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Isocitrate Dehydrogenase Kinase/Phosphatase: Identification of Mutations Which Selectively Inhibit Phosphatase Activity

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Mutations in *aceK*, the gene encoding isocitrate dehydrogenase kinase/phosphatase, which selectively inhibit phosphatase activity have been isolated. These mutations yield amino acid substitutions within a 113-residue region of this 578-residue protein. These mutations may define a regulatory domain of this protein.

In *Escherichia coli*, the Krebs cycle enzyme isocitrate dehydrogenase (IDH) is regulated by reversible phosphorylation (1, 3). The phosphorylation of IDH controls the partitioning of isocitrate between the Krebs cycle and the glyoxylate bypass. The flow of isocitrate through this bypass is essential for growth on acetate, since it avoids the quantitative loss of the acetate carbons as CO_2 in the Krebs cycle (6, 7). During growth on acetate, ca. 70% of the IDH is maintained in the inactive, phosphorylated form, forcing isocitrate through the glyoxylate bypass (2, 8, 11, 13, 14). In most genetic backgrounds, phosphorylation of IDH appears to be essential for use of the glyoxylate bypass, since mutations which eliminate IDH kinase prevent growth on acetate (13).

The phosphorylation and dephosphorylation of IDH are catalyzed by a bifunctional protein, IDH kinase/phosphatase, expressed from the *aceK* gene (5, 9, 10). The IDH kinase and IDH phosphatase reactions appear to occur at the same active site, a conclusion supported by the observation that a mutation at the consensus ATP binding site eliminates both activities (18).

One of the approaches which we have taken to examine the structure of IDH kinase/phosphatase has been the random mutagenesis of *aceK*, the gene which encodes this protein. Our strategy has been to screen for alleles which have lost the ability to complement a null mutation in *aceK* and then assay the products of these candidate alleles for IDH kinase and IDH phosphatase activities. In a previous report (4), we described the isolation of two alleles of *aceK* (*aceK3* and *aceK4*) whose products retain IDH kinase activity but have suffered drastic reductions in their IDH phosphatase activities. We have subsequently isolated three additional alleles whose products have similar defects (Table 1). Four of these alleles (*aceK3*, *aceK4*, *aceK5*, and *aceK7*) were generated by replication of a plasmid bearing $aceK^+$ in the mutator strain W3550 (*mutD5*) (15). To increase the range of possible mutations, we have also employed chemical mutagenesis with hydroxylamine (16). This method resulted in the generation of an additional allele whose product has selectively lost IDH phosphatase activity, *aceK6*. In order to ensure that each allele had resulted from a single point mutation, we employed relatively mild conditions for mutagenesis. Replication in strain W3550 yielded 0.3% noncomplementing alleles, while treatment with hydroxylamine generated these alleles in only 0.1% of transformants.

The locations of the mutations were approximated by testing the abilities of fragments of $aceK^+$ to direct repair of these mutations (17). In this procedure, each mapping plasmid (which carries a fragment of $aceK^+$) was linearized by restriction digestion and then was mixed with a plasmid carrying a mutant allele of *aceK* which had been linearized by digestion at a different site. Circular heteroduplexes were formed between the linearized mutant and mapping plasmids by denaturation and gradual renaturation. The heteroduplex plasmids were selected, since they could transform recipient strains (because they were circular) while the original plasmids could not (because they were linear). The success of the repair process was tested by examining the abilities of the resulting clones to restore strain IL2 (Δ*aceK*) (18) to wild-type growth on acetate medium. All of the mutations fell within the region of *aceK* between nucleotides 1093 and 1492 relative to the start codon (Fig. 1).

Sequence analyses of the regions which had been delineated by the initial mapping procedure identified a single-base substitution in each of the mutant alleles (Table 2). The resulting amino acid substitutions all occur within a 113-amino-acid segment of IDH kinase/phosphatase, a protein which has a total of 578 residues (5).

While our mapping and sequencing results identified only one mutation in each allele, there was a possibility that other mutations remained undetected. To ensure that each of these mutations was uniquely responsible for the selective elimination of IDH phosphatase activity, we recloned restriction fragments containing the mutations into a wild-type allele. In

TABLE 1. Enzymatic activities of IDH kinase/phosphatase derivatives[a]

Allele	Enzyme activity (mU/mg)[b]	
	IDH kinase	IDH phosphatase
$aceK^+$	184	125
aceK3	297	0.5
aceK4	110	1.9
aceK5	174	0.0
aceK6	634	0.0
aceK7	507	0.5

[a] Restriction fragments containing the indicated mutations were recloned into $aceK^+$ plasmids to ensure that only a single mutation was present in each allele (see text). Data presented here, which were derived from the reconstructed alleles, were indistinguishable from those obtained with the original isolates.

[b] Assayed in sonicated extracts as described previously (10, 11).

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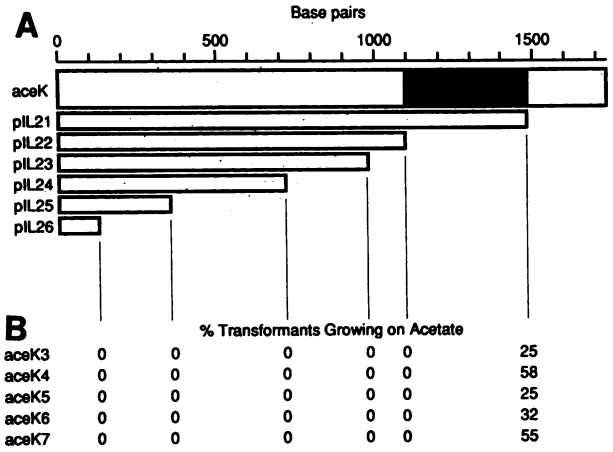

FIG. 1. Location of mutations by heteroduplex analysis. (A) The sequences from *aceK*$^+$ carried by each mapping plasmid are indicated with open boxes. The shaded area is the region of *aceK*$^+$ required for repair of all of the mutant alleles. (B) Results of the attempts to repair the point mutations using the fragments of *aceK*$^+$ carried by the mapping plasmids. The percentages of transformants capable of growth on acetate are indicated.

each case, the entire nucleotide sequence of the transferred fragments had been determined. Complementation testing (not shown) and assay for enzymatic activities (Table 1) of the resulting clones demonstrated that the mutations which had been identified were uniquely responsible for the observed effects.

Our working model for IDH kinase/phosphatase proposes that the kinase and phosphatase reactions occur within the same active site and that IDH phosphatase activity results from the kinase back reaction tightly coupled to ATP hydrolysis (for a review, see reference 12). If the IDH kinase and IDH phosphatase reactions occur at the same active site, then it seems unlikely that the mutations which selectively eliminate phosphatase activity are defining a domain of the protein which is unique to IDH phosphatase. Rather, these mutations may define a domain which participates in the regulation of the relative activities of IDH kinase and IDH phosphatase. Consistent with this possibility, the mutations in *aceK3* and *aceK4* have substantially altered the effector sensitivities of the residual IDH phosphatase activities of their products: the activation constants for 3-phosphoglycerate, pyruvate, and AMP have been increased from 10- to >100-fold, while the relative activation has increased from 5- to >10-fold compared with that of the wild-type protein (4a).

Although the identified mutations may define a regulatory domain, the selective inhibitions of IDH phosphatase activity which they produce result from changes in the structure of the active site. These structural differences may affect any of a variety of kinetic processes, such as competition between the kinase, phosphatase, and ATPase activities of these proteins. While the mutations which we have identified have had similar effects on the kinetic behaviors of IDH kinase/phosphatase, their effects are not identical. For example, the IDH kinase activities of the products of *aceK6* and *aceK7* are much greater than those of the wild-type protein and the other three mutant proteins. Correlation of these structural, regulatory, and catalytic effects should provide considerable insight into the properties of the wild-type protein.

TABLE 2. Summary of mutations

Allele	Nucleotide substitution[a]	Amino acid substitution[b]
aceK3	A(1118)G	Q(373)R
aceK4	A(1241)G	Y(414)C
aceK5	G(1414)T	V(472)F
aceK6	G(1429)A	D(477)N
aceK7	C(1093)T	R(365)C

[a] The format used is N_1 (position) N_2, where N_1 and N_2 are the wild-type and substituted bases and the number in parentheses indicates the position relative to the first base of the initiation codon.

[b] The format used is A_1 (position) A_2, where A_1 and A_2 are the wild-type and substituted amino acid residues and the number in parentheses indicates the position relative to the N-terminal methionine in the primary translation product.

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