the results for these higher concentrations are not shown in the upper part of the figure. (A) The incubation medium was analyzed by SDS−PAGE, and the stained gel is shown. The enzyme used is indicated on the left side of each panel, the lower band corresponding to the IDH substrate. (B) Autoradiogram of the gel shown in (A), with the position of phospho-IDH indicated. (C) The intensities of phosphorylation obtained in (B) were analyzed by densitometry, and the values were fitted as a function of ATP concentration by using the MacCurveFit (version 1.0.8) software. (○) Wild-type IDHK/P; (●) Asn377Ala mutant; (□) Asp403Ala mutant; (■) Glu439Ala mutant. Three independent experiments were performed, and the typical results obtained for one experiment are shown here.

Table 1: Apparent Fitted K_m for ATP and Magnesium of Wild-Type and Mutants IDHK/P[a]

	K_m for ATP (μM)	K_m for Mg^{2+} (mM)
wild-type	6.9 ± 1.5	0.1 ± 0.02
Asn377Ala mutant	14.7 ± 3	0.6 ± 0.2
Asp403Ala mutant	8.7 ± 1.9	0.8 ± 0.3
Glu439Ala mutant	9.8 ± 4	0.2 ± 0.05

[a] Values were obtained from Figures 5 and 6.

some structural constraints on the binding of either ATP or its alkylating analogue, thus preventing the labeling of Lys-336 by 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine.

The results obtained here with the Asp-371 mutants raise the possibility that this residue might be the catalytic base in IDHK/P. However, we cannot draw definite conclusions about its role in catalysis since the purification of each mutant was unsatisfactory, probably due to some intrinsic structural instability. The corresponding putative catalytic base of the protein tyrosine kinase Csk has also been mutated into Ala, Asn, and Glu residues, and the former two mutants were reported to be poorly expressed (*61*). In contrast, the Glu mutant was purified and shown to be essentially devoided of activity. On the other hand, the mutation into an Ala residue of the equivalent Asp residue in the EGF protein tyrosine kinase allowed the purification of the mutated, though inactive, protein (*62*). Clearly, depending on the nature of the protein kinase, mutation of the putative catalytic base produces a more or less drastic effect on the stability of the protein, even if the same amino acid substitution is made. It is important to mention that the participation of a catalytic base in the phosphorylation mechanism of ePKs is still a matter of debate (*63*, *64*). Hence, it has been recently proposed that in PKA, Asp-166 does not act as a catalytic base, but instead maintains the conformation of the active site (*65*). Asp-166 notably interacts, either directly through a salt bridge in PKA (*65*) or indirectly through a network of hydrogen bonding in phosphorylase kinase (*64*), with its neighboring Lys-168. This critical residue is totally conserved in the serine/threonine ePKs where it contacts the γ-phosphate of ATP, presumably to stabilize the intermediate reaction steps (*21*). It is noteworthy that, in the partial

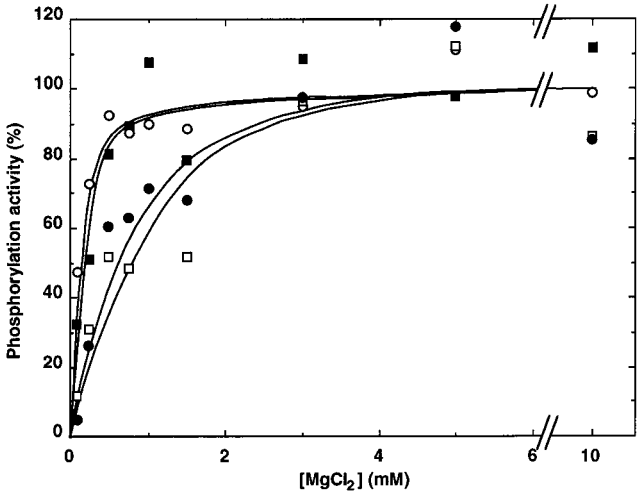

FIGURE 6: Kinase activity of wild-type and mutant IDHK/P, in the presence of increasing concentrations of $MgCl_2$. Purified recombinant IDH was incubated for 30 s at 37 °C in a phosphorylation mixture containing 50 μM ATP and either wild-type IDHK/P, Asn377Ala, or Asp403Ala mutants, in the presence of increasing concentrations of $MgCl_2$ as indicated. The incubation medium was submitted to SDS−PAGE, and the stained gel was autoradiographed; the intensities of phosphorylation were analyzed as described in Figure 5. (○) Wild-type IDHK/P; (●) Asn377Ala mutant; (□) Asp403Ala mutant; (■) Glu439Ala mutant. Three independent experiments were performed, and the typical results obtained for one experiment are shown here.

sequence alignement shown in Figure 1, Lys-168 of PKA is aligned with Gln-373 of IDHK/P. A spontaneous mutation of the latter into an Arg residue provoked a marked decrease of the phosphatase activity without affecting the kinase activity of the IDHK/P mutant (*66*, *67*). This observation led the authors to suggest that the Gln-373 residue is somehow involved in the switch between the kinase and phosphatase activities of IDHK/P, a proposal in agreement with our new sequence alignment.

The putative catalytic base is part of a loop, known as the "catalytic loop" in ePKs (*1*, *21*). In this loop, Asp-166 is separated by four residues from the invariant Asn-171 involved in the ligation of one of the two magnesium ions in PKA. Although a similar spacing is found in most of the ePKs (*18*, *22*), insertion is permitted in this loop without affecting the overall positioning of the critical residues (*40*). Also, in the structure of a phosphoinositide kinase which adopts a nucleotide-binding fold similar to ePKs, a deletion is observed in this loop including in particular the invariant Asn residue (*68*). Therefore, the insertion of one residue in the equivalent loop of IDHK/P without affecting the overall structure of the loop seems quite possible.

The involvement of Asn-171 and Asp-184 residues in the binding of magnesium ions has been emphasized by the crystal structure of several ePKs. However, depending on the nature of the enzyme, one or two magnesium (or manganese) ion(s) is (are) bound in the structure. Hence, a high-affinity ion interacts with the β- and γ-phosphates of nucleotide and is chelated by Asp-184, whereas a second ion interacts, with low affinity, with the α- and γ-phosphates of nucleotide and is chelated by both Asn-171 and Asp-184 in either PKA, phosphorylase kinase, or mitogen-activated protein kinase (*26*, *50*, *69*). In contrast, only one magnesium ion is found in the structure of either casein kinase 1 or casein kinase 2, and it corresponds to the second, low-affinity, site of the former class of ePKs (*51*, *53*). The reason for this difference is currently unknown (*21*), but might be related to the diversity in catalytic efficiency of each enzyme. For instance, while magnesium binding to the high-affinity site is required for the catalysis of PKA, binding to the low-affinity site is associated with enzyme inhibition (*70*). Since Asp-184 is the only residue that chelates the magnesium ion bound to the high-affinity site, this might explain why its substitution by an Ala residue totally abolished the activity of the yeast PKA (*54*). Here, the putatively equivalent Asp residue of IDHK/P was replaced by an Ala residue which resulted essentially in a decreased affinity for magnesium. Therefore, it might be argued that this residue is not the only one to be involved in magnesium binding in IDHK/P; this would rather be in favor of a binding site where both equivalent residues of Asn-171 and Asp-184 might chelate the magnesium ion.

In conclusion, the results presented here suggest that the catalytic mechanism of phosphorylation by IDHK/P bears some resemblance with that of ePKs. On the other hand, IDHK/P recognizes the tertiary structure of its IDH substrate for the phosphorylation to occur (*71*), in contrast to ePKs where the primary structure is the key element for the recognition of the protein substrate (*72*), allowing the latter to phosphorylate even small peptides (*73*). For this reason, it seems likely that the structure of the protein substrate binding site of IDHK/P is going to be somewhat different from that in ePKs. The molecular basis for this apparent dilemma will have to await for the 3-D structure of the IDHK/P to be understood. However, crystallization attempts of IDHK/P have been so far unsuccessful (*74*), and site-directed mutagenesis remains a useful approach to unravel the functioning of this complex bifunctional enzyme.

REFERENCES

1. Taylor, S. S., Knighton, D. R., Zheng, J., Sowadski, J. M., Gibbs, C. S., and Zoller, M. J. (1993) *Trends Biochem. Sci. 18*, 84−89.
2. Saier, M. H., Jr., Wu, L. F., and Reizer, J. (1990) *Trends Biochem. Sci. 15*, 391−395.
3. Meadow, N. D., Fox, D. K., and Roseman, S. (1990) *Annu. Rev. Biochem. 59*, 497−542.
4. Deutscher, J., Fischer, C., Charrier, V., Galinier, A., Lindner, C., Darbon, E., and Dossonnet, V. (1997) *Folia Microbiol. 42*, 171−178.
5. Cozzone, A. J. (1993) *J. Cell. Biochem. 51*, 7−13.
6. Fabret, C., Feher, V. A., and Hoch, J. A. (1999) *J. Bacteriol. 181*, 1975−1983.
7. Cozzone, A. J. (1998) *Biochimie 80*, 43−48.
8. Cozzone, A. J. (1988) *Annu. Rev. Microbiol. 42*, 97−125.
9. Vincent, C., Doublet, P., Grangeasse, C., Vaganay, E., Cozzone, A. J., and Duclos, B. (1999) *J. Bacteriol. 181*, 3472−3477.
10. Ilan, O., Bloch, Y., Frankel, G., Ullrich, H., Geider, K., and Rosenshine, I. (1999) *EMBO J. 18*, 3241−3248.
11. LaPorte, D. C. (1993) *J. Cell. Biochem. 51*, 14−18.
12. Cozzone, A. J. (1998) *Annu. Rev. Microbiol. 52*, 127−64.
13. Holms, H. (1996) *FEMS Microbiol. Rev. 19*, 85−116.
14. Saier, M. H., Jr., Ramsaier, T. M., and Reizer, J. (1996) in *Escherichia coli and Salmonella typhimurium, Cellular and Molecular Biology* (Neidhardt, F. C., Ed.) pp 1325−1343, ASM Press, Washington, DC.
15. LaPorte, D. C., and Koshland, D. E., Jr. (1982) *Nature 300*, 458−460.
16. Stueland, C. S., Eck, K. R., Stieglbauer, K. T., and LaPorte, D. C. (1987) *J. Biol. Chem. 262*, 16095−16099.

17. LaPorte, D. C., Stueland, C. S., and Ikeda, T. P. (1989) *Biochimie 71*, 1051−1057.
18. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) *Science 241*, 42−52.
19. Johnson, L. N., Lowe, E. D., Noble, M. E., and Owen, D. J. (1998) *FEBS Lett. 430*, 1−11.
20. Knighton, D. R., Zheng, J. H., Ten Eyck, L. F., Ashford, V. A., Xuong, N. H., Taylor, S. S., and Sowadski, J. M. (1991) *Science 253*, 407−414.
21. Bossemeyer, D. (1995) *FEBS Lett. 369*, 57−61.
22. Hanks, S. K., and Quinn, A. M. (1991) *Methods Enzymol. 200*, 38−62.
23. Bossemeyer, D. (1994) *Trends Biochem. Sci. 19*, 201−205.
24. Hemmer, W., McGlone, M., Tsigelny, I., and Taylor, S. S. (1997) *J. Biol. Chem. 272*, 16946−16954.
25. Johnson, L. N., Noble, M. E., and Owen, D. J. (1996) *Cell 85*, 149−158.
26. Lowe, E. D., Noble, M. E., Skamnaki, V. T., Oikonomakos, N. G., Owen, D. J., and Johnson, L. N. (1997) *EMBO J. 16*, 6646−6658.
27. Zheng, J., Knighton, D. R., ten Eyck, L. F., Karlsson, R., Xuong, N., Taylor, S. S., and Sowadski, J. M. (1993) *Biochemistry 32*, 2154−2161.
28. Herberg, F. W., Doyle, M. L., Cox, S., and Taylor, S. S. (1999) *Biochemistry 38*, 6352−6360.
29. Shi, L., Potts, M., and Kennelly, P. J. (1998) *FEMS Microbiol. Rev. 22*, 229−253.
30. Leonard, C. J., Aravind, L., and Koonin, E. V. (1998) *Genome Res. 8*, 1038−1047.
31. Kennelly, P. J., and Potts, M. (1996) *J. Bacteriol. 178*, 4759−4764.
32. Stueland, C. S., Ikeda, T. P., and LaPorte, D. C. (1989) *J. Biol. Chem. 264*, 13775−13779.
33. Hon, W. C., McKay, G. A., Thompson, P. R., Sweet, R. M., Yang, D. S., Wright, G. D., and Berghuis, A. M. (1997) *Cell 89*, 887−895.
34. Rittinger, K., Negre, D., Divita, G., Scarabel, M., Bonod-Bidaud, C., Goody, R. S., Cozzone, A. J., and Cortay, J. C. (1996) *Eur. J. Biochem. 237*, 247−254.
35. Oudot, C., Jault, J. M., Jaquinod, M., Negre, D., Prost, J. F., Cozzone, A. J., and Cortay, J. C. (1998) *Eur. J. Biochem. 258*, 579−585.
36. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
37. Oudot, C., Jaquinod, M., Cortay, J. C., Cozzone, A. J., and Jault, J. M. (1999) *Eur. J. Biochem. 262*, 224−229.
38. McClure, M. A., Vasi, T. K., and Fitch, W. M. (1994) *Mol. Biol. Evol. 11*, 571−592.
39. Blanchet, C., Combet, C., Geourjon, C., and Deléage, G. (2000) *Bioinformatics 16*, 286−287.
40. Steinbacher, S., Hof, P., Eichinger, L., Schleicher, M., Gettemans, J., Vandekerckhove, J., Huber, R., and Benz, J. (1999) *EMBO J. 18*, 2923−2939.
41. Smith, C. M., Shindyalov, I. N., Veretnik, S., Gribskov, M., Taylor, S. S., Ten Eyck, L. F., and Bourne, P. E. (1997) *Trends Biochem. Sci. 22*, 444−446.
42. Bairoch, A., and Apweiler, R. (2000) *Nucleic Acids Res. 28*, 45−48.
43. Nelson, K., Wang, F. S., Boyd, E. F., and Selander, R. K. (1997) *Genetics 147*, 1509−1520.
44. Rost, B., and Sander, C. (1993) *J. Mol. Biol. 232*, 584−599.
45. Geourjon, C., and Deleage, G. (1995) *Comput. Appl. Biosci. 11*, 681−684.
46. King, R. D., and Sternberg, M. J. (1996) *Protein Sci. 5*, 2298−2310.
47. Garnier, J., Gibrat, J. F., and Robson, B. (1996) *Methods Enzymol. 266*, 540−553.
48. Frishman, D., and Argos, P. (1996) *Protein Eng. 9*, 133−142.
49. Levin, J. M. (1997) *Protein Eng. 10*, 771−776.
50. Bossemeyer, D., Engh, R. A., Kinzel, V., Ponstingl, H., and Huber, R. (1993) *EMBO J. 12*, 849−859.
51. Xu, R. M., Carmel, G., Sweet, R. M., Kuret, J., and Cheng, X. (1995) *EMBO J. 14*, 1015−1023.
52. Robinson, M. J., Harkins, P. C., Zhang, J., Baer, R., Haycock, J. W., Cobb, M. H., and Goldsmith, E. J. (1996) *Biochemistry 35*, 5641−5646.
53. Niefind, K., Guerra, B., Pinna, L. A., Issinger, O. G., and Schomburg, D. (1998) *EMBO J. 17*, 2451−2462.
54. Gibbs, C. S., and Zoller, M. J. (1991) *J. Biol. Chem. 266*, 8923−8931.
55. Wülfing, C., Lombardero, J., and Plückthun, A. (1994) *J. Biol. Chem. 269*, 2895−2901.
56. Kennelly, P. J., and Potts, M. (1999) *Front. Biosci. 4*, D372−385.
57. Zoller, M. J., Nelson, N. C., and Taylor, S. S. (1981) *J. Biol. Chem. 256*, 10837−10842.
58. Kamps, M. P., Taylor, S. S., and Sefton, B. M. (1984) *Nature 310*, 589−592.
59. Russo, M. W., Lukas, T. J., Cohen, S., and Staros, J. V. (1985) *J. Biol. Chem. 260*, 5205−5208.
60. Komatsu, H., and Ikebe, M. (1993) *Biochem. J. 296*, 53−58.
61. Cole, P. A., Grace, M. R., Phillips, R. S., Burn, P., and Walsh, C. T. (1995) *J. Biol. Chem. 270*, 22105−22108.
62. Cheng, K., and Koland, J. G. (1998) *Biochem. J. 330*, 353−359.
63. Zhou, J., and Adams, J. A. (1997) *Biochemistry 36*, 2977−2984.
64. Skamnaki, V. T., Owen, D. J., Noble, M. E., Lowe, E. D., Lowe, G., Oikonomakos, N. G., and Johnson, L. N. (1999) *Biochemistry 38*, 14718−14730.
65. Hutter, M. C., and Helms, V. (1999) *Protein Sci. 8*, 2728−2733.
66. Ikeda, T. P., Houtz, E., and LaPorte, D. C. (1992) *J. Bacteriol. 174*, 1414−1416.
67. Miller, S. P., Karschnia, E. J., Ikeda, T. P., and LaPorte, D. C. (1996) *J. Biol. Chem. 271*, 19124−19128.
68. Rao, V. D., Misra, S., Boronenkov, I. V., Anderson, R. A., and Hurley, J. H. (1998) *Cell 94*, 829−839.
69. Bellon, S., Fitzgibbon, M. J., Fox, T., Hsiao, H. M., and Wilson, K. P. (1999) *Struct. Fold. Des. 7*, 1057−1065.
70. Armstrong, R. N., Kondo, H., Granot, J., Kaiser, E. T., and Mildvan, A. S. (1979) *Biochemistry 18*, 1230−1238.
71. McKee, J. S., Hlodan, R., and Nimmo, H. G. (1989) *Biochimie 71*, 1059−1064.
72. Pearson, R. B., and Kemp, B. E. (1991) *Methods Enzymol. 200*, 62−81.
73. Kemp, B. E., and Pearson, R. B. (1991) *Methods Enzymol. 200*, 121−134.
74. Finer-Moore, J., Tsutakawa, S. E., Cherbavaz, D. R., LaPorte, D. C., Koshland, D. E., Jr., and Stroud, R. M. (1997) *Biochemistry 36*, 13890−13896.
75. Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994) *Nucleic Acids Res. 22*, 4673−4680.
76. Kabsch, W., and Sander, C. (1983) *Biopolymers 22*, 2577−2637.

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