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Structural Basis of the Substrate Specificity of Bifunctional Isocitrate Dehydrogenase Kinase/Phosphatase

Susan P. Yates,[†] Thomas E. Edwards,[‡,§] Cassie M. Bryan,[‡,∥] Adam J. Stein,[‡,§] Wesley C. Van Voorhis,[‡,∥] Peter J. Myler,[‡,⊥,@] Lance J. Stewart,[‡,§] Jimin Zheng,[#] and Zongchao Jia*[,†,#]

[†]Department of Biomedical and Molecular Sciences, Queen's University, Kingston, ON K7L 3N6, Canada

[‡]Seattle Structural Genomics Center for Infectious Disease (SSGCID), Seattle Biomedical Research Institute, Seattle, Washington 98109-5240, United States

[§]Emerald BioStructures Inc., 7869 NE Day Road West, Bainbridge Island, Washington 98110, United States

[∥]Department of Medicine, Division of Allergy and Infectious Diseases, MS 356423, School of Medicine, University of Washington, Seattle, Washington 98195-6423, United States

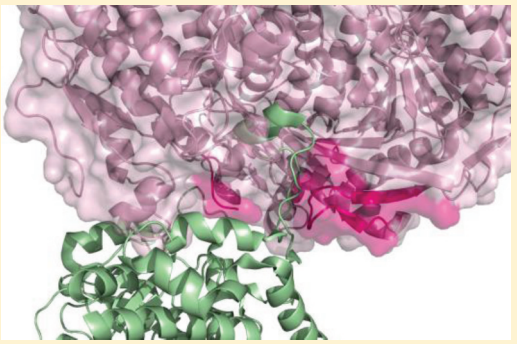
[⊥]Seattle Biomedical Research Institute, 307 Westlake Avenue North, Suite 500, Seattle, Washington 98109, United States

[@]Department of Global Health and Department of Medical Education and Biomedical Informatics, University of Washington, Seattle, Washington 98195, United States

[#]College of Chemistry, Beijing Normal University, Beijing 100865, China

Supporting Information

ABSTRACT: Isocitrate dehydrogenase kinase/phosphatase (AceK) regulates entry into the glyoxylate bypass by reversibly phosphorylating isocitrate dehydrogenase (ICDH). On the basis of the recently determined structure of the AceK−ICDH complex from *Escherichia coli*, we have classified the structures of homodimeric $NADP^+$-ICDHs to rationalize and predict which organisms likely contain substrates for AceK. One example is *Burkholderia pseudomallei* (Bp). Here we report a crystal structure of Bp-ICDH that exhibits the necessary structural elements required for AceK recognition. Kinetic analyses provided further confirmation that Bp-ICDH is a substrate for AceK. We conclude that the highly stringent AceK binding sites on ICDH are maintained only in Gram-negative bacteria.



The enzyme isocitrate dehydrogenase (ICDH) participates in the Krebs cycle converting isocitrate to α-ketoglutarate in a two-step mechanism whereby a dehydrogenation reaction requiring the reduction of $NAD(P)^+$ to $NAD(P)H^+$ is followed by a decarboxylation reaction.[1] ICDH exists in all domains of life (Bacteria, Eukarya, and Archaea), yet it has evolved to differ in its cofactor specificity and its oligomeric state. On the basis of primary sequence, the family is classified into three subfamilies. Subfamily I includes archaeal and bacterial $NADP^+$-ICDHs; subfamily II consists mainly of eukaryotic $NADP^+$-ICDHs with some bacterial exceptions, and subfamily III includes hetero-oligomeric NAD^+-ICDHs.[2] Isocitrate dehydrogenase kinase/phosphatase (AceK) acts directly on ICDH and is the metabolic switch between the energy-generating Krebs cycle and gluconeogenesis requiring glyoxylate bypass in response to nutrient availability.[3] Notably, AceK exists in only certain bacteria (discussed later). AceK is a bifunctional enzyme with protein kinase, phosphatase, and ATPase activities all remarkably shared at the same active site[3] and is a rare example of reversible prokaryotic protein phosphorylation, acting as a "primitive" protein kinase with opposing phosphatase activity. Despite being the very founding member of prokaryotic protein phosphorylation,[4,5] a full understanding of AceK has remained elusive until the structures of AceK and its complex with ICDH from *Escherichia coli* (Ec) were recently unveiled.[6]

Many protein kinases are able to use peptide substrates that are based on the sequence surrounding the phosphorylation site (P-site), albeit with a reduced affinity. Substrate specificity is often increased by docking motifs, like in MAPKs where the D domain on the MAPK target binds the docking groove on MAPK distal to the catalytic site.[7,8] Some kinase−substrate interactions require scaffold proteins, particularly in intracellular signaling pathways.[7] However, AceK forms an intimate association with only homodimic ICDH (Figure 1A) and cannot phosphorylate either proteolytic fragments derived from

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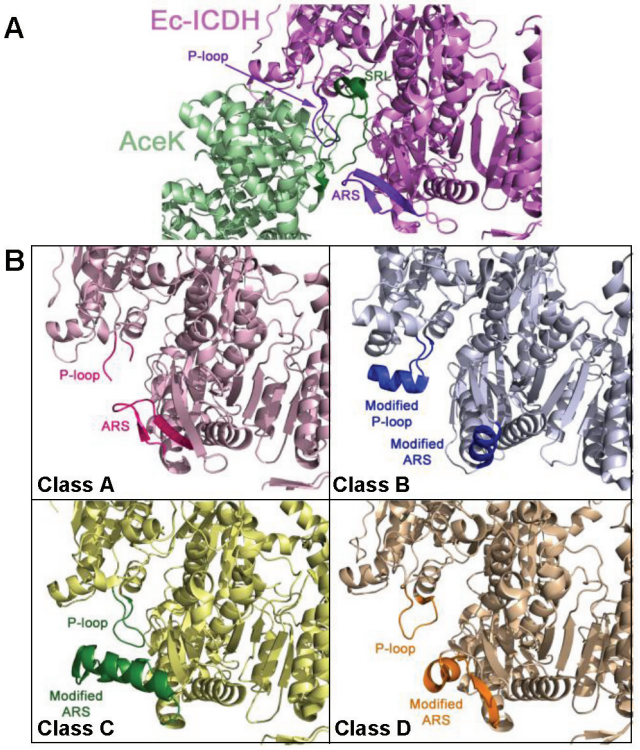

Figure 1. Structural classification of ICDH based on AceK binding. (A) AceK–Ec-ICDH complex. The SRL (green) of AceK (teal) extends into the Ec-ICDH (purple) structure. The main binding elements, ARS and P-loop (blue), for AceK on ICDH are shown. (B) Classification of ICDH based on the structural elements in the corresponding P-loop and ARS regions. For the sake of clarity, only one representative structure is shown for each class (class A, Bp-ICDH; class B, Pc-ICDH; class C, Bs-ICDH; class D, Ap-ICDH).

ICDH or synthetic peptides corresponding to the sequence around the phosphorylation site. Kinases that require globular domains for recognition do exist;[9,10] however, AceK uniquely requires the ICDH dimer.[6] The substrate recognition loop (SRL; residues 484–510) of AceK deeply extends ~32 Å into the active site cleft of ICDH with a short α-helix at its tip. An extensive array of interactions between the SRL and both molecules of dimeric ICDH exists, including hydrophobic packing, salt bridges, and hydrogen bonds.[6] Because AceK recognizes the tertiary structure of homodimeric ICDH to form a "productive" complex with one substrate site, a peptide is simply an inadequate interaction epitope for AceK[11,12] and cannot substitute for the complexity the dimer creates.

From the structure of the *E. coli* AceK–ICDH complex, the regions of protein–protein interaction were identified. To understand possible reasons why AceK is unable to bind and act on most ICDHs, we compared and searched for putative AceK binding sites on other ICDHs. Because ICDH from *E. coli*, a known target of AceK, is an $NADP^+$-dependent homodimer, we limited our comparisons to homodimeric $NADP^+$-ICDHs whose crystal structures have been determined and deposited in the Protein Data Bank. ICDH from *Bacillus subtilis* (Bs), *Burkholderia pseudomallei* (Bp; this work), *Aeropyrum pernix* (Ap), and *Archaeoglobus fulgidus* (Af) and a hypothetical ICDH from *Sulfolobus tokodaii* (St) are subfamily I members, and Pc-ICDH (porcine heart mitochondrial), Hc-ICDH (human cytosolic), Sc-ICDH (*Saccharyomyces cerevisiae*), Dp-ICDH (*Desulfotalea psychrophila*), Tm-ICDH (*Thermotoga maritima*), and Mc-ICDH (mouse cytosolic) are subfamily II members. Although the level of sequence identity is quite low (18–23% vs Ec-ICDH) between these two subfamilies, their structures are remarkably similar, including the catalytic active site residues. Using DaliLite Pairwise comparison of protein structures,[13] the regions that likely contact AceK were evaluated. On the basis of these alignments and the structure of the AceK–ICDH complex, we further classified these ICDHs according to the proposed AceK binding motifs (Figure 1B and Figure S1 of the Supporting Information). Group A consists of Ec- and Bp-ICDH, group B Pc-, Hc-, Dp-, Tm-, Sc-, and Mc-ICDHs, group C Bs-ICDH, and group D Ap-, Af-, and St-ICDHs. Ser113 from Ec-ICDH, the target of phosphorylation by AceK,[3] is structurally conserved among all ICDHs.

Only Bp-ICDH was classified with Ec-ICDH in group A, showing remarkable similarities overall and particularly in their AceK binding sites. To reveal the detailed structural elements for AceK recognition and binding, we determined the structure of Bp-ICDH at 1.65 Å resolution (see the Supporting Information). Bp- and Ec-ICDH are 75% identical in sequence and align with a Cα root-mean-square deviation (rmsd) of 0.57 Å over 410 similar residues, indicating that these enzymes are highly similar in sequence and structure (Figure S2 of the Supporting Information). From the structure of the AceK–ICDH complex, two discontinuous regions on ICDH contact AceK. These include the P-loop from monomer 1 and the twisted antiparallel β-sheet from monomer 2 (ARS; AceK recognition segment), in the homodimeric structure of ICDH. Similar structural elements are present in Bp-ICDH (Figure 1 and Figure S1), and as in Ec-ICDH, no apparent structural clashes would occur if AceK bound to Bp-ICDH (Figure S3 of the Supporting Information).

Group B is comprised of subfamily II ICDHs, and the most notable difference is the P-loop, which compared to other bacterial and archaeal ICDHs contains an insertion of several residues forming a short helix (Figure 1). This insertion may limit access to the active site, but it would also interfere with and prevent AceK binding,[14] conflicting with the SRL and the helix adjacent to the SRL on AceK both sterically and through electrostatic repulsion due to the inherent negative charge of these elements. A novel self-regulatory mechanism is proposed in Hc-ICDH that mimics the phosphorylation by AceK.[15] Currently, no evidence of phosphorylation of ICDH exists, and no AceK protein or homologues have been identified in eukaryotes. During phosphorylated regulation by AceK, the phosphate moiety attached to the serine inhibits binding of isocitrate to the active site. Alternatively, in the inactive form of Hc-ICDH, a helix unwinds into a loop allowing Asp279 to interact with Ser94 (analogous to Ser113 of Ec-ICDH), causing inhibition of isocitrate binding without phosphorylation.[15] Evidence of a self-regulatory mechanism, however, is not supported by the structure of Tm-ICDH, a thermophilic bacterium,[16] meaning it is not universal to all subfamily II members.

Bs-ICDH from *B. subtilis*, a Gram-positive bacterium, represents class C and overall is 71 and 75% identical to and completely conserved near the phosphorylation sites of Ec-ICDH and Bp-ICDH, respectively. However, Bs-ICDH is a poor substrate for AceK.[17] Previous AceK kinetic studies reported the K_M values for the kinase and phosphatase reactions to be 60- and 3450-fold greater than those for Ec-ICDH, respectively.[17] Thus, Bs-ICDH is not an ideal candidate to be

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phosphorylated by AceK. This is not surprising given that no observations exist for the phosphorylation of Bs-ICDH in vivo, nor has a corresponding Bs-AceK protein been identified.[18] The key difference is the 13-residue insert (residues 246−276) in the small domain. This insert consists of a turn, a single β-strand, and two α-helices and projects 8 Å outward, restricting access to the active site, and would partially clash with the SRL of AceK (Figure 1).[6] This region corresponds to the ARS in Ec-ICDH, which we propose is important for AceK recognition. The unique insert is also observed in *Acidithiobacillus thiooxidans* ICDH;[19] however, it is an NAD^+-dependent homodimeric ICDH, but as in *B. subtilis*, no AceK proteins have been identified in this species.

Lastly, class D consists of the archaeal subfamily I ICDHs, which are structurally very similar to Ec-ICDH with a Cα rmsd of 1.7 Å. In the region of the ARS, the second strand is substituted with an α-helix, further supporting the possibility that the ARS is a critical binding determinant for AceK. A homology search of all available archaeal genomes reveals that no ORFs encode known or putative AceK proteins.[20] Phosphorylation of the corresponding serine in archaeal ICDHs has not been observed either.[21] Unfortunately, all the ICDH structures described above, with the exception of that of class A, represent those species that do not encode any known or putative AceK as dictated by BLAST comparisons and searches of all available genomic databases. In contrast, *Bk. pseudomallei* from class A indeed encodes AceK (Uniprot entry Q63Y16).

Ec-AceK phosphorylates (and dephosphorylates) Ec-ICDH, and on the basis of our structural classification, Ec-AceK is likely to act on Bp-ICDH (class A) with comparable efficiency. To investigate this structure-based prediction further, kinetic studies were undertaken to evaluate the ability of Bp-ICDH to be a substrate for both the kinase and phosphatase activities of AceK. The AceK kinase assay is coupled to ICDH activity, whereby the reduction of $NADP^+$ to NADPH is monitored. When AceK phosphorylates ICDH, the activity of ICDH is inhibited; hence, higher ICDH activity corresponds to lower AceK kinase activity.[6] Both Ec- and Bp-ICDH reduce $NADP^+$ in the absence of AceK with similar efficiencies (Figure 2).

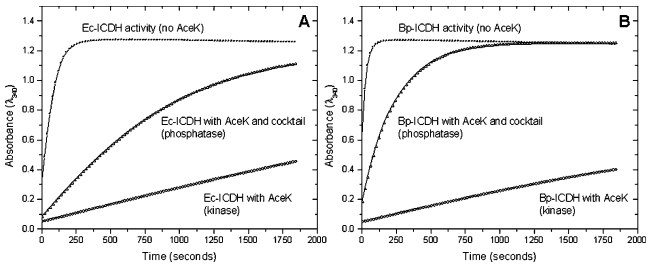

Figure 2. AceK kinase and phosphatase activity against (A) Ec-ICDH and (B) Bp-ICDH. ICDH activity in the absence of AceK acts as a reference, and phosphatase activity was measured in the presence of a phosphatase activator/kinase inhibitor cocktail.

When AceK is present, only 3% of Ec-ICDH activity is retained, and as predicted, Bp-ICDH was similarly affected, retaining only 2% activity. This loss of ICDH catalytic function infers that AceK phosphorylated both ICDH proteins. We have further shown the direct phosphorylation of both Ec- and Bp-ICDH by AceK using a dot-blot assay (Figure S4 of the Supporting Information). Because AceK exhibits normal kinase function against both ICDH proteins, we proceeded to evaluate the phosphatase activity. AceK initially phosphorylates ICDH, which is followed by the addition of a phosphatase activator/kinase inhibitor cocktail (containing AMP and pyruvate that inhibit kinase and activate phosphatase activities of AceK[22]). The dephosphorylation of ICDH by AceK removes the inhibition of ICDH activity, so that higher AceK phosphatase activity leads to higher observed ICDH activity. Addition of the cocktail increased ICDH activity (indicative of phosphatase function) to 14 and 32% of the normal ICDH activity for Ec- and Bp-ICDH, respectively (Figure 2). Our structural analysis predicted that Bp-ICDH is a substrate for AceK, and this prediction is supported by the kinetic results. Both Bp- and Ec-ICDH are true substrates for this bifunctional enzyme. Therefore, our newly determined Bp-ICDH structure contains the necessary binding elements for the productive recognition by AceK and provides biochemical evidence that the P-loop and ARS are critical for this protein−protein interaction. It also illustrates the possibility of cross-species reactivity, as they are interchangeable as substrate proteins. Likewise, we predict that AceK enzymes from the two species are also interchangeable.

A search of all known or putative AceK proteins revealed that its existence is limited to Gram-negative bacteria. The structural comparison of Bp- and Ec-ICDHs, both from Gram-negative bacteria, clearly shows conservation of the AceK binding sites, whereas in all other ICDHs, these sites are modified. In addition, ICDHs from species that encode an AceK protein show remarkable sequence conservation with no insertions (Figure S5 of the Supporting Information); likely any of these ICDH proteins (like Ec- and Bp-ICDHs) would be interchangeable as substrates for AceK. It also demonstrates the highly stringent nature of this protein−protein interaction as even small structural changes eliminate this interaction.

AceK serves as the gatekeeper to the glyoxylate bypass. The bypass consists of the enzymes isocitrate lyase (ICL) and malate synthase (MS). ICL competes with ICDH for isocitrate; however, ICDH has a higher affinity for this metabolite. AceK assists by temporarily shutting down ICDH, allowing ICL the opportunity to convert isocitrate to glyoxylate and then to malate by MS.[23] This bypass compromises the decarboxylation steps of the Krebs cycle, which is critical for survival on two-carbon compounds preventing the loss of acetyl-CoA as CO_2. Therefore, the four-carbon metabolic intermediates, succinate and malate, continue to be produced but at the cost of generating energy. The glyoxylate bypass has been observed in bacteria, protists, plants, fungi, and nematodes. The existence of this bypass in Metazoa remains controversial despite some evidence of ICL and/or MS activities.[24] It is apparent that Gram-negative bacteria are the only organisms to use AceK as the on−off switch for this pathway. The genomes of *B. subtilis* and other Gram-positive bacteria (e.g., *Staphylococcus*, *Streptococcus*, *Enterococcus*, and *Clostridium*) lack the entire glyoxylate bypass and its enzymes, rationalizing the absence of AceK, and despite evidence for ICL and MS in some low-G+C Gram-positive bacteria, AceK remains absent from their genomes.[18] Evidence of the glyoxylate pathway in Archaea does exist;[25] however, with no AceK[20] to turn on the bypass or any evidence of phosphorylated regulation of ICDH,[21] another regulatory mechanism must exist to funnel isocitrate into the bypass, perhaps analogous to the self-regulatory mechanism of Hc-ICDH.[15]

AceK is as unique to Gram-negative bacteria as are the specific structural binding elements on ICDH. Did the absence

or loss of an encoded AceK protein coincide with the modification of the AceK recognition sites on ICDHs? Perhaps the differences in ICDH structure were a consequence of the lack of selective pressure to maintain these elements in the absence of AceK or the ICDH proteins evolved to modify the corresponding P-loop and ARS regions to circumvent host–pathogen interactions to protect them from pathogenic Gram-negative bacteria producing AceK. Thus, alterations in ICDH structure were a protective adaptation requiring alternative means of regulating ICDH under conditions of poor nutrient availability.

In summary, taking advantage of the recently determined structure of the *E. coli* ICDH–AceK complex, we have reclassified ICDHs and predicted that Bp-ICDH is a substrate for Ec-AceK. Indeed, our Bp-ICDH structure displays all the essential structural elements required for Ec-AceK recognition and interaction. Our kinetic experiments further support this prediction. On the basis of the sequence and structural analyses, we conclude that AceK is specific for ICDHs from Gram-negative bacteria.

■ ASSOCIATED CONTENT

Supporting Information

Methods and supplementary tables and figures. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: (613) 533-6277. Fax: (613) 533-2497. E-mail: jia@queensu.ca.

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