Isocitrate Binding at Two Functionally Distinct Sites in Yeast NAD\(^+\)-specific Isocitrate Dehydrogenase*

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Yeast NAD\(^+\)-specific isocitrate dehydrogenase (IDH) is an octamer containing two types of homologous subunits. Ligand-binding analyses were conducted to examine effects of residue changes in putative catalytic and regulatory isocitrate-binding sites respectively contained in IDH2 and IDH1 subunits. Replacement of homologous serine residues in either subunit site, S98A in IDH2 or S92A in IDH1, was found to reduce by half the total number of holoenzyme isocitrate-binding sites, confirming a correlation between detrimental effects on isocitrate binding and respective kinetic defects in catalysis and allosteric activation by AMP. Replacement of both serine residues eliminates isocitrate binding and measurable catalytic activity. The putative isocitrate-binding sites of IDH1 and IDH2 contain five identical and four nonidentical residues. Reciprocal replacement of the four nonidentical residues in either or both subunits (A108R, F136Y, T241D, and D252N in IDH1 and/or R114A, Y142F, D248T, and D252N in IDH2) was found to be permissive for isocitrate binding. This provides further evidence for two types of binding sites in IDH, although the authentic residues have been shown to be necessary for normal kinetic contributions. Finally, the mutant enzymes with residue replacements in the IDH1 site were found to be unable to bind AMP, suggesting that allosteric activation is dependent both upon binding of isocitrate at the IDH1 site and upon the changes in the enzyme normally elicited by this binding.

Mitochondrial NAD\(^+\)-specific isocitrate dehydrogenase catalyzes a key regulatory step in the tricarboxylic acid cycle in eucaryotic cells. Complex allosteric responses include activation of the mammalian enzyme by ADP and of the Saccharomyces cerevisiae enzyme by AMP (1–3). Yeast NAD\(^+\)-specific isocitrate dehydrogenase (IDH)\(^{3}\) was the focus of detailed kinetic and ligand-binding analyses by Atkinson and co-workers (2–5), who proposed that this enzyme regulates metabolic flux in response to relative cellular levels of adenine nucleotides, expressed as \([\text{ATP}] + 0.5 \times [\text{ADP}] + [\text{AMP}]\). Barnes et al. (4) purified IDH and reported that the holoenzyme is composed of eight apparently identical subunits (molecular weight \(\approx 39,000\)). Kinetic and ligand-binding analyses (4, 5) suggested significant cooperativity in subunit interactions and complex interdependent interactions of the enzyme with various ligands. Equilibrium dialysis experiments (6) provided evidence for four isocitrate-binding sites but for only two binding sites each for the other ligands required for catalysis, Mg\(^{2+}\) and NAD\(^+\), and for two binding sites for the allosteric activator AMP. These and other results led to the proposal that the enzyme contains both catalytic (isocitrate/Mg\(^{2+}\)) and noncatalytic isocitrate-binding sites.

More recently, we and others (6, 7) provided evidence that IDH is an octamer composed equally of two different types of subunits, and our subsequent cloning of the yeast genes (8, 9) confirmed that the enzyme contains two subunits, IDH1 and IDH2, that are similar in size (respective molecular weights of 38,001 and 37,755) and sequence (42% residue identity). The mammalian enzyme is also an octamer but is composed of three subunits, in a ratio of \(\alpha_2\beta_2\gamma_2\), that are similarly related to each other and to the yeast enzyme subunits (10–12). Yeast IDH1 and IDH2 also share \(\sim 32\%\) sequence identity with Escherichia coli isocitrate dehydrogenase (13), a nonallosteric homodimeric enzyme that uses NADP\(^+\) as a cofactor (14, 15).

Sequence comparisons with the bacterial enzyme, which has been analyzed in great detail in crystallographic studies (16–19), led to the proposal that both yeast subunits could contain isocitrate-binding sites. All nine of the residues implicated as essential for isocitrate/Mg\(^{2+}\) binding in the catalytic site of the bacterial enzyme (18) are contained in the putative site of IDH2, whereas only five of the nine residues are conserved in the putative site of IDH1. This suggested that the IDH2 site may support catalysis, whereas the IDH1 site may bind isocitrate for purposes other than catalysis.

We have previously examined and defined different functions for IDH1 and IDH2 using targeted mutagenesis and kinetic analyses. Initial studies (20) focused on serine residues in the putative isocitrate-binding sites of each subunit that are apparent homologues of the E. coli active site Ser-113 residue. This residue is the site for phosphorylation and inactivation of the bacterial enzyme (13), a reversible regulatory mechanism for controlling relative rates of flux through the tricarboxylic acid and glyoxylate cycles in vivo (21–23). Replacement of the analogous serine residue (S98A) of yeast IDH2 resulted in a dramatic decrease in \(V_{\text{max}}\) but had little effect on cooperativity with respect to isocitrate or on allosteric activation by AMP, whereas replacement of the serine residue (S92A) of IDH1 produced dramatic defects in these regulatory properties but much less of an effect on catalytic activity. These results were consistent with differential function of the subunits and suggested that the residue replacements detrimentally affect isocitrate binding at each site. The current study addresses isocitrate-binding properties of these mutant enzymes and of a mutant enzyme containing substitutions in both subunit sites. Consistent with a model of isocitrate/Mg\(^{2+}\) binding for catalysis.

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Isocitrate-binding Sites of Yeast IDH

by IDH2 and of isocitrate binding for regulation by IDH1, results of other mutagenesis and kinetic studies (24) suggested that the binding site for NAD\(^+\) is primarily contained in IDH2 and that the analogous nucleotide-binding site in IDH1 binds AMP.

Another study of the putative isocitrate-binding sites (25) examined effects of replacement of the four nonidentical residues of nine in each IDH subunit site with the corresponding residues from the other subunit site (A108R, F136Y, T241D, and N245D in IDH1 or R114A, Y142F, D248T, and D252N in IDH2). These changes were designed to disrupt the normal kinetic results of isocitrate binding by each site but to be potentially permissive for isocitrate binding per se (26). The four residue replacements in IDH2, as expected, reduced \(V_{\text{max}}\) by \( \geq 150\)-fold, but the mutant enzyme retained kinetic cooperativity with respect to isocitrate and allosteric activation by AMP. The four residue replacements in IDH1 reduced \(V_{\text{max}}\) by 17-fold, and the mutant enzyme displayed no cooperativity or allosteric activation. These results are consistent with the presumed functions of the two subunits. However, actual effects on isocitrate binding were not assessed prior to the current study, in which we also examine kinetic and isocitrate-binding properties of a mutant enzyme containing the four reciprocal residue substitutions in both subunits.

Despite the apparent differences in primary contributions of each subunit, it is also clear that interactions between IDH1 and IDH2 are essential for holoenzyme structure and function. Both subunits are required for catalytic activity in vivo, because yeast mutants lacking either or both subunits exhibit the same growth phenotypes (8, 9, 25), including an inability to grow with acetate as a carbon source. Both subunits are required for oligomeric structure, because the independent subunits expressed in yeast appear to be monomers (20). Two-hybrid assays also indicate strong heteromeric but not homomeric interactions between subunits (27). Finally, based on the three-dimensional model for the active site of the homodimeric E. coli enzyme (18), mutagenesis was used to show that of the nine residues in the putative isocitrate-binding sites of each IDH subunit, two are contributed by the other subunit (27). Thus, within the octameric enzyme, the basic structural/functional unit appears to be a heterodimer of IDH1 and IDH2 subunits. However, as discussed in more detail below, a simple model with a core heterodimer containing one catalytic subunit and one regulatory subunit is inadequate because it leads to a prediction of twice the number of ligand-binding sites than are actually measured for the octameric holoenzyme.

The current study assesses the isocitrate-binding properties of mutant forms of IDH to test hypotheses developed from previous kinetic evaluations. In addition, we examine a mechanism proposed by Kuehn et al. (5) for allosteric activation of IDH. Based on their inability to detect binding of AMP by IDH in the absence of isocitrate, they speculated that binding of the allosteric activator requires that isocitrate be bound by a non-catalytic site of the enzyme. The availability of mutant enzymes with defects in the putative noncatalytic site in IDH1 provides a direct experimental test of this proposal.

**EXPERIMENTAL PROCEDURES**

*Host Yeast Strain and Plasmid Constructions—*Wild-type and mutant forms of IDH were expressed in a yeast strain, IDH1Δ21 (MATa ade2–1 can1–100 his3–11,15 leu2–3,112 trpl–1 ura3–1 Δidh1::LEU2 Δidh2::HIS3), containing deletion/disruption mutations in chromosomal IDH1 and IDH2 loci (28). Transformants of this strain were selected and maintained on agar plates containing YNB medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, pH 6.5) with 2% glucose and nutrient supplements as necessary for growth and selection.

Codon changes in yeast IDH1 or IDH2 genes to produce the mutant enzymes used in this study were introduced by site-directed mutagenesis as previously described (20, 26). The codon changes were designed to replace single (S92A) or multiple residues (A108R, F136Y, T241D, and N245D) of IDH1 and single (S98A) or multiple residues (R114A, Y142F, D248T, and D252N) of IDH2. For expression in previous studies of centromere-based plasmids, the mutant sites carrying both IDH1 and IDH2 genes were used, with one of the genes containing coding region mutations and the other gene containing a pentahistidine codon tag at the 3' end of the coding region. For current experiments, subcloning was used to construct plasmids containing paired mutations in both IDH1 and IDH2 genes, and site-directed mutagenesis was used when necessary to introduce additional residue changes. The current studies required high levels of enzyme expression, subcloning was used to transfer 6.4-kbp Sph/HindIII DNA fragments containing desired pairs of genes into a multicopy 2-μm based plasmid (pRS426; Ref. 29) containing the yeast UR3 gene for selection. Independent plasmids in the resulting collection encode the wild-type enzyme (IDH1/Wild/IDH2) and each of the following mutant enzymes: IDH1/S92A/IDH2/S98A, IDH1/S92A,R114A/Y142F,D248T,D252N/IDH2/S98A, IDH1/S92A, R114A,Y142F,D248T,D252N, and IDH1/S92A,R114A,Y142F,D248T,D252N/IDH2/R114A,Y142F,D248T,D252N. Yeast transformations were conducted using a lithium acetate protocol (30).

*Enzyme Expression and Purification—*For purification of wild-type and mutant forms of IDH, transformant colonies were recovered from plates and grown for 16 h in 5 ml of YP medium (2% Bacto-peptone) containing 2% glucose, then transferred and grown for 24 h in 100 ml of YNB glucose medium lacking uracil to select for plasmid replication. These cultures were used to inoculate 1-liter cultures of YP medium containing 2% ethanol as the carbon source to stimulate cell growth and to maximize levels of IDH expression (31). After growth for 36 h at 30 °C, cells were harvested for enzyme purification. From 1–2 liters of cultures for kinetic analyses or from 6 liters of cultures for ligand-binding analyses, cell pellets were resuspended (1 ml/5 g cells) in a buffer containing 50 mM potassium phosphate, pH 7.0, 5 mM potassium citrate, and 50% glycerol, frozen by dripping into liquid nitrogen, and stored at −70 °C. Preparation of cell extracts by breaking with glass beads and affinity purification using Ni\(^{2+}\)-nitritroacetic acid chromatography were conducted as previously described (24). Yields were 2.5–3.3 mg of purified enzyme/8 g of cells/liter of culture. The purified enzymes were stored at 4 °C in affinity column elution buffer (50 mM sodium phosphate, pH 7.5, 300 mM NaCl, and 200 mM imidazole) prior to kinetic or ligand-binding assays, which were performed within 24 h following purification. Concentrations of purified enzymes were calculated from absorbance measurements made at 280 nm using a molar extinction coefficient of 168,820 M\(^{-1}\)cm\(^{-1}\) (32). Purities of >95% were assessed following gel electrophoresis and staining with Coomassie Blue. Mutant enzymes in this study examined by high performance liquid chromatography (25) and by sedimentation velocity ultracentrifugation\(^2\) exhibit elution and sedimentation properties characteristic of the wild-type enzyme.

*Kinetic and Ligand-binding Assays—*IDH activity was routinely measured using assays containing 40 mM Tris-HCl, pH 7.4, 4 mM MgCl\(_2\), 0.5 mM NAD\(^+\), and 2.0 mM DL-isocitrate. The concentration of DL-isocitrate was calculated as 50% of the total concentration of DL-isocitrate. For measurement of some kinetic parameters, n-isocitrate concentrations ranged from 0 to 2.0 mM and AMP was added to 100 μM. Other variations in assay conditions are described in the text. A unit of activity is defined as production of 1 nmol of NADH/min at 24 °C.

Procedures for ligand-binding assays were adapted for IDH from an ultrafiltration method described by Dean et al. (33). We have previously found wild-type IDH to be stable, with respect to retention of kinetic and allosteric properties, when stored for several weeks at 4 °C in affinity column elution buffer. To avoid removal of purified enzymes from this buffer, which contains high concentrations of NaCl and imidazole, we empirically determined conditions for concentration of the purified enzyme in this buffer followed by direct dilution into a binding assay buffer more compatible with kinetic assay conditions. Specifically, the purified enzymes were concentrated in affinity column elution buffer by ultrafiltration (Centricon YM-50, Amicon) to obtain concentrations of ~10 mg/ml. Aliquots of 100 μl of concentrated enzyme were added to 200 μl of binding buffer containing 100 mM NaCl and the test ligand in the top chambers of Centricon YM-50 columns (Amicon). The enzyme/ligand assay mixes were incubated for 10 min at room temperature, then centrifuged briefly (45 s at 4000 rpm in a Beckman SS34 rotor at room temperature) to obtain ~125 μl of ultrafiltration concentrate.
filtrations in the lower chambers of the columns. No enzyme was detectable in these ultrafiltrates using activity or protein assays. Parallel assays at each ligand concentration were conducted with columns loaded with 100 μl of affinity column elution buffer and 900 μl of binding buffer. For isocitrate ligand-binding assays, the binding buffer contained 40 mM Tris-HCl, pH 7.4, 4 mM MgCl₂, and d-isocitrate concentrations ranging from 0 to 1.0 mM. These assays were conducted in the absence or in the presence of 100 μM AMP. For AMP ligand-binding assays, the buffer contained 40 mM Tris-HCl, pH 7.4, 4 mM MgCl₂, 1.0 mM d-isocitrate, and AMP concentrations ranging from 0 to 0.4 mM.

Concentrations of isocitrate in ultrafiltrates were measured enzymatically using IDH and calculated using a standard curve of activity versus known isocitrate concentrations measured on the same day. AMP concentrations in ultrafiltrates were measured spectrophotometrically (absorbance at 260 nm) by comparison with a standard curve derived with known concentrations of AMP dissolved in affinity column buffer and diluted 1:10 in the appropriate binding buffer. The concentration of bound ligand was determined by subtracting the concentration of ligand in the ultrafiltrate of the assay containing enzyme from the concentration in the paired ultrafiltrate of the assay lacking enzyme. Binding is expressed as moles of bound ligand/mol of IDH holoenzyme (molecular weight = 303,024), and each value represents an average from two independent experimental determinations.

RESULTS

Isocitrate-binding Properties of IDH—To assess ligand-binding properties of wild-type and mutant forms of IDH, we adapted an ultrafiltration method used by Dean et al. (33) for analysis of E. coli isocitrate dehydrogenase. Conditions for ligand binding were empirically developed using affinity-purified wild-type IDH as described under “Experimental Procedures.” The results are reproducible with different preparations of the enzyme and are compatible with those previously obtained by Kuehn et al. (5) using the conventionally purified enzyme in equilibrium binding analyses.

Analysis of isocitrate binding by the wild-type enzyme was conducted in the absence or presence of 100 μM AMP, the allosteric activator of IDH, to compare effects on S₀.₅ values with those obtained in kinetic analyses. The binding assays were conducted with d-isocitrate concentrations ranging from 0 to 1.0 mM and with 1.0 mg of affinity-purified enzyme/assay. Mg²⁺ was included in the binding assay buffer at the same concentration used in kinetic assays, whereas NAD⁺ was omitted to prevent catalysis. The amount of isocitrate bound at each concentration (mole/mole of IDH) was calculated after measuring the concentration of free isocitrate in the ultrafiltrate using enzyme assays as described under “Experimental Procedures.” As illustrated in Fig. 1, the isocitrate-binding curves obtained for IDH (panel A) are quite similar to the velocity saturation curves obtained with kinetic assays (panel B). Both types of analyses demonstrate the dramatic positive effect of AMP on the affinity of IDH for isocitrate. The ligand-binding assays indicate four binding sites for isocitrate/holoenzyme as previously reported (5).

The parameters obtained from kinetic and ligand-binding assays of IDH are compared in Table I. The S₀.₅ values for isocitrate measured in the absence of AMP are essentially identical with both methods, but, in the presence of AMP, a slightly higher S₀.₅ value is obtained with binding assays (0.15 mM) than with kinetic assays (0.09 mM). Thus, AMP reduces the isocitrate S₀.₅ value by ~5-fold in these kinetic analyses and by ~3-fold in these binding analyses. Hill coefficients obtained with both methods are quite similar, with values of ~4 in the absence or presence of AMP. In Table I, parameters obtained in current studies with the affinity-purified enzyme are compared with those obtained in previous studies with the conventionally purified enzyme. The previous kinetic analyses by Barnes et al. (4) produced lower S₀.₅ values for isocitrate and a differential of 16-fold for the AMP effect. The previously reported values for kinetic cooperativity with respect to isocitrate were lower than the current values, suggesting that current rapid purification techniques may facilitate retention of this allosteric property. Results of the previous equilibrium binding analysis by Kuehn et al. (5) for IDH binding of isocitrate in the presence of AMP are similar to ours with respect to number of binding sites and Hill coefficients, but they reported a lower S₀.₅ value. Availability of sufficient purified enzyme precluded their measurements of isocitrate binding in the absence of AMP. These differences in values for parameters of IDH for isocitrate are minor, given the significant differences in methods for purification and for kinetic and ligand-binding assays.

As an additional control for isocitrate-binding analyses, we purified and analyzed yeast mitochondrial NADP⁺-specific isocitrate dehydrogenase (IDP1). In addition to cofactor specificity, this enzyme differs from IDH in that IDP1 is a homodimer with no known allosteric properties (31). As illustrated in Table I, we find with kinetic and binding analyses that IDP1 has an affinity for isocitrate similar to that of IDH measured in the presence of AMP. Additionally, IDP1 has two binding sites for isocitrate and there is no indication of cooperativity in binding from either kinetic or ligand-binding assays. Both properties are consistent with predictions based on the structure of this and related enzymes (14, 31). These results indicate that our analyses can discriminate differences in the number of and degree of cooperativity between isocitrate-binding sites.

Isocitrate Binding by Mutant Forms of IDH—As described above, we have previously constructed and kinetically analyzed mutant forms of IDH with targeted residue changes in the presumed sites for catalytic binding of isocitrate/Mg²⁺ in IDH2.

V. Contreras, K. I. Minard, and L. McAlister-Henn, unpublished observations.
Isocitrate-binding Sites of Yeast IDH

Table I

<table>
<thead>
<tr>
<th>Enzyme</th>
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<th>Binding assays</th>
<th>Kinetic assays</th>
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<tr>
<td></td>
<td>~AMP/~AMP</td>
<td>S&lt;sub&gt;0.5&lt;/sub&gt;</td>
<td>Hill coefficient</td>
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<tr>
<td>IDH1&lt;sup&gt;His&lt;/sup&gt;/IDH2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>3.9/3.8</td>
<td>0.52/0.15</td>
<td>4.4/3.6</td>
</tr>
<tr>
<td>IDH1/IDH2&lt;sup&gt;His&lt;/sup&gt;</td>
<td>/3.9</td>
<td>/0.05</td>
<td>/3.7</td>
</tr>
<tr>
<td>IDP&lt;sup&gt;1His&lt;/sup&gt;</td>
<td>2.2</td>
<td>0.06</td>
<td>1.1</td>
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</tbody>
</table>

*For affinity-purified IDH, parameters for ligand binding are averages of two or three independent determinations, and kinetic parameters were previously reported (25). Binding and kinetic assays were conducted in the absence or presence of 100 μM AMP.

* Data for the conventionally purified enzyme are from equilibrium binding studies reported by Kuehn et al. (5) and from kinetic analyses reported by Barnes et al. (4). Isocitrate-binding assays were conducted in the presence of 1 mM AMP, and kinetic assays were conducted in the absence or presence of 1 mM AMP.

* Isocitrate-binding and kinetic data were obtained using an affinity-purified form of yeast mitochondrial NADP<sup>+</sup>-specific isocitrate dehydrogenase (IDP1). Isocitrate assays were conducted with 50 mM potassium phosphate (pH 7.75), 5 mM MgCl<sub>2</sub>, 0.5 mM NADP<sup>+</sup>, and n-isocitrate concentrations ranging from 0 to 0.5 mM.

and for regulatory binding of isocitrate in IDH1. For analysis of effects on ligand binding, we have focused on two pairs of mutant enzymes predicted to be the most informative. As illustrated in Fig. 2, the predicted isocitrate-binding sites of IDH2 and of IDH1 differ in four residue positions (indicated by rectangles). Each mutant enzyme in the first pair (IDH1<sup>His</sup>/IDH2<sup>2His</sup>/IDH1<sup>A108R</sup>/F136Y/T241D/N245D/IDH2<sup>His</sup>) contains replacements of these four residues in one subunit with the corresponding residues located in the other subunit site. This pair was originally designed to potentially retain the ability to bind isocitrate but to eliminate the normal functional responses elicited by isocitrate binding in each subunit site (26). For current studies, we also constructed and expressed a mutant form of IDH (IDH1<sup>A108R</sup>/F136Y/T241D/N245D/IDH2<sup>S98A/His</sup>) containing the reciprocal residue replacements in both subunits. In the second pair of mutant enzymes (IDH1<sup>His</sup>/IDH2<sup>His</sup>/IDH1<sup>S92A</sup>/IDH2<sup>His</sup>), each contains an alanine substitution for the “active site” serine residue (indicated by ovals in Fig. 2). This pair was originally designed to discern subunit function by interfering with isocitrate binding at each site (20), based on the function of a homologous serine residue in E. coli isocitrate dehydrogenase catalytic site (33). For current ligand-binding analyses, we also constructed a mutant form of IDH (IDH1<sup>A108R</sup>/F136Y/T241D/N245D/IDH2<sup>R114A/Y142F/D248T/D252N/His</sup>) containing alanine replacements for active site serine residues in both subunits.

Both sets of three mutant enzymes were expressed using multicyclic plasmids in a ΔIDH1ΔIDH2 yeast strain as described under “Experimental Procedures.” The enzymes were affinity-purified based on the presence of a pentahistidine tag on the carboxyl terminus of one of the two subunits. Enzyme activity was assessed to confirm that kinetic parameters corresponded with those previously reported for the mutant enzymes containing residue substitutions in single subunits (20, 25) and to analyze the parameters of mutant enzymes containing residue substitutions in both subunits. The parameters for isocitrate binding in the absence or in the presence of AMP were then determined for each of the mutant enzymes using the ultrafiltration method described above.

As illustrated in Fig. 3, isocitrate binding was measurable for all of the mutant enzymes except the IDH1<sup>S92A</sup>/IDH2<sup>His</sup> enzyme (panel F). In terms of the number of binding sites estimated at saturation, the results confirm fundamental predictions about substitutions in the putative sites in IDH1 and IDH2, e.g., the panels on the left in Fig. 3 indicate that mutant enzymes containing reciprocal active site residue substitutions (IDH1<sup>A108R</sup>/F136Y/T241D/N245D/IDH2<sup>His</sup> in panel A, IDH1<sup>His</sup>/IDH2<sup>R114A/Y142F/D248T/D252N</sup> in panel B, and IDH1<sup>A108R</sup>/F136Y/T241D/N245D/IDH2<sup>2His</sup> in panel C) all retain the capacity for binding 4 mol of holoenzyme as was demonstrated for the wild-type enzyme. Thus, replacement of four residues in the IDH2 site with the residues that differ in the IDH1 site, and/or replacement of the four residues in the IDH1 site with the residues that differ in the IDH2 site, appears to be fully permissive for isocitrate binding. In contrast, saturation curves for mutant enzymes containing active site serine residue replacements (panels on the right in Fig. 3) indicate that the total number of isocitrate-binding sites is reduced to two per holoenzyme by the S92A replacement in IDH1 (panel D) or by the S98A replacement in IDH2 (panel E). Binding of isocitrate by the mutant enzyme containing both serine residue replacements (panel F) is essentially eliminated.

Also obvious from the plots in Fig. 3 are differences among mutant enzymes with respect to the effects of AMP upon isocitrate binding. The mutant enzymes containing residue replacements in the IDH1 site (IDH1<sup>A108R</sup>/F136Y/T241D/N245D/IDH2<sup>His</sup>) in panel A and IDH1<sup>S92A</sup>/IDH2<sup>2His</sup> in panel D) are unresponsive to AMP, i.e. the isocitrate-binding curves are essentially identical for assays conducted in the absence or in the presence of AMP. As expected, the mutant enzyme containing reciprocal residue replacements in both active sites (IDH1<sup>A108R</sup>/F136Y/T241D/N245D/IDH2<sup>2His</sup>) in panel C) is also unresponsive to AMP. These results suggest that binding of isocitrate by IDH1 (blocked by the S92A residue replacement) is necessary for positive allosteric response to AMP and that, in addition to the binding of isocitrate at the IDH1 site, which is apparently unaffected in the IDH1<sup>A108R</sup>/F136Y/T241D/N245D/IDH2<sup>2His</sup> enzyme, the four residues unique to the IDH1 site (or some subset thereof) are important for eliciting the appropriate response to AMP. The effects of IDH1 residue replacements on AMP interactions are examined in more detail below. In contrast with the IDH1 mutant enzymes, AMP does increase the affinities for isocitrate of the enzymes containing residue replacements in the IDH2 active site (IDH1<sup>A108R</sup>/IDH2<sup>R114A/Y142F/D248T/D252N/His</sup>) in panel B and IDH1/IDH2<sup>S98A/His</sup> in panel E).

These and other parameters for binding of isocitrate are quantitatively compared with kinetic parameters determined for the mutant enzymes in Table II. With respect to general characteristics of all mutant enzymes in this study, two aspects of isocitrate binding were unexpected. First, the S<sub>0.5</sub> values for isocitrate binding measured in the absence of AMP are uniformly lower than that measured for the wild type enzyme, implying a higher basal affinity for the mutant enzymes. This difference is ~3-fold for the IDH1<sup>S92A</sup>/IDH2<sup>His</sup> enzyme and ~2-fold for the other mutant enzymes that demonstrate measurable binding. In comparison, the S<sub>0.5</sub> values for isocitrate binding measured for mutant enzymes in the presence of AMP range from essentially equivalent to ~2-fold higher than the
value measured for the wild-type enzyme. Thus, affinity for isocitrate of the mutant enzymes relative to the wild-type enzyme is higher in the absence of AMP and equivalent or lower in the presence of AMP. This suggests that, in the absence of AMP, the wild-type enzyme may normally exist in a low affinity state for isocitrate, and that the residue changes in this study affect this state of the enzyme by increasing basal affinity.

The second unexpected result is the absence of detrimental effects of any of the residue substitutions on cooperativity with respect to isocitrate binding (Table II). No significant effect on binding cooperativity was expected for mutant enzymes with residue substitutions only in the IDH2 site because catalytic cooperativity is retained in those enzymes. However, the results of kinetic analyses clearly show that the residue substitutions in the IDH1 isocitrate site (i.e. in the IDH1A108R,F136Y,T241D,N245D/IDH2His, IDH1R114A,Y142F,D248T,D252N/IDH2His, and IDH1S98A/IDH2His enzymes) eliminate cooperativity in catalysis, because the Hill coefficients are \( <1 \) in the presence or absence of AMP. For the same mutant enzymes, isocitrate-binding assays produce Hill coefficients ranging from \( 3 \) to \( 7 \) under similar conditions. Thus, for these enzymes, cooperativity measured in ligand-binding assays does not correlate with cooperativity measured in kinetic analyses. For the IDH1A108R,F136Y,T241D,N245D/IDH2His enzyme, this result suggests that cooperative binding of isocitrate alone is insufficient for kinetic cooperativity. However, there is an added layer of complexity for the mutant enzymes containing active site serine residue substitutions, suggesting that this combination of residue changes does effectively eliminate isocitrate binding at both subunit sites. Additionally, no activity was obtained under standard assay conditions for the mutant enzyme (IDH1A108R,F136Y,T241D,N245D/IDH2R114A,Y142F,D248T,D252N/His) containing reciprocal residue replacements in both active sites. However, based on previous results obtained with mutant enzymes containing subsets of these residue changes (25), we tested and found that higher concentrations of Mg\(^{2+}\) in the assay mix produce trace activity for the latter enzyme. Although the parameters obtained under these conditions for the IDH1A108R,F136Y,T241D,N245D/IDH2R114A,Y142F,D248T,D252N/His mutant enzyme and previously for the IDH1A108R,F136Y,T241D,N245D/IDH2His mutant enzyme (shown in italics in Table II) are not directly comparable with those obtained for other mutant enzymes in this study, the residual activities are sufficient to show loss of AMP activation and loss of cooperativity. As originally predicted, the IDH1A108R,F136Y,T241D,N245D/IDH2S98A,Y142F,D248T,D252N/His mutant enzyme appears to retain the capacity for binding isocitrate at both interconverted IDH1 and IDH2 sites. 

AMP Binding by Wild-type and IDH1 Mutant Enzymes—Kuehn et al. (5) previously reported that IDH binding of the allosteric activator AMP requires the presence of isocitrate. They speculated that isocitrate binding at a noncatalytic site

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**FIG. 2. Models for the isocitrate-binding sites in IDH2 and IDH1 subunits.** Putative isocitrate-binding sites for yeast IDH subunits are based on amino acid sequence alignments and structural fitting (34, 39) with the primary and three-dimensional structures of *E. coli* isocitrate dehydrogenase (13, 18). The IDH2 site contains residues identical with those in the catalytic site of the bacterial enzyme and is proposed to bind isocitrate and Mg\(^{2+}\) for catalysis. Residues that differ in the IDH2 and IDH1 sites are indicated by rectangles, and serine residues in each site that apparently greater affinity of the mutant enzymes for isocitrate measured in the absence of AMP, as noted above.

We were unable to measure catalysis or substrate binding for the mutant enzyme (IDH1A108R,F136Y,T241D,N245D/IDH2His) containing both active site serine residue substitutions, suggesting that this combination of residue changes does effectively eliminate isocitrate binding at both subunit sites. Additionally, no activity was obtained under standard assay conditions for the mutant enzyme (IDH1A108R,F136Y,T241D,N245D/IDH2S98A,Y142F,D248T,D252N/His) containing reciprocal residue replacements in both active sites. However, based on previous results obtained with mutant enzymes containing subsets of these residue changes (25), we tested and found that higher concentrations of Mg\(^{2+}\) in the assay mix produce trace activity for the latter enzyme. Although the parameters obtained under these conditions for the IDH1A108R,F136Y,T241D,N245D/IDH2S98A,Y142F,D248T,D252N/His mutant enzyme and previously for the IDH1A108R,F136Y,T241D,N245D/IDH2His mutant enzyme (shown in italics in Table II) are not directly comparable with those obtained for other mutant enzymes in this study, the residual activities are sufficient to show loss of AMP activation and loss of cooperativity. As originally predicted, the IDH1A108R,F136Y,T241D,N245D/IDH2S98A,Y142F,D248T,D252N/His mutant enzyme appears to retain the capacity for binding isocitrate at both interconverted IDH1 and IDH2 sites. 

**AMP Binding by Wild-type and IDH1 Mutant Enzymes—**Kuehn et al. (5) previously reported that IDH binding of the allosteric activator AMP requires the presence of isocitrate. They speculated that isocitrate binding at a noncatalytic site
might facilitate binding of AMP with subsequent positive allosteric effects on the catalytic isocitrate-binding site. We wished to examine AMP binding by the affinity-purified wild-type enzyme and to investigate this hypothesis using mutant enzymes with defects in the IDH1 isocitrate-binding site.

AMP binding by the affinity-purified wild-type enzyme was tested using absorbance measurements to quantitate concentrations of free ligand in ultrafiltrates. Initial experiments tested the requirement of IDH for isocitrate to obtain AMP binding. As illustrated in Fig. 4A, no AMP binding was measurable in the absence of isocitrate, whereas binding of 2 mol of AMP/mol of holoenzyme was obtained with D-isocitrate concentrations 0.25 mM.

We also examined IDH activity as a function of AMP concentration at several isocitrate concentrations. As illustrated in Fig. 4B and as tabulated in Table III, similar $V_{\text{max}}$ values are obtained at saturating concentration of AMP with assays containing 0.1, 0.25, or 1.0 mM D-isocitrate. However, an $M_{0.5}$ value for AMP of $-79 \mu M$ was obtained with 0.1 mM isocitrate and an $-10$-fold lower value was obtained with 0.25 mM isocitrate. This dramatic effect of isocitrate on $M_{0.5}$ values for AMP is consistent with a primary effect of isocitrate on IDH affinity for AMP. Hill coefficients of $-1.5$ were obtained with both lower concentrations of isocitrate, suggesting some cooperativity in AMP effects on catalysis. Values for these parameters are generally consistent with those previously obtained for the conventionally purified enzyme (Ref. 5, Table III). At the nearly saturating concentration of 1.0 mM D-isocitrate, velocities approximate $V_{\text{max}}$ and are essentially unaffected by AMP (Fig. 4B). We therefore chose this concentration of isocitrate to measure binding of AMP by IDH.

As illustrated in Fig. 5 (closed circles), AMP binding to IDH in the presence of 1.0 mM D-isocitrate is saturable, and the data indicate two sites per holoenzyme. There is some indication of cooperativity in AMP binding (Hill coefficient $-1.6$), consistent with kinetic data (Table III). An $M_{0.5}$ value for AMP of $-50 \mu M$ was measured in these binding assays. This value is higher than previously reported (5) or than expected based on kinetic assays. At present, the reason for this discrepancy is unknown, although we do find that $M_{0.5}$ values measured for AMP in binding assays are extremely sensitive to differences in concentrations of isocitrate (data not shown).

The data presented above in Fig. 3 and Table II for the IDH1A108R,F136Y,T241D,N245D/IDH2His and the IDH1S92A/
Isocitrate-binding Sites of Yeast IDH

**TABLE II**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Binding assays</th>
<th>Kinetic assays*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Binding sites</td>
<td>Hill coefficient</td>
</tr>
<tr>
<td></td>
<td>−AMP/AMP</td>
<td>−AMP/AMP</td>
</tr>
<tr>
<td></td>
<td>mm</td>
<td>mm</td>
</tr>
<tr>
<td>IDH1His/IDH2</td>
<td>3.9/3.8</td>
<td>4.4/3.6</td>
</tr>
<tr>
<td>IDH1A108R,F136Y,T241D,N245D/IDH2His</td>
<td>(± 0.04/0.17)</td>
<td>(± 0.06/0.04)</td>
</tr>
<tr>
<td>IDH1His/IDH2R114A,Y142F,D248T,D252N</td>
<td>4.0/4.1</td>
<td>5.4/6.5</td>
</tr>
<tr>
<td>IDH1S92A/IDH2S98A/His</td>
<td>(± 0.01/0.04)</td>
<td>(± 0.01/0.01)</td>
</tr>
<tr>
<td>IDH1S92A/IDH2S98A/His</td>
<td>4.4/4.3</td>
<td>4.8/5.4</td>
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<tr>
<td>IDH1A108R,F136Y,T241D,N245D/IDH2R114A,Y142F,D248T,D252N/His</td>
<td>(± 0.02/0.1)</td>
<td>(± 0.02/0.01)</td>
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<tr>
<td>IDH1His/IDH2R114A,Y142F,D248T,D252N/His</td>
<td>4.5/4.5</td>
<td>3.5/3.3</td>
</tr>
<tr>
<td>IDH1S92A/IDH2S98A/His</td>
<td>(± 0.13/0.25)</td>
<td>(± 0.03/0.03)</td>
</tr>
<tr>
<td>IDH1A108R,F136Y,T241D,N245D</td>
<td>2.0/2.1</td>
<td>7.3/7.1</td>
</tr>
<tr>
<td>IDH1A108R,F136Y,T241D,N245D</td>
<td>(± 0.33/0.21)</td>
<td>(± 0.00/0.02)</td>
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<tr>
<td>IDH1His/IDH2R114A,Y142F,D248T,D252N/His</td>
<td>2.0/2.2</td>
<td>6.9/3.3</td>
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<tr>
<td>IDH1A108R,F136Y,T241D,N245D</td>
<td>(± 0.26/0.05)</td>
<td>(± 0.05/0.03)</td>
</tr>
<tr>
<td>IDH1His/IDH2R114A,Y142F,D248T,D252N/His</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IDH1S92A/IDH2S98A/His</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>IDH1His/IDH2R114A,Y142F,D248T,D252N/His</td>
<td>ND</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Kinetic parameters were determined as previously reported (25).

**DISCUSSION**

Ligand-binding studies described in this report confirm previous predictions based on kinetic analyses of mutant enzymes that yeast IDH contains two types of isocitrate-binding sites (Fig. 2). The catalytic isocitrate-binding site comprises primarily residues from IDH2. A replacement of the active site serine residue in IDH2 (S98A) results in a dramatic decrease in catalytic activity (20, 25) because of an apparent elimination of isocitrate binding at this site. For another mutant enzyme, replacement of four of nine residues in the IDH2 site (R114A, Y142F, D248T, and D252N) with the four residues that differ in the IDH1 site was found to be compatible with binding of isocitrate but not for catalysis. Based upon the correspondence of residues in the IDH2 site with residues in the catalytic site of *E. coli* isocitrate dehydrogenase (18, 33, 35) and upon kinetic analyses of mutant yeast enzymes (20, 25), Ser-98 and Arg-114 are presumed to form hydrogen bonds with isocitrate, Tyr-142 is believed to be important for the dehydrogenation step in catalysis, and Asp-248 plus Asp-252 are likely to be involved in binding of the divalent cation required for activity.

The other IDH isocitrate-binding site comprises primarily residues from IDH1 and appears to be a noncatalytic site with a role in allosteric regulation. The existence of this site was previously proposed by Kuehn et al. (5) to explain aspects of the kinetic and ligand-binding properties of the wild-type enzyme. Replacement of the active site serine residue in IDH1 (S92A) has a relatively moderate effect on *V* max (20, 25) but apparently eliminates isocitrate binding at this site as well as holoenzyme binding and activation by AMP. Additionally, replacement of the four residues in IDH1 (A108R, F136Y, T241D, and N245D) with the four residues that differ in the IDH2 site is permissive for isocitrate binding but is nonpermissive for normal function of this site, i.e. for promotion of AMP binding and activation.

Mutant enzymes containing corresponding pairs of residue replacements in both subunits further confirm these results. The IDH1His/IDH2S98A/His mutant enzyme exhibits no measurable binding of isocitrate and no measurable catalytic activity. The IDH1A108R,F136Y,T241D,N245D/IDH2R114A,Y142F,D248T,D252N/His enzyme retains the wild-type number of four binding sites for isocitrate/mol of holoenzyme, but the mutant enzyme exhibits very little catalytic activity and no allosteric regulatory properties.

These results suggest that the catalytic isocitrate-binding sites of yeast and bacterial isocitrate dehydrogenases have
Isocitrate-binding Sites of Yeast IDH

### Table III

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Kinetic assays</th>
<th>Binding assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$V_{max}$ (units/mg)</td>
<td>$K_{m,5}$ ($\mu$M)</td>
</tr>
<tr>
<td>IDH1&lt;sup&gt;His&lt;/sup&gt;/IDH2&lt;sup&gt;His&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.1 mM d-isocitrate</td>
<td>33.9</td>
<td>78.9</td>
</tr>
<tr>
<td>0.25 mM d-isocitrate</td>
<td>33.0</td>
<td>7.4</td>
</tr>
<tr>
<td>1.0 mM d-isocitrate</td>
<td>33.0</td>
<td>NA&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>IDH1/IDH2&lt;sup&gt;His&lt;/sup&gt;</td>
<td>35.6</td>
<td>6.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> NA, not applicable. Velocity is near $V_{max}$ at this concentration of isocitrate.

### Fig. 5

AMP binding by wild-type and mutant forms of IDH. AMP-binding assays were conducted in the presence of 1.0 mM d-isocitrate with the affinity-purified wild-type enzyme (○), the IDH1<sup>S92A</sup>/IDH2<sup>His</sup> enzyme (●), and the IDH1<sup>A108R,F136Y,T241D,N245D</sup>/IDH2<sup>His</sup> enzyme (▼).

It has been highly conserved, despite substantial differences between these enzymes with respect to oligomeric structure, kinetic and physiological regulation, and cofactor specificity. In addition, the two subunits of the yeast enzyme have evolved to preserve crucial features of isocitrate binding, but residues in the IDH1 site have apparently diverged to confer regulatory properties that impact catalysis in the IDH2 site.

One previous prediction not supported by current results is that the loss of kinetic cooperativity observed for mutant enzymes containing residue replacements in the IDH1 isocitrate site (20, 25) is because of a loss of cooperativity in isocitrate binding. Loss of kinetic cooperativity is observed for the IDH1<sup>S92A</sup>/IDH2<sup>His</sup>, IDH1<sup>A108R,F136Y,T241D,N245D</sup>/IDH2<sup>His</sup>, and IDH1<sup>A108R,F136Y,T241D,N245D</sup>/IDH2<sup>His</sup> enzymes, but all retain substantial cooperativity (Hill coefficients >3) in isocitrate-binding analyses (Table II). The altered IDH1 site in the IDH1<sup>A108R,F136Y,T241D,N245D</sup>/IDH2<sup>His</sup> and IDH1<sup>A108R,F136Y,T241D,N245D</sup>/IDH2<sup>His</sup> enzymes retains the capacity for binding isocitrate. Thus, the negative effect on catalytic cooperativity is analogous to the negative effect upon AMP binding by these residue substitutions and suggests that the authentic residues, although not essential for isocitrate binding or for cooperativity in binding, are essential for both properties of catalytic cooperativity and allosteric activation by AMP. Retention of binding cooperativity by the IDH1<sup>S92A</sup>/IDH2<sup>His</sup> mutant enzyme is less easily interpreted, because this IDH1 site has apparently lost the capacity for binding isocitrate. A simple conclusion, however, from both types of residue replacements in IDH1 is that binding of isocitrate at the IDH1 site and the subsequent effect of this binding on the enzyme are essential for catalytic cooperativity.

The retention of strong cooperativity in binding of isocitrate exhibited by the IDH1<sup>S92A</sup>/IDH2<sup>His</sup> and IDH1/IDH2<sup>S98A/His</sup> mutant enzymes is difficult to explain with current knowledge about IDH quaternary structure. Both enzymes exhibit Hill coefficients of ≥4 for isocitrate binding in the absence of AMP, despite the overall measurement of only two binding sites for each mutant holoenzyme. In ligand-binding assays, the Hill coefficient is generally accepted to be an indication of the strength of interactions among binding sites and is used as an estimate of the minimum number of binding sites for a given ligand (36). However, other models suggest that this coefficient can overestimate the number of binding sites if an enzyme undergoes a slow conformational change between bound and unbound states (37, 38). If we apply these models to current results, the implication is that replacement of these serine residues in either IDH1 or IDH2 may temporarily stabilize a form(s) of the enzyme with apparently stronger cooperative interactions among residual isocitrate-binding sites.

Another complication in assessing interactions among isocitrate-binding sites of IDH is that our current working structural model for the enzyme overestimates the number of ligand-binding sites measured for the holoenzyme. This model is based upon the existence of two identical isocitrate-binding catalytic sites in the homodimer of the E. coli enzyme (18) and upon our cumulative kinetic data (24, 27), suggesting that a heterodimer of IDH2 and IDH1 could similarly form two isocitrate-binding sites, one being catalytic and the other regulatory. However, this would predict for the octameric enzyme a total of eight isocitrate-binding sites and four each for Mg<sup>2+</sup>, NAD<sup>+</sup>, and AMP. For each of these ligands, this is twice the number of sites actually measured in ligand-binding analyses (Ref. 5 and this report). Thus, either half of the ligand-binding sites are normally occluded in the wild-type holoenzyme or a model invoking a more complex heteromeric core unit may be more valid. Obviously, these aspects of the architecture of this enzyme and the organization of binding sites require solution of the quaternary structure.

With respect to affinity, the specific observation of similar $S_{0.5}$ values measured in binding assays with the IDH1<sup>S92A</sup>/IDH2<sup>His</sup> and IDH1/IDH2<sup>S98A/His</sup> enzymes suggests that the respective residual catalytic IDH2 and regulatory IDH1 isocitrate-binding sites have similar affinities for isocitrate. Kuehn et al. (5) predicted this finding based on analyses of the wild-type enzyme. A more general observation is, relative to the wild-type enzyme, the mutant enzymes analyzed in this study exhibit 2–3-fold increases in binding affinity for isocitrate (measured in the absence of AMP). In contrast, kinetic analyses of these enzymes indicate significant reductions in apparent affinity for isocitrate (20, 25). Thus, as was the case for cooperativity, apparent affinities estimated by kinetic analyses do not necessarily reflect binding affinities. Importantly, these results from isocitrate-binding analyses suggest that wild-type IDH may have evolved to maintain a relatively low affinity...
state, and that current residue changes within the isocitrate-binding sites of either subunit affect this state by increasing overall holoenzyme affinity. In vivo, the low affinity state of the wild-type enzyme could provide a more dramatic differential for allosteric activation by AMP and may be important in directing metabolic utilization of isocitrate. A precedent for the metabolic importance of affinity for isocitrate is provided by E. coli isocitrate dehydrogenase. In bacteria, this value is sufficiently high to theoretically preclude utilization of the common substrate by the glyoxylate cycle enzyme, isocitrate lyase, which exhibits much lower affinity for isocitrate (21). Thus, under certain environmental conditions, a physiological mechanism (phosphorylation) is necessary for inactivation of isocitrate lyase. As noted in Table I, the latter enzyme in yeast (IDP1) exhibits a much higher apparent affinity for isocitrate than does IDH in the absence of AMP. The presence of AMP makes the apparent affinity of IDH more comparable with that of IDP1, suggesting a possible mechanism for control of branchpoint flux based on allosteric control.

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Isocitrate Binding at Two Functionally Distinct Sites in Yeast NAD⁺-specific Isocitrate Dehydrogenase
An-Ping Lin and Lee McAlister-Henn

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