Isocitrate dehydrogenase (IDH) of Escherichia coli is regulated by a bifunctional protein, IDH kinase/phosphatase. In addition to the kinase and phosphatase activities, this protein catalyzes an intrinsic ATPase reaction. The initial velocity kinetics of these activities exhibited extensive similarities. IDH kinase and phosphatase both yielded intersecting double-reciprocal plots. In addition, we observed similar values for the kinetic constants describing interactions of the kinase and phosphatase with their protein substrates and the interactions of all three activities with ATP. In contrast, while the maximum velocities of IDH kinase and IDH phosphatase were nearly equal, they were 10-fold less than the maximum velocity of the ATPase. Although the IDH phosphatase reaction required either ATP or ADP, it was not supported by the nonhydrolyzable ATP analogue 5'-adenylylimidodiphosphate.

The kinetic properties of wild-type IDH kinase/phosphatase were compared with those of two mutant derivatives of this protein. The mutations in these proteins selectively inhibit IDH phosphatase activity. Inhibition of IDH phosphatase resulted from three factors: decreases in the maximum velocities, reduced affinities for phospho-IDH, and a loss of coupling between ATP and phospho-IDH. These mutations also affected the properties of IDH kinase, increasing the maximum velocities and decreasing the affinities for ATP and phospho-IDH. The intrinsic ATPase activities also exhibited reduced affinity for ATP. These results are discussed in the context of a model which proposes that all three activities occur at the same active site.

In Escherichia coli, the Krebs cycle enzyme isocitrate dehydrogenase (IDH)\(^1\) is regulated by phosphorylation (1, 2). The phosphorylation and dephosphorylation of IDH are catalyzed by a bifunctional protein, IDH kinase/phosphatase. IDH kinase/phosphatase is expressed from a single gene, aceK, and both activities reside on the same polypeptide (3–5). The phosphatase activity of this protein has an absolute requirement for ATP or ADP (5). IDH kinase/phosphatase also catalyzes an ATPase reaction. This ATPase activity occurs in the absence of the protein substrates and is only partially inhibited when these substrates are present (6).

One approach that we have taken to examine IDH kinase/phosphatase has been the random mutagenesis of aceK. We previously described the isolation of a class of mutant alleles whose products retain IDH kinase activity but have dramatically reduced IDH phosphatase activity. The products of these alleles have suffered single amino acid substitutions that fall within a 113-amino acid region of this 578-residue protein (7, 8).

The regulation of IDH kinase/phosphatase has been under study for nearly 15 years (reviewed in Ref. 9). In contrast, relatively little attention has been given to the enzymology of this unusual protein. This paper presents the results of an initial characterization of the kinetic properties of IDH kinase/phosphatase and of the products of two of the mutant derivatives: AceK3 (Gln\(^{373} \rightarrow \) Arg) and AceK4 (Tyr\(^{114} \rightarrow \) Cys) (7, 8).

**EXPERIMENTAL PROCEDURES**

Materials—[\(^{32P}\)]ATP was obtained from DuPont NEN and further purified by the method of Axelson et al. (10). Adenylylimidodiphosphate was purchased from Sigma and purified by the method of Olson et al. (7). All reagents were of the purest grades available.

Plasmid Construction—Plasmids pDL9, pDL25, and pDL30 express the aceK\(^{-}\), aceK3, and aceK4 genes, respectively, from the tac promoter (7). To maintain plasmid selection during high-density fermentation, these plasmids were constructed which contain the tet\(^{-}\) gene for selection. Briefly, the 1.6-kilobase Ssp-I-Aval fragment of plasmid pBR322 containing the tet\(^{-}\) gene was isolated, filled in with Klenow, and BglII linkers were attached. The resulting fragment was digested with BglII and then ligated into the BamHI site of pDL9, pDL25, and pDL30 generating the plasmids pEKL1, pEKL5, and pEKL3, respectively.

Protein Purification—IDH was overexpressed in E. coli strain Y1090 harboring the multicopy plasmid pTK509 which carries the gene encoding IDH (4). IDH was purified by a modification of the method of Garnack and Reeves (11).

Phospho-IDH was prepared in vitro using purified IDH and wild-type IDH kinase/phosphatase. Phospho-IDH was then separated from IDH kinase/phosphatase by chromatography on an Affi-Gel blue column (12). Wild-type IDH kinase/phosphatase was purified from E. coli strain ST2010R harboring the plasmid pEK1. The mutant AceK3 and AceK4 proteins were purified from the E. coli strain SL1R (AceK3::Kan\(^{+}\)) harboring either plasmid pEKL5 or pEKL3, respectively. All strains were grown at 37°C in L broth (1% tryptone, 1% NaCl, 0.5% yeast extract) with 40 \(\mu\)g/ml tetracycline in a 3.5-liter high-density fermentor. Expression of the aceK genes was induced with 3 \(\mu\)l isopropyl-\(\beta\)-D-thiogalactopyranoside during the mid-log phase of growth and then the strains were grown for an additional 3–4 h. The cells were harvested by centrifugation and stored at –80°C. The purification of IDH kinase/phosphatase has been described previously (5).

Protein concentrations were determined by the method of Lowry et al. (13) using bovine serum albumin as the standard.

Enzyme Activity Determinations—The activity of IDH phosphatase was measured by following the release of \(^{32P}\) phosphate from \(^{32P}\)-phospho-IDH as described previously (12). The standard reaction (50 \(\mu\)l) contained 25 mM MOPS (pH 7.5), 100 mM NaCl, 5 mM MgCl\(_2\), 0.5 mM ATP, 5 mM ATP, and 5 mM ADP.

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Kinetics of IDH Kinase/Phosphatase

Initial Velocity Kinetics of Wild-type IDH Phosphatase—Measurements of the initial velocity of wild-type IDH phosphatase produced intersecting patterns on double-reciprocal plots (Fig. 1A). Such plots are typical of a sequential mechanism (i.e. ordered or random). Sequential mechanisms can be described by Equation 1,

\[ v = \frac{V_{\text{max}}AB}{K_{A} + K_{B} + K_{A}B + K_{A}A + AB} \]  (Eq. 1)

where A and B are the substrate concentrations, v is the initial velocity, \( V_{\text{max}} \) is the maximum velocity, \( K_{A} \) is the apparent dissociation constant for A, and \( K_{B} \) and \( K_{AB} \) are the Michaelis constants for A and B, respectively (16). Estimates of the kinetic parameters were determined from Eq. 1 using the computer program SEQUENL developed by Cleland (17). The results are summarized in Table I. The apparent dissociation constant of wild-type IDH phosphatase for ATP (\( K_{A} \text{ATP} = 98 \mu M \)) was much greater than the corresponding Michaelis constant (6 \( \mu M \)). A similar relationship was observed for the kinetic constants for phospho-IDH (6 and 0.4 \( \mu M \), respectively). The ratio of these constants, \( \alpha = K_{m}/K_{p} \), provides a measure of the apparent substrate interaction, where \( \alpha = 1 \) indicates independent binding. The value of \( \alpha \) for wild-type IDH phosphatase suggests that substrate binding is coupled (i.e. the binding of one substrate enhances the binding of the other substrate).

Initial Velocity Kinetics of the Mutant IDH Phosphatases—Like wild-type IDH phosphatase, the residual IDH phosphatase of the AceK3 protein produced intersecting double-reciprocal plots (Fig. 1B). Similar patterns were observed with the AceK4 IDH phosphatase (data not shown). However, the single amino acid substitutions in the mutant proteins have produced striking changes in the kinetic properties of the residual IDH phosphatase activities.

The maximal velocities of both mutant IDH phosphatases were decreased relative to the wild-type enzyme. The \( V_{\text{max}} \) of the AceK3 phosphatase was decreased by a factor of 2.5, while the AceK4 phosphatase had suffered a 5-fold reduction in \( V_{\text{max}} \) (Table I).

The AceK3 and aceK4 mutations have also produced dramatic changes in the kinetic behavior of IDH phosphatase with respect to ATP (Table I). The \( K_{m} \text{ATP} \) of AceK3 IDH phosphatase increased 9-fold, while the value of \( K_{p} \text{ATP} \) decreased by a factor of 2 when compared with that of the wild-type enzyme. The AceK4 product exhibited increases in the \( K_{m} \text{ATP} \) and \( K_{p} \text{ATP} \) values of 20- and 3-fold, respectively.

The mutant IDH phosphatases also exhibited dramatic changes in the kinetic parameters for phospho-IDH (Table I). The \( K_{m} \text{IDH-P} \) and \( K_{p} \text{IDH-P} \) values of AceK3 IDH phosphatase were increased 52- and 3-fold, respectively, relative to those of the wild-type protein, while the AceK4 protein showed a 48-fold increase in \( K_{m} \text{IDH-P} \) and 7-fold increase in \( K_{p} \text{IDH-P} \).

The coupling between ATP and phospho-IDH that we observed for wild-type IDH phosphatase has also been affected by these mutations. The AceK4 protein exhibited a small residual coupling, while the AceK3 protein exhibited no coupling at all.

Initial Velocity Kinetics of the IDH Kinases—Like IDH phosphatase, wild-type IDH kinase exhibited intersecting patterns in double-reciprocal plots (Fig. 2A). We observed strong coupling between ATP and dephospho-IDH, which is also similar to the behavior of wild-type IDH phosphatase (Table I).

The initial velocity patterns of the AceK3 (Fig. 2B) and AceK4 (data not shown) IDH kinases resembled those of the wild-type kinase. The mutant kinases retained affinity for dephospho-IDH and coupling between this substrate and ATP.
The analyses of the kinetic characteristics of IDH kinase and IDH phosphatase. The maximum velocities of the kinases have increased, while those of the phosphatase have decreased. The kinase activities retained their affinity for the protein substrate (dephospho-IDH), while the residual phosphatase activities exhibited reduced affinity for its protein substrate (phospho-IDH). Finally, the AceK3 and AceK4 IDH kinases retained positive coupling between ATP and the protein substrate while their phosphatase activities did not.

Inhibition of Wild-type and Mutant IDH Kinase by Phospho-IDH—Since the aceK3 and aceK4 mutations alter the affinity of IDH phosphatase for phospho-IDH, we also examined the effect of these mutations on the affinity of IDH kinase for phospho-IDH. The affinities for phospho-IDH were estimated from product inhibition studies.

The bifunctional nature of wild-type IDH kinase/phosphatase posed inherent complications for analysis of the inhibition of IDH kinase by phospho-IDH. For example, the sequential dephosphorylation and rephosphorylation of the added phospho-IDH could have resulted in the incorporation of $^{32}$P into the phospho-IDH pool. To evaluate this source of interference, we performed IDH kinase assays in the absence of dephospho-IDH (Fig. 3A, solid circles). We observed a low level of $^{32}$P incorporation into protein, and this effect was saturated at the lowest concentration of phospho-IDH, which we employed. The precise mechanism responsible for $^{32}$P incorporation into IDH under these conditions is uncertain, but this effect must be considered when evaluating the ability of phospho-IDH to act as an inhibitor of IDH kinase.

Wild-type IDH kinase was inhibited by phospho-IDH (Fig. 3A, open circles). The precise value of the inhibition constant cannot be determined since $^{32}$P incorporation into the phospho-IDH pool contributed to the observed IDH kinase activity (see above). However, we were able to calculate a range for this constant by subtracting or ignoring the incorporation observed in the absence of dephospho-IDH (Fig. 3B and Table I). The range of this constant, 8–15 $\mu$M, is negligible compared with the effects that the aceK3 and aceK4 mutations had on the affinities of the mutant IDH kinases for phospho-IDH (see below).

We have also examined inhibition of the mutant IDH kinases by phospho-IDH. In contrast with wild-type IDH kinase/phosphatase, label was not incorporated into IDH in the absence of dephospho-IDH. (This was the expected result since the AceK3 and AceK4 proteins retain very little IDH phosphatase activity.) To aid the comparison of wild-type and mutant IDH kinases, we employed concentrations of ATP and dephospho-IDH, which were in the pseudo-first order range for all three enzymes. The mutant activities exhibited dramatic increases in the apparent $K_i$ values for phospho-IDH relative to the wild-type protein (Table I). These values were at least 20-fold higher for the AceK3 and AceK4 kinases. The effective $K_{i,phosphatase}$ of the IDH phosphatases were increased to similar extents at this concentration of ATP: 30-fold for AceK3 and 47-fold for AceK4.

**Fig. 2. Initial velocity patterns with wild-type and AceK3 IDH kinase.** The kinase assay was performed as described under "Experimental Procedures." $V_{max}$ were measured as a function of dephospho-IDH and MgATP concentrations. A, wild-type IDH kinase. Left panel, $V_{max}$ versus 1/[MgATP] at the following fixed concentrations: 0.5 ( ), 0.7 ( ), 1.2 ( ), and 4.9 ( ) $\mu$M. Right panel, $V_{max}$ versus 1/[dephospho-IDH] at the following fixed MgATP concentrations: 21 ( ), 31 ( ), 52 ( ), and 206 ( ) $\mu$M. B, AceK3 IDH kinase. Left panel, $V_{max}$ versus 1/[MgATP] at 0.5 ( ), 0.7 ( ), 1.2 ( ), and 4.9 ( ) $\mu$M dephospho-IDH. Right panel, $V_{max}$ versus 1/[dephospho-IDH] at 41 ( ), 61 ( ), 100 ( ), and 410 ( ) $\mu$M MgATP.

**Table I**

| Activity | Protein     | $V_{max}$ | $K_{m,ATP}$ | $K_{ATP}$ | $K_{m,phospho-IDH}$ | $K_{phospho-IDH}$ | $\alpha = K_i/K_m$ | $K_{IDH-phospho-IDH}$ |
|----------|-------------|-----------|-------------|-----------|---------------------|-------------------|-------------------|---------------------|
| Phosphatase | Wild-type | 60        | 6           | 98        | 0.4                 | 6                 | 17                |                     |
|          | AceK3      | 24        | 53          | 51        | 21                  | 20                | 1                 |                     |
|          | AceK4      | 12        | 120         | 270       | 19                  | 40                | 2                 |                     |
| Kinase   | Wild-type  | 90        | 16          | 170       | 0.23                | 2.4               | 11                | 8–15                |
|          | AceK3      | 450       | 360         | 580       | 0.25                | 1.4               | 6                 | >100                |
|          | AceK4      | 500       | 320         | 5300      | 0.23                | 4.2               | 17                | >100                |
| ATPase   | Wild-type  | 590       | 230         | 580       | 0.25                | 1.4               | 6                 | >100                |
|          | AceK3      | 710       | 770         | 200       | 0.25                | 1.4               | 6                 | >100                |
|          | AceK4      | 650       | 2900        |           |                     |                   |                   |                     |

$^a$ The values are given in terms of nmol/mg/min.

$^b$ $K_m$ = Michaelis constant. The values are given in $\mu$M concentrations.

$^c$ $K_i$ = apparent dissociation constant. The values are given in $\mu$M concentrations.

$^d$ $K_i$ = effective inhibition constant; IDH-P = phospho-IDH. The data were obtained using 100 $\mu$M ATP and 0.5 $\mu$M IDH. The values are given in $\mu$M concentrations.
In addition to the IDH kinase and IDH phosphatase activities, although the great exceeded the corresponding values for the kinase and mutant ATPase activities were estimated from double reciprocal plots of product inhibition of IDH kinase activity by phospho-IDH. The assay was performed as described under “Experimental Procedures” using 100 μM MgATP and the indicated concentration of phospho-IDH. The assays were performed in the presence (●) and absence (○) of 0.5 μM dephospho-IDH. B, Dixon plots of product inhibition of wild-type and mutant IDH kinase activity by phospho-IDH. The kinetic assay was performed as described under “Experimental Procedures” using 100 μM MgATP and 0.5 μM IDH. Inhibition of the wild-type (●), AceK3 (○), and AceK4 (□) IDH kinase activities was measured as a function of varied phospho-IDH concentration. Parallel assays were performed without dephospho-IDH for the wild-type enzyme, and the observed 32P incorporation was subtracted from that observed with dephospho-IDH.

Initial Velocity Kinetics of the Wild-type and Mutant ATPase Activities—In addition to the IDH kinase and IDH phosphatase reactions, IDH kinase/phosphatase exhibits an intrinsic ATPase activity (6). The kinetic constants of the wild-type and mutant ATPase activities were estimated from double reciprocal plots (Table I). The maximum velocities of all three proteins greatly exceeded the corresponding values for the kinase and phosphatase activities. Although the Vmax values of the mutant and wild-type proteins were similar, the Km values of the AceK3 and AceK4 ATPase activities are increased 3- and 12-fold, respectively.

A Nonhydrolyzable ATP Analogue Does Not Support the IDH Phosphatase Reaction—IDH phosphatase exhibits an intrinsic ATPase activity. The model explains the unusual nucleotide requirement of IDH phosphatase: this activity has an absolute dependence on either ATP or ADP (5). Our working model proposes that the actual substrate for IDH phosphatase is ADP but that ATP can be converted to ADP by the ATPase activity (see “Discussion”). To test this hypothesis, we examined the ability of a nonhydrolyzable analogue of ATP, 5’-adenylyl imidodiphosphate (AMPP(NH)P), to support the wild-type IDH phosphatase reaction. This nucleotide clearly binds to IDH phosphatase, since it inhibited this reaction in the presence of ATP (Table II). However, in the absence of ATP, AMPP(NH)P did not support IDH phosphatase activity. This observation is consistent with the proposal that ATP must be converted to ADP before it can participate in the IDH phosphatase reaction.

DISCUSSION

IDH kinase/phosphatase possesses three activities: IDH kinase, IDH phosphatase, and an ATPase. The initial velocity behaviors of these activities were similar for the wild-type protein. For example, the apparent dissociation constants for the protein substrates for IDH kinase (Km) and IDH phosphatase (Km) were 2.4 and 6 μM. The apparent dissociation constant for ATP of the kinase and phosphatase and the Km of the ATPase activity were also similar. The kinase and phosphatase exhibited strong coupling (α) between ATP and the protein substrates. In contrast, although the maximum velocities of IDH kinase and phosphatase were nearly identical, the Vmax of the ATPase was about an order of magnitude greater. The significance of this difference is discussed below.

We previously reported the isolation of mutant derivatives of IDH kinase/phosphatase (7, 8). These proteins retain IDH kinase activity but had suffered striking reductions in IDH phosphatase activity. Our kinetic analyses provide insights into the mechanism responsible for this selective loss of IDH phosphatase activity of two of these proteins: AceK3 (Gln373 → Arg) and AceK4 (Tyr414 → Cys). Three factors have contributed to this loss: decreases in the maximum velocities, reduced affinities for the protein substrate (phospho-IDH), and a loss of coupling between ATP and phospho-IDH. These proteins retained IDH kinase activity because binding of dephospho-IDH and coupling between ATP and dephospho-IDH were unaffected. In addition, the decrease in the affinity of these kinase activities for ATP was offset by an increase in the maximum velocities.

We have developed a working model that describes IDH kinase/phosphatase. This model proposes that the kinase, phosphatase, and ATPase reactions occur in the same active site. This active site can exist in two conformations, one favoring IDH kinase and the other favoring IDH phosphatase. The phosphatase reaction results from the kinase back reaction tightly coupled to the ATPase reaction (Fig. 4). Although this model is speculative, it is supported by a variety of observations.

The model explains the unusual nucleotide requirement of IDH phosphatase: this activity has an absolute dependence on either ATP or ADP. In contrast, this activity is not supported by a nonhydrolyzable analogue of ATP. Our model proposes that ADP acts as a transient phosphate acceptor. A similar role is played by the active site serine of E. coli alkaline phosphatase (18).

This model also explains the unusual ATPase activity of IDH kinase/phosphatase. Weak ATPase activities are often associated with kinases in the absence of the normal phosphate acceptor. In contrast, the Vmax of the ATPase activity of IDH kinase/phosphatase is almost an order of magnitude greater than that of either the kinase or phosphatase (Table I). Fur-
Furthermore, while the ATPase activities of other kinases are completely inhibited by the normal phosphate acceptor, the ATPase of IDH kinase/phosphatase is only partially inhibited by either dephospho- or phospho-IDH (6).

A number of observations support the existence of a single ATP binding site for IDH kinase/phosphatase. The sequence of IDH kinase/phosphatase includes a single region that matches the consensus for ATP binding sites (19). Mutation of the “Invariant” lysine of this motif eliminated both IDH kinase and IDH phosphatase without destabilizing the protein (13). The results of affinity labeling experiments are also consistent with a single ATP binding site: azido-ATP produced parallel inhibition of IDH kinase and IDH phosphatase yet labeled a single peptide (20).

Our working model provides a framework for interpretation of the kinetic characteristics of the mutant proteins. We suspect that the mutations in these proteins have shifted the conformational equilibrium toward the kinase conformation at the expense of IDH phosphatase. However, it should be noted that this is not the only model that could explain the results presented in this paper.

The IDH kinase and IDH phosphatase activities of the mutant protein exhibited reciprocal changes in their maximum velocities: the $V_{\text{max}}$ of the kinase was increased while that of the phosphatase was decreased. This might have resulted from a shift in the conformational equilibrium toward the kinase form.

The IDH kinase and phosphatase activities exhibited a parallel loss of affinity for phospho-IDH. This observation tends to favor a single binding site for phospho-IDH, a feature of our model.

Effects of these mutations on the $K_{\text{ATP}}$ values for IDH kinase and IDH phosphatase and on the $K_{\text{p,ATP}}$ value for the ATPase are more ambiguous. In the simplest case, these values should be roughly equal if all three activities occur at the same active site. These values were similar for the wild-type protein, consistent with the evidence that favors a single ATP binding site (see above). However, while the values for the kinase and ATPase agreed for the mutant proteins, the value of $K_{\text{ATP}}$ for the IDH phosphatase activities did not. If our model is correct, this discrepancy might reflect differences in the properties of the kinase and phosphatase conformations of the mutant proteins. However, this issue remains to be resolved.

Why would IDH kinase/phosphatase catalyze the phosphorylation and dephosphorylation of IDH at the same active site? Such an arrangement would allow these reactions to employ some of the same catalytic groups. According to our model, IDH kinase/phosphatase employs the kinase back reaction as part of the IDH phosphatase reaction. In addition, the protein substrates of IDH kinase and phosphatase are nearly identical, differing only in the phosphorylation of Ser113. Catalysis of both reactions at the same site would allow many of the same amino acids to be used for substrate recognition. The ultimate resolution of these issues will require the crystal structure of IDH kinase/phosphatase complexed with its substrates.

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