Homologous Binding Sites in Yeast Isocitrate Dehydrogenase for Cofactor (NAD$^{+}$) and Allosteric Activator (AMP)*

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Yeast NAD$^{+}$-specific isocitrate dehydrogenase (IDH) is an allosterically regulated octameric enzyme composed of two types of homologous subunits designated IDH1 and IDH2. Based on sequence comparisons and structural models, both subunits are predicted to have adenosine nucleotide binding sites. This was tested by alanine replacement of residues in putative sites in each subunit. Targets included adjacent aspartate/isoleucine residues implicated as important for determining cofactor specificity in related dehydrogenases and a residue in each IDH subunit in a position occupied by histidine in other cofactor binding sites. The primary kinetic effects of D286A/I287A and of H281A replacements in IDH2 were found to be a dramatic reduction in apparent affinity of the holoenzyme for NAD$^{+}$ and a concomitant reduction in $V_{\text{max}}$. Ligand binding assays also showed that the H281A mutant enzyme fails to bind NAD$^{+}$ under conditions that are saturating for the wild-type enzyme. In contrast, the primary effect of corresponding D279A/D280A and of R274A replacements in IDH1 is a reduction in holoenzyme binding of AMP, with concomitant alterations in kinetic and isocitrate binding properties normally associated with activation by this allosteric effector. These results suggest that the nucleotide cofactor binding site is primarily contributed by the IDH2 subunit, whereas the homologous nucleotide binding site in IDH1 has evolved for regulatory binding of AMP. These results are consistent with previous studies demonstrating that the catalytic isocitrate binding sites are comprised of residues primarily contributed by IDH2, whereas sites for regulatory binding of isocitrate are contributed by analogous residues of IDH1. In this study, we also demonstrate that a prerequisite for holoenzyme binding of NAD$^{+}$ is binding of isocitrate/Mg$^{2+}$ at the IDH2 catalytic site. This is comparable to the dependence of AMP binding upon binding of isocitrate at the IDH1 regulatory site.

Mitochondrial NAD$^{+}$-specific isocitrate dehydrogenase (IDH) catalyzes a rate-liming step in the tricarboxylic acid cycle and is subject to complex allosteric regulation. In particular, because of allosteric activation of the mammalian enzyme by ADP (1) and of the yeast enzyme by AMP, IDH is proposed to regulate metabolic flux in response to energy needs of the cell (2). Saccharomyces cerevisiae IDH is an octamer composed of four each of two homologous subunits, IDH1 and IDH2 (3). The mature polypeptides are similar in size (349 and 354 amino acid residues, respectively) and share 42% residue sequence identity (4, 5). Both subunits are essential for holoenzyme structure and function, although, as described below, catalytic function has been primarily attributed to IDH2 whereas regulatory functions have been assigned to IDH1 (6–8). Mammalian IDH contains three different types of subunits that share significant homology with those of yeast IDH, an $\alpha$-subunit with catalytic functions similar to those of yeast IDH2, and $\beta$- and $\gamma$-subunits that are presumed to impart regulatory properties (9–10).

Although crystallographic data are unavailable for yeast and mammalian IDHs, these enzymes share substantial similarity in sequence with several bacterial decarboxylating dehydrogenases for which three-dimensional structures are available. As a family, these dehydrogenases are unique in that they lack the classic Rossman fold described for other enzymes that bind NAD(P)$^{+}$ (11). Particularly useful for analyses of yeast IDH are structures reported for Escherichia coli isocitrate dehydrogenase (12–14) and for Thermus thermophilus 3-isopropylmalate dehydrogenase (15). The former is a homodimeric NAD$^{+}$-specific enzyme that functions in the bacterial tricarboxylic acid cycle, but that is regulated by phosphorylation rather than by allosterly (16). The latter is a homodimeric NAD$^{+}$-specific enzyme in the leucine biosynthetic pathway. Consistent with catalysis of similar reactions, these bacterial enzymes share some similarity in primary structure (25% residue identity) and substantial similarity in three-dimensional structure. Differences between residues in catalytic sites of the enzymes have been instructive for analyses of substrate and cofactor specificity (17–19).

Both yeast IDH1 and IDH2 subunits share residue sequence identities of ~32% with E. coli isocitrate dehydrogenase. Based on sequence alignments and modeling, the catalytic isocitrate/Mg$^{2+}$ binding site of yeast IDH was predicted to be primarily composed of residues from IDH2 (6), because IDH2 contains identities for nine key residues in the catalytic site of the E. coli enzyme (12). IDH1, however, contains identities for only five of these nine residues, and was proposed to bind but not catalytically alter isocitrate. These predictions have been confirmed by results summarized in Table I of site-directed mutagenesis studies (6, 20–22). Among shared residues in isocitrate binding sites, IDH1 and IDH2 each contain a serine residue analogous to bacterial Ser-113. The latter is the site for phosphorylation of the E. coli enzyme in vivo (16), a modification that inactivates the enzyme by preventing binding of isocitrate (23). Alanine replacement of the analogous Ser-98 in IDH2 was found to profoundly reduce catalysis. Similar replacement in Ser-92 of IDH1 had much less of an effect on catalytic capacity; however, it dramatically reduced cooperativity and allosteric activation.

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‡ The abbreviations used are: IDH, isocitrate dehydrogenase; NTA, nitrilotriacetic acid.

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by AMP (6, 20). Replacement of the serine residue in either yeast subunit eliminated half of the holoenzyme isocitrate binding sites, and the combination of these residue replacements in both subunits prevented isocitrate binding (Ref. 21 and Table I). Thus, both the IDH1 and IDH2 sites bind isocitrate but for different kinetic functions. In other studies, the four of nine residues that differ in each of the putative IDH1 and IDH2 isocitrate binding sites were replaced by the corresponding residues in the other subunit site (22). Mutant enzymes containing these reciprocal residue replacements in IDH1 (A108R/F136Y/T241D/N245D) and/or in IDH2 (R114A/Y142F/D248T/D252N) were found to retain the wild-type number of isocitrate binding sites (Ref. 21 and Table I), indicating that these replacements were permissive for binding at each site. However, the mutant enzyme with residue replacements in IDH2 retained very little catalytic activity, and the mutant enzyme with residue replacements in IDH1 exhibited no allosteric activation by AMP nor any binding of AMP (20, 21). Thus, the unique residues in each subunit isocitrate binding site are essential for different functions in catalysis (IDH2 site) or in allosteric regulation (IDH1 site).

Based on these and other results, the homologous yeast IDH subunits appear to be an exceptional model for divergent evolution. The catalytic isocitrate/Mg\(^{2+}\) binding site in IDH2 has been highly conserved, and a similar isocitrate binding site in IDH1 has evolved for regulatory function. In the current study, we use mutagenesis to investigate putative nucleotide binding sites in each subunit. Our hypothesis is that residues important for cofactor NAD\(^+\) binding are primarily contributed by IDH2, and that the AMP binding site is comprised of analogous residues of IDH1. A corollary is that residues in the putative NAD\(^+\) binding site of IDH2 are likely to be evolutionarily conserved with residues in other cofactor binding sites, whereas analogous residues in the putative AMP binding site in IDH1 are likely to have diverged for binding of the structurally related allosteric activator.

Yeast IDH also displays complex interdependencies among various ligands for binding to the enzyme (24). For example, the presence of isocitrate is a prerequisite for AMP binding, and we have shown the specific nature of this requirement is binding of isocitrate by the regulatory IDH1 site (21). In this report, we also use mutant and wild-type enzymes to investigate the dependence upon citrate or isocitrate to obtain NAD\(^+\) binding and the dependence upon Mg\(^{2+}\) for binding of other ligands of the enzyme.

### Experimental Procedures

**Mutagenesis and Enzyme Expression**—For expression of wild-type and mutant forms of IDH in yeast, multicopy prS426 plasmids (25) carrying both IDH1 and IDH2 genes were used. In these plasmids, each gene is preceded by authentic promoter sequences, and one of the 2-subunit genes contains codons for five histidine residues at the 3'-end of the coding region (7). We have previously shown that the histidine tag on the carboxyl terminus of either subunit facilitates affinity purification of holoenzyme and has no apparent effect on kinetic properties of the enzyme (7).

Site-directed mutagenesis was conducted using a QuikChange™ site-directed mutagenesis kit (Stratagene) to replace codons for IDH1 Arg-274 and for IDH2 His-281 with codons for alanine. Mutagenesis was conducted using a pBR322 plasmid carrying the IDH1 gene and a pRS316 plasmid carrying the IDH2 gene. Mutagenic oligonucleotides (with their complementary oligonucleotides) were 5'-CTTGGAGAG- GCTTCCCCATGTTGTTAGATATTAAG for the R274A replacement in IDH1 and 5'-CATCGTTGAAGGTGCTTGTCGGCTGG- CGCTGCTATTGG for the H281A replacement in IDH2. DNA sequence analyses was conducted to ascertain the presence of only the desired mutations. DNA fragments containing the altered IDH1 and IDH2 genes were subcloned into prS426 plasmids described above for expression with the wild-type gene for the other subunit.

Mutagenesis of IDH1 and IDH2 genes for alanine replacement of adjacent Asp-279 and Ile-280 residues in IDH1 and of adjacent Asp-286 and Ile-287 residues in IDH2 was previously described (7). For current studies, IDH1D279A/D286A/IDH2 and IDH1/IDH2D286A/IDH2A were transferred on 6.4-kbp SstII/HindIII DNA fragments from centromere-based plasmids into prS426. Some experiments also used pRS426 plasmids constructed as previously described (6, 20, 22) for expression of the following mutant forms of IDH: IDH1S92A/IDH2, IDH1/IDH2S98A, IDH1S92A/IDH2, and IDH1/IDH2S98A.

Plasmids were transferred into yeast strain IDH3Δ2 (MATα ade2-1 can1-100 his3-11,15 leu2-3,112 trp101 ura3-1); cells were transferred on 6.4-kbp SstII/HindIII DNA fragments from centromere-based plasmids into prS426. Some samples also used equivalent protein concentrations, determined by Bradford assays using bovine serum albumin as the standard, were loaded onto 10% polyacrylamide/niuclide dodecyl sulfate gels. Following electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane for immunoblot analysis using an anti-yeast IDH antisemur (3). The enhanced chemiluminescence method (ECL, Amer sham Biosciences) was used for detection.

**Enzyme Purification**—For purification of wild-type and mutant forms of IDH, yeast transformants were grown as previously described in VP yeast medium (21). Cell pellets were harvested from 1–2 liters of culture to obtain enzyme for kinetic assays or from 6–8 liters of culture to obtain enzyme for ligand binding assays. Cell pellets were stored at −70 °C prior to breaking. Affinity purification was conducted as previously described (7) using NTA-NTA resin (Qiagen). Concentrations of purified enzymes were measured using absorbance values at A\(_{280}\) nm and a molar extinction coefficient for the octameric holoenzyme of 168820 M\(^{-1}\) cm\(^{-1}\) (29). Purity of affinity-purified enzymes was assessed by electrophoresis as described above followed by staining with Coomassie Blue. Yields of purified enzymes (0.4–1.0 mg/g of cell pellet) were comparable for the wild-type and for mutant enzymes with the exception of the IDH1/IDH2S98A, IDH2A mutant enzyme. Yields of the latter enzyme were ~100-fold lower despite apparently equivalent cellular levels of expression as described in the text. Modifications to the purification procedure, including addition of a variety of protease inhibitors during cell breakage and conducting column chromatography...
binding of the cofactor. In contrast to the NADP\(^+\)-specific enzymes, NAD\(^-\)-specific dehydrogenases including the *T. thermophilus* enzyme and both subunits of IDH contain adjacent aspartate and isoleucine residues in analogous residue positions (Fig. 1). The importance of these adjacent residues in determining cofactor specificity was confirmed by Chen et al. (17), who reported that altering the cofactor specificity of *E. coli* isocitrate dehydrogenase requires seven residue replacements including K344D and Y345I. To test the roles of adjacent Asp-286 and Ile-287 residues in the yeast IDH2 subunit and of the yeast IDH1 subunit, the analogous residue positions are occupied by His-281 in IDH2 and by Arg-274 in IDH1 (Fig. 1). In the *E. coli* enzyme crystal structure, the analogous His-339 is located near the adenine ring (13, 18). In the yeast IDH subunits, the analogous residue position is occupied by His-281 in IDH2 and by Arg-274 in IDH1 (Fig. 1). We therefore constructed mutant enzymes containing alanine residues for each of these residues to test effects on kinetics and nucleotide binding properties of the enzyme.

Mutagenesis was conducted as described under “Experimental Procedures.” Wild-type and mutant enzymes were expressed using multicopy plasmids carrying both *IDH1* and *IDH2* genes in a yeast strain containing disruptions of the endogenous *IDH1* and *IDH2* loci. Expressed enzymes contain pentahistidine tags on the carboxyl terminus of one of the two subunits (7). As illustrated in Fig. 2A, cellular protein extracts from yeast transformants contain approximately equivalent immunochemical levels of wild-type enzymes (*lanes a* and 1) and of mutant enzymes (*lanes 2–5*). With enzymes carrying the histidine tag on *IDH1*, *IDH1*, and *IDH2* subunits are electrophoretically distinct (*lanes 1–3 and 5*), but with enzymes carrying the histidine tag on *IDH2*, the subunits comigrate (*lanes a and 4*). The enzymes were purified from cellular extracts using Ni\(^2+\)-NTA column chromatography. As illustrated in Fig. 2B, the wild-type enzyme used in this study (*IDH1*/*IDH2*).
and mutant enzymes including IDH1$^{R274A}$/IDH2 (lane 1), IDH1/IDH2$^{R281A}$ (lane 2), and IDH1$^{D286A,I287A}$/IDH2 (lane 3) were similarly purified. However, despite apparently normal cellular levels of expression of the IDH1/IDH2$^{D286A,I287A}$ enzyme (Fig. 2A, lane 5), exceptionally low relative yields of this enzyme (Fig. 2B, lane 5) were obtained during purification procedures conducted under a variety of conditions. This suggests that the structural integrity of this mutant enzyme is highly compromised, producing a susceptibility to proteolysis or disassembly during cell breakage and purification. Our inability to purify sufficient amounts of the IDH1/IDH2$^{D286A,I287A}$ enzyme precluded ligand binding analyses.

Kinetic Parameters and Isocitrate Binding Properties—To obtain saturation curves for isocitrate with affinity-purified wild-type and mutant enzymes, kinetic assays, and ligand binding assays were conducted in the absence or in the presence of 100 μM AMP. Examples of these curves are shown in Fig. 3. Saturation velocity curves for NAD$^+$ were also obtained with kinetic assays conducted in the presence of AMP. Kinetic parameters for isocitrate and NAD$^+$ are presented in Table II.

Kinetic data from isocitrate saturation curves for the IDH1/IDH2$^{R281A}$ and IDH1/IDH2$^{D286A,I287A}$ enzymes (Fig. 3A and Table II) show that the major effect of residue substitutions in the putative NAD$^+$ binding site of the enzyme is a decrease in apparent $V_{max}$ values. This value is ~9-fold lower for the IDH1/IDH2$^{R281A}$ enzyme and ~300-fold lower for the IDH1/IDH2$^{D286A,I287A}$ enzyme relative to that of the wild-type enzyme. Despite a significant difference in the degree of catalytic dysfunction, both mutant enzymes retain essentially wild-type characteristics of cooperativity with respect to isocitrate (Hill coefficients of 3.5–4.1) and of allosteric activation by AMP. The latter property is evident by an ~5-fold decrease in the $S_{0.5}$ value for isocitrate when measured in the presence of AMP. Kinetic data from NAD$^+$-saturation curves (Table II) further
suggest that the major effect of these residue substitutions in IDH2 is on affinity for NAD\(^+\). The \(S_{0.5}\) values for NAD\(^+\) are increased \(-30\)-fold for the IDH1/IDH2\(^{H281A}\) enzyme and \(-40\)-fold for the IDH1/IDH2\(^{D286A,I287A}\) enzyme. However, with high concentrations of cofactor, the apparent \(V_{\text{max}}\) value for the IDH1/IDH2\(^{H281A}\) enzyme is essentially equivalent to that of the wild-type enzyme, whereas the velocity of the IDH1/IDH2\(^{D286A,I287A}\) enzyme is unaffected. These data suggest that both mutant enzymes with residue substitutions in IDH2 have reduced affinity for NAD\(^+\), but that the D286A/I287A substitutions are much more detrimental than the H281A substitution to cofactor binding and to catalytic function. These kinetic data further suggest that both IDH2 mutant enzymes retain wild-type characteristics with respect to binding of isocitrate and of AMP. The isocitrate binding properties of the IDH1/IDH2\(^{H281A}\) enzyme were analyzed and found to be quite similar to those of the wild-type enzyme (Fig. 3B and Table III). Both mutant and wild-type enzymes have four isocitrate binding sites, and an equivalent AMP effect (an \(-3.5\)-fold decrease in the \(K_D\) value for isocitrate) is observed. As described above, we were unable to obtain ligand binding data for the IDH1/IDH2\(^{D286A,I287A}\) enzyme.

More moderate effects on \(V_{\text{max}}\) in isocitrate saturation curves are produced by the R274A and D279A/I280A residue substitutions in the putative AMP binding sites of IDH1 (Fig. 3A and Table I). The IDH1\(^{R274A}/IDH2\) enzyme exhibits an \(-8\)-fold decrease in velocity in the absence of AMP, comparable to that produced with the corresponding H281A substitution in IDH2. However, the decrease in velocity of the IDH1\(^{R274A}/IDH2\) enzyme is less (\(-5\)-fold) when measured in the presence of AMP and, in addition, an apparent \(V_{\text{max}}\) value only \(-2\)-fold lower than wild-type is obtained in NAD\(^+\)—isocitrate binding saturation curves (Table II). Thus, high concentrations of NAD\(^+\) have a substantial effect on velocity of the IDH1\(^{R274A}/IDH2\) enzyme, which also exhibits an \(-4\)-fold increase in the \(S_{0.5}\) value for NAD\(^+\). In comparison, the apparent \(V_{\text{max}}\) values for the IDH1\(^{D279A,I280A}/IDH2\) mutant enzyme measured under several conditions are consistently \(-2\)-fold less than wild-type values. This relatively mild effect of D279A/I280A substitutions in IDH1 on catalytic competence sharply with the dramatic decrease in velocity obtained with corresponding D286A/I287A substitutions in IDH2.

Both types of residue substitutions in IDH1 produce defects in AMP activation measured in kinetic and ligand binding saturation curves with isocitrate. For the IDH1\(^{R274A}/IDH2\) enzyme, the apparent affinity for isocitrate in kinetic assays is actually less in the presence than in the absence of AMP (Fig. 3A and Table II). For the IDH1\(^{D279A,I280A}/IDH2\), AMP produces only a \(-1.5\)-fold decrease in the kinetic \(S_{0.5}\) value for isocitrate. Ligand binding assays conducted with both mutant enzymes (Fig. 3B and Table III) indicate the loss of any effect of AMP on isocitrate binding. Thus, a primary defect in both IDH1 mutant enzymes is loss of AMP activation. However, there are several significant differences between these mutant enzymes. The IDH1\(^{R274A}/IDH2\) enzyme exhibits a substantial loss of cooperativity with respect to isocitrate binding and kinetics (Hill coefficients of \(-1\)--\(-2\)). This is not observed for the IDH1\(^{D279A,I280A}/IDH2\) enzyme. Also, as described above, the apparent \(V_{\text{max}}\) of the IDH1\(^{R274A}/IDH2\) enzyme, but not that of the IDH1\(^{D279A,I280A}/IDH2\) enzyme, is affected by the presence of AMP or of increasing concentrations of NAD\(^+\). Thus, with respect to isocitrate kinetic and ligand binding properties, the primary defect associated with the D279A/I280A substitutions appears to be loss of AMP activation, whereas defects associated with the R274A substitution are more pleiotropic but include a loss of AMP activation.

### Table II

| Enzyme                        | Isocitrate | NAD\(^+\) |
|-------------------------------|------------|------------|
|                               | \(V_{\text{max}}\) | \(S_{0.5}\) | Hill coefficient |
| IDH1/IDH2\(^{R274A}/IDH2\)   | 31.6/31.6 (+1.4/1.5) | 0.46/0.09 (+0.04/0.01) | 3.73/4.4 (+0.5/0.1) |
| IDH1/IDH2\(^{D286A,I287A}\)  | 3.5/3.5 (+0.2/0.4) | 0.47/0.10 (+0.09/0.02) | 4.05/3.5 (+0.2/0.4) |
| IDH1/IDH2\(^{H281A}\)        | 0.1/0.1 (+0.0/0) | 0.42/0.07 (+0.14/0.04) | 4.0/0.11 (+0.9/0.8) |
| IDH1\(^{R274A}/IDH2\)        | 3.9/6.8 (+0.5/1.1) | 1.37/2.11 (+0.37/0.17) | 1.2/0.1 (+0.2/0.3) |
| IDH1\(^{D279A,I280A}/IDH2\)  | 14.3/15.6 (+0.3/0.4) | 3.20/0.89 (+0.20/0.12) | 4.3/0.4 (+0.1/0.4) |

\(^{a}\) Data for the wild-type enzyme are comparable to those previously reported (20).
Nucleotide Binding Sites of Yeast IDH

TABLE III

| Enzyme         | Isocitrate | NAD⁺ | AMP |
|----------------|------------|------|-----|
|                | Binding sites | Kᵢ | Hill coefficient | Binding sites | Kᵢ | Hill coefficient | Binding sites | Kᵢ | Hill coefficient |
| IDH1/IDH2      | 3.9/3.8     | 0.52 | 4.4/3.6        | 1.8           | 0.24 | 1.4              | 2.0           | 0.03 | 1.4             |
| IDH1/IDH2H281A | 3.9/4.1     | 0.39/0.11 | 3.9/3.8 | 0.2           | 0.07 | 0.04              | 0.01           | 0.01 | 0.01             |
| IDH1R274A/IDH2 | 4.1/4.2     | 0.38/0.39 | 2.1/1.9        | 1.9           | 0.67 | 1.4              | 0.1           |       | 0               |
| IDH1D279A,I280A/IDH2 | 4.1/4.0 | 0.26/0.26 | 3.8/3.8 | 2.0           | 0.25 | 1.3              | 0             |       | 0               |

a Data for isocitrate binding by the wild-type enzyme were previously reported (21).

AMP (21). We further utilized mutant forms of IDH to examine this requirement. We found that, under conditions producing saturable binding of AMP by the wild-type enzyme (Fig. 5B, ○), an IDH1R281A/IDH2 mutant enzyme, which is deficient in binding of isocitrate at the regulatory site (Ref. 21 and Table 1), also fails to bind AMP (Fig. 5B, ▼). These results suggested that prerequisites for binding of allosteric activator include both binding of isocitrate at the regulatory site in IDH1 and, in addition, subsequent changes in the enzyme normally elicited by the binding of isocitrate by authentic residues (i.e. Ala-108, Phe-136, Thr-241, and Asp-245) in the regulatory site.

In the current study, we similarly used mutant enzymes to investigate the reported (iso)citrate requirement for NAD⁺ binding by IDH (24). Citrate is used as an analogue for isocitrate in these binding assays to preclude catalysis. As illustrated in Fig. 5C, no NAD⁺ is bound in the absence of citrate, and concentrations of citrate ≥500 μM are necessary to obtain saturable binding of NAD⁺. Since this requirement presumably involves (iso)citrate binding at the catalytic IDH2 site, we examined NAD⁺ binding using mutant enzymes with residue replacements in this site. As illustrated in Fig. 5D, an IDH1/IDH2R281A mutant enzyme (○), which is deficient in isocitrate binding at the IDH2 site (Ref. 21 and Table 1), fails to bind NAD⁺ under conditions producing saturable binding by the wild-type enzyme (●). This is not the case for a IDH1/IDH2R114A,Y142F,D248T,D252N mutant enzyme, which contains reciprocal replacements for unique residues in the IDH2 isocitrate binding site with corresponding residues from the IDH1 isocitrate binding site. This mutant enzyme, although essentially inactive (20), retains the wild-type number of four isocitrate binding sites (Ref. 21 and Table 1). As shown in Fig. 5D (▼), the IDH1/IDH2R114A,Y142F,D248T,D252N mutant enzyme also retains essentially wild-type characteristics of NAD⁺ binding. Thus, in contrast to the prerequisites described above for AMP binding, (iso)citrate binding at the IDH2 site appears to be sufficient for cofactor binding, i.e., the authentic residues (i.e. Arg-114, Tyr-142, Asp-248, and Asp-252) in the IDH2 site are not essential for this function.

We have further analyzed the reported dependence upon the presence of Mg²⁺ for binding of other ligands by wild-type IDH (24). Catalysis requires a divalent cation (32), and a complex of isocitrate/Mg²⁺ is bound to the catalytic site of the E. coli enzyme (13), suggesting that isocitrate/Mg²⁺ is the substrate that binds to the IDH2 catalytic site. However, residue differences suggest that the IDH1 isocitrate binding site binds isocitrate, but not Mg²⁺ (21). Also, Kuehn et al. (24) found that yeast IDH has twice as many isocitrate as Mg²⁺ binding sites. We therefore compared isocitrate binding (≥100 μM AMP) by the wild-type enzyme in the presence and absence of Mg²⁺. As illustrated in Fig. 6A and as summarized in Table IV, the total number of isocitrate binding sites/holoenzyme is reduced from four to two in the absence of Mg²⁺. However, the overall affinity of the enzyme for isocitrate and the AMP effect on the Kᵢ value for isocitrate are largely unaffected by the absence of Mg²⁺. These results suggest that the divalent cation is required for binding of isocitrate by half of the isocitrate binding...
sites (presumably the catalytic IDH2 sites) but not for binding to other sites (presumably the regulatory sites provided by IDH1).

Since (iso)citrate binding subsequently affects binding of other ligands, we also tested the requirements for Mg\textsuperscript{2+} for binding of nucleotides by IDH. As shown in Fig. 6B, IDH exhibits no binding of NAD\textsuperscript{+} in the absence of Mg\textsuperscript{2+}. This is presumably because of the dependence of cofactor binding on the binding of (iso)citrate at the catalytic site which, in turn, requires the presence of divalent cation. In contrast, binding of AMP by IDH is largely unaffected by the absence of Mg\textsuperscript{2+} (Fig. 6C and Table IV). This observation is consistent with the assumption that isocitrate binding at regulatory sites in IDH1 is independent of divalent cation, and that binding of isocitrate alone at this site supports subsequent binding of the allosteric activator.

**DISCUSSION**

Kinetic and ligand binding analyses described in this report suggest that yeast IDH has distinct but homologous nucleotide binding sites for NAD\textsuperscript{+} and for AMP. The cofactor binding site contains residues of the IDH2 subunit that are homologous with those in cofactor binding sites of other NAD\textsuperscript{+}-specific decarboxylating dehydrogenases (Fig. 1). Adjacent Asp-286 and Ile-287 residues of IDH2 apparently correspond with adjacent Asp-279 and Ile-280 residues of *T. thermophilus* 3-isopropylmalate dehydrogenase, residues implicated as important for cofactor specificity (17, 18). In NADP\textsuperscript{+}-specific isocitrate dehydrogenases, equivalent positions are occupied by adjacent or nearby lysine (or arginine) and tyrosine residues. Thus, replacement of IDH2 Asp-286 and Ile-287 with alanine residues produces a dramatic reduction in apparent affinity for NAD\textsuperscript{+} and a concomitant reduction in the catalytic capacity of IDH. Similar effects on affinity for NADP\textsuperscript{+} and on velocity were reported for mutant enzymes containing replacements for Arg-314 and Tyr-316 residues of mammalian mitochondrial NADP\textsuperscript{+}-specific isocitrate dehydrogenase (30). In addition, His-281 of IDH2 is important for cofactor binding, since the most dramatic effect of alanine replacement of this residue is a reduction in affinity for NAD\textsuperscript{+}. His-281 thus appears to be the functional homologue of a specific histidine residue in the cofactor binding sites of the *T. thermophilus* enzyme (His-274, Ref. 15), of the mammalian enzyme mentioned above (His-309, Ref. 31), and of *E. coli* isocitrate dehydrogenase (His-339, Refs. 13 and 18). Unlike the aspartate/isoleucine or lysine/tyrosine pairs described above, this histidine residue apparently functions in binding NAD\textsuperscript{+} or NADP\textsuperscript{+} but is not a determinant of cofactor specificity.

The yeast IDH1 subunit also contains an aspartate/isoleucine pair at residue positions 279 and 280, but contains an arginine in residue position 274 that aligns with the histidine in position 281 of IDH2. We have shown that the primary effect associated with alanine replacements for both types of residues in IDH1 is a significant reduction in holoenzyme affinity for AMP. The consequence is a defect in allosteric activation, i.e. for these mutant enzymes, isocitrate binding is unaffected by the presence of AMP. Despite similar effects on holoenzyme
affinity for AMP, the D279A/I280A and R274A replacements in IDH1 have different kinetic effects. The latter replacement has a greater effect on apparent $V_{\text{max}}$ values and significantly reduces cooperativity with respect to isocitrate. These results suggest that, in addition to participating in binding of AMP, IDH1 Arg-274 may function in communication between regulatory and catalytic sites.

Overall, results obtained in this study suggest that related but unique sites have evolved in the homologous subunits to facilitate binding of different nucleotide ligands of IDH. The differential functions of IDH1 and IDH2 subunits in nucleotide binding are consistent with previous results showing that both subunits also contribute isocitrate binding sites (6–8, 20, 21), but that the site comprised primarily of residues from IDH2 is catalytic whereas the site comprised primarily of residues from IDH1 supports regulatory properties of the holoenzyme. Thus, the catalytic isocitrate/Mg$^{2+}$- and NAD$^+$ binding sites are contributed by IDH2, whereas the regulatory isocitrate- and AMP binding sites are contributed by IDH1. As might be expected, the IDH2 catalytic site(s) exhibits a more significant conservation of residues in catalytic sites of related enzymes (20). In the case of IDH1, some residue conservation is observed for binding of structurally related ligands, but key residue differences appear to be important elements in the evolution of regulatory properties of this complex allosteric enzyme.

A key to the communication between catalytic and regulatory sites in IDH is that, while each ligand binding site is primarily comprised of residues from one type of subunit, a few residues are apparently contributed by the other type of subunit. For example, results of previous mutagenesis studies (8) are consistent with the conclusion that of nine residues in the IDH2 catalytic isocitrate/Mg$^{2+}$ binding site, two are contributed by IDH1 and, reciprocally, the analogous two residues of nine in the IDH1 regulatory isocitrate binding site are contributed by IDH2. These and other results from yeast two-hybrid studies (8) support a model for a heterodimer of IDH1 and IDH2 subunits as the basic structural-functional unit of the holoenzyme. Consistent with reciprocal subunit contributions to isocitrate binding sites, we have also reported kinetic analyses of other mutant enzymes that indicate similar potential intersubunit contributions to nucleotide binding sites (7). These studies will be expanded by replacement of other residues with similar putative intersubunit functions and by direct ligand binding analyses.

In current and previous studies (21), we have also evaluated some of the complex interrelationships among various ligands for binding by yeast IDH. Cumulative results suggest the following conclusions: (A) Despite residue differences in the isocitrate binding sites, the catalytic site in IDH1 and the regulatory site in IDH1 form isocitrate with similar affinity. Binding at catalytic and regulatory sites is independent, since isocitrate binding at either site is not affected by loss of isocitrate binding at the other site (21). (B) Nucleotide binding sites are also independent of each other, because residue replacements examined in this study primarily affect either NAD$^+$ or AMP binding (Fig. 4). (C) Binding of isocitrate by the catalytic IDH1 site, but not by the regulatory IDH1 site, requires Mg$^{2+}$. Thus, the substrate bound by the enzyme is a complex of isocitrate/Mg$^{2+}$. Furthermore, that isocitrate binding by the IDH1 site is independent of Mg$^{2+}$ is additional evidence for evolutionary divergence of this site for regulatory rather than catalytic function. (D) A prerequisite for binding of NAD$^+$ is binding of the

![Fig. 6. Requirements for Mg$^{2+}$ for binding of other ligands of wild-type IDH. A, saturation binding curves for isocitrate in the absence (circles) or presence (triangles) of 100 $\mu$M AMP were conducted in the absence (closed symbols) or presence (open symbols) of 4 mM MgCl$_2$. B, NAD$^+$ binding assays were conducted in the absence (○) or presence (△) of 4 mM MgCl$_2$. C, AMP binding assays were conducted in the absence (●) or presence (○) of 4 mM MgCl$_2$.]

![Table IV: Effect of Mg$^{2+}$ on ligand binding parameters of IDH](https://example.com/table-iv)
(iso)citrate/Mg\(^{2+}\) complex by the catalytic IDH2 site. No binding of cofactor is observed in the absence of either (iso)citrate or Mg\(^{2+}\). (E) A prerequisite for binding of AMP is binding of isocitrate by the IDH1 regulatory binding site. An additional prerequisite for binding of AMP is some subsequent change(s) in the holoenzyme elicited by isocitrate binding at the IDH1 site, since replacement of the four non-identical of nine residues in the IDH1 isocitrate binding site with corresponding residues from the IDH2 site is permissive for isocitrate binding but not for AMP binding (21).

Collectively, these results suggest that only isocitrate binding by the regulatory IDH1 site occurs in the absence of other ligands of the enzyme. The complex prerequisites for binding of other ligands may reflect mechanisms for tight control of IDH in vivo, e.g., to ensure that binding and sequestering of a common tricarboxylic acid cycle cofactor occurs only in the presence of sufficient substrate (isocitrate/Mg\(^{2+}\)), and to ensure that allosteric activation by AMP occurs only when concentrations of isocitrate are sufficiently elevated.

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