Effect of AMP on mRNA Binding by Yeast NAD$^+$-Specific Isocitrate Dehydrogenase†

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ABSTRACT: Yeast mitochondrial NAD$^+$-specific isocitrate dehydrogenase (IDH) has previously been shown to bind specifically to 5′-untranslated regions of yeast mitochondrial mRNAs, and transcripts containing these regions have been found to allosterically inhibit activity of the enzyme. This inhibition is relieved by AMP, an allosteric activator of this regulatory enzyme of the tricarboxylic acid cycle. We further investigated these enzyme/ligand interactions to determine if binding of RNA and AMP by IDH is competitive or independent. Gel mobility shift experiments indicated no effect of AMP on formation of an IDH/RNA complex. Similarly, sedimentation velocity ultracentrifugation experiments used to analyze interactions in solution indicated that AMP alone had little effect on the formation or stability of an RNA/IDH complex. However, when these sedimentation experiments were conducted in the presence of isocitrate, which has been shown to be essential for binding of AMP by IDH, the proportion of RNA sedimenting in a complex with IDH was significantly reduced by AMP. These results suggest that AMP can affect the binding of RNA by IDH but that this effect is apparent only in the presence of substrate. They also suggest that the catalytic activity of IDH in vivo may be subject to complex allosteric control determined by relative mitochondrial concentrations of mRNA, isocitrate, and AMP. We also found evidence for binding of 5′-untranslated regions of mitochondrial mRNAs by yeast mitochondrial NADP$^+$-specific isocitrate dehydrogenase (IDP1) but not by the corresponding cytosolic isozyme (IDP2). However, this appears to be a nonspecific interaction since no evidence was obtained for any effect on the catalytic activity of IDP1.

Yeast NAD$^+$-specific isocitrate dehydrogenase (IDH)[1] is one of a growing list of enzymes shown to have dual functions. This mitochondrial enzyme is proposed to regulate flux through the tricarboxylic acid cycle primarily via positive allosteric response to AMP (*1*, *2*). IDH has also been demonstrated to bind specifically to regions in the 5′-untranslated regions of the eight major yeast mitochondrial mRNAs (*3*). De Jong et al. (*4*) recently reported evidence supporting a role for this interaction in preventing inappropriate translation of the mRNAs. We have also found that addition of RNA transcripts containing these 5′-untranslated sequences dramatically inhibits IDH activity (*5*). Furthermore, this inhibition of activity is relieved by the presence of the allosteric activator, AMP. Collectively, these observations suggest a potential mechanism for coordinate control of metabolite flux through oxidative pathways and mitochondrial gene expression.

The catalytically active form of yeast IDH is a heterooctamer composed of four each of two subunits designated IDH1 and IDH2 (*6*). The two subunits, with respective molecular masses of 38000 and 37800 Da, share 42% primary sequence identity. On the basis of sequence alignments with related bacterial enzymes (isocitrate dehydrogenase and isopropylmalate dehydrogenase) that have been analyzed by crystallographic methods (*7*−*9*) and of results of targeted mutagenesis studies (*10*−*13*), the IDH2 subunit is proposed to contribute most of the residues in the catalytic isocitrate/Mg^{2+} and NAD$^+$ binding sites, whereas the IDH1 subunit has apparently evolved similar sites for cooperative binding of isocitrate and allosteric binding of AMP. However, despite these differential functions, each subunit contributes residues to the isocitrate-binding "active site" of the other subunit, suggesting that the basic structural/functional unit within the octamer is a heterodimer (*12*). This is consistent with gene disruption studies indicating that both subunits are essential for enzyme activity in vitro and in vivo (*14*, *15*) and that both subunits are essential for RNA binding (*16*). This is also consistent with two-hybrid analyses that detected heteromeric (IDH1 with IDH2) but not homomeric (IDH1 with IDH1 or IDH2 with IDH2) interactions (*12*).

Phenotypes associated with disruption of *IDH1* and/or *IDH2* genes are consistent with multiple or complex functions for the enzyme. The inability of yeast mutant strains

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[1] Abbreviations: IDH, NAD$^+$-specific isocitrate dehydrogenase; IDP1, mitochondrial NADP$^+$-specific isocitrate dehydrogenase; IDP2, cytosolic NADP$^+$-specific isocitrate dehydrogenase; HPLC, high-performance liquid chromatography.

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containing these gene disruptions to grow with acetate as a carbon source (*15*) is a phenotype shared with several yeast mutants containing disruptions in genes encoding other tricarboxylic acid cycle enzymes (*17*−*19*). Other phenotypes are more unique to IDH mutants. For example, IDH gene disruption mutants exhibit slow growth with glycerol as a carbon source, a phenotype that can be suppressed by mutations resulting in loss of citrate synthase or one of several other enzymes with oxidative functions (*20*). IDH gene disruption mutants also exhibit an increase in production of respiratory deficient cells, a phenotype that has been attributed to loss of mRNA binding and resulting effects on mitochondrial translation (*21*).

The RNA-binding properties of IDH have been examined in detail by Grivell and colleagues (*3*, *22*). Using gel shift binding and competition experiments, they demonstrated the specificity of IDH binding to the 5′-untranslated regions of yeast mitochondrial mRNAs relative to other types of RNA. They also identified specific regions within these mitochondrial transcripts that share possible secondary structural features that may direct binding of IDH. More recently, de Jong et al. (*4*) demonstrated that rates of mitochondrial translation are increased 2−3-fold in yeast strains lacking IDH and that mitochondrial translation products in these strains exhibit a similar fold reduction in half-lives. Their interpretation of these results is that IDH may modulate translation by preventing initiation until mitochondrial transcripts are in the vicinity of mRNA-specific membrane-bound translational activators (*21*, *23*, *24*). This control is believed to ensure correct localization of the translation products, which are primarily inner membrane-localized components of the electron transport chain including three subunits of ATP synthase, three subunits of cytochrome *c* oxidase, and one subunit of apocytochrome *b*. A soluble mitochondrial ribosomal protein is the other major mitochondrial translation product.

To determine if this regulatory phenomenon might be reciprocal, we investigated the effects of mitochondrial transcripts on IDH activity (*5*). Transcripts containing IDH-binding sites in *COX2* and *ATP9* mRNAs dramatically inhibited IDH activity, whereas other nonmitochondrial RNAs of similar size had much less of an effect. To study this phenomenon in more detail, we have concentrated on the 5′-untranslated region of the C*OX2* mRNA, which has been used as a model for analysis of translational control (*25*−*28*). An RNA transcript containing this region of *COX2* was found to be an allosteric inhibitor, producing significant increases in the $S_{0.5}$ value for isocitrate. All inhibitory effects of the RNA could be eliminated by addition of 25−100 μM AMP, which allosterically activates IDH by decreasing the $S_{0.5}$ value for isocitrate. The reciprocal allosteric effects of RNA and of AMP are illustrated in Figure 1. Significantly, the concentrations of AMP and of RNA capable of producing these allosteric effects are quite similar to the concentrations calculated for these molecules in vivo (*29*−*31*), suggesting that this regulation may be physiologically important. Thus, it seems possible that the interaction of IDH with the mitochondrial mRNAs could provide a mechanism for coordinate control of metabolite flux through the tricarboxylic acid cycle and of mitochondrial gene expression at the level of translation. In the current study, we have further investigated these interactions by examining effects of AMP on binding of the *COX2* transcript by IDH and by examining the specificity of the RNA/enzyme interaction with respect to the enzyme.

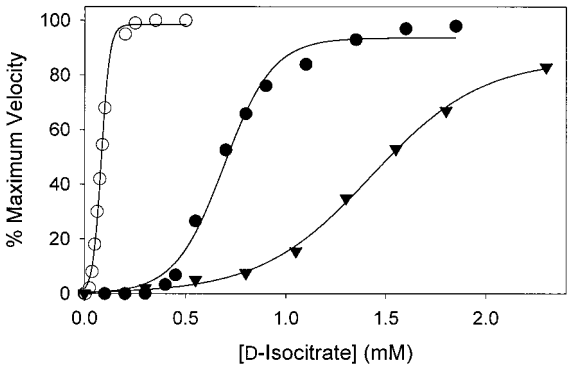

FIGURE 1: Allosteric effects of AMP and the *COX2* transcript on yeast IDH activity. Saturation velocity kinetic analyses for IDH as previously reported (*5*) were conducted with assays containing increasing concentrations of isocitrate with no allosteric effector (●), with 100 μM AMP (○), or with 0.1 μM RNA (▼). Respective millimolar values calculated from these data for isocitrate $S_{0.5}$ are 0.69 (no effector), 0.08 (with AMP), and 1.42 (with RNA).

EXPERIMENTAL PROCEDURES

Yeast Strains and Growth Conditions. Histidine-tagged enzymes were expressed in yeast strains (*15*, *32*, *33*) containing deletion/disruption mutations in corresponding genomic loci (*idh1::LEU2* plus *idh2::HIS3*, *idp1::URA3*, or *idp2::URA3*) of the parental strain S173-6B (*MATa leu2-3,112 his 3-1 ura3-52 trp1-289*; *34*). Minimal YNB medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, pH 6.5) containing supplements added to 20 μg/mL to satisfy auxotrophic requirements and 2% glucose as the carbon source was used in agar plates for storage and in liquid form for precultures of transformed strains. For preparation of enzymes, YNB glucose precultures were diluted into rich YP medium (1% yeast extract, 2% Bacto-peptone) containing 2% glycerol and 2% lactate as carbon sources. Cells were harvested when cultures reached $A_{600nm} = 1.5-2.0$ and stored as pellets at −20 °C prior to use.

Preparation of RNA Transcripts. DNA templates for transcription were generated by polymerase chain reaction (PCR) as previously described (*5*). The templates contained T7 promoter sequences and were designed to produce transcripts of uniform size (∼130 nucleotides) containing the IDH-binding sites in the 5′-untranslated regions of *COX2* and *ATP9* mRNAs. The *COX2* transcript contained the 54-nucleotide 5′-untranslated region and a portion of the coding region. The *ATP9* transcript contained nucleotides −643 to −520 relative to the AUG translational initiation site. For synthesis of unlabeled RNAs, transcription reactions were conducted using a T7-MEGAshortscript in vitro transcription kit (Ambion). Unincorporated nucleotides were removed with ProbeQuant G-50 Micro Columns (Amersham Pharmacia Biotech). Product yields (∼30 μg of transcript/200 ng of DNA template) were quantitated, and RNA transcripts were stored in aliquots at −70 °C. [α-^{32}P]UTP-labeled transcripts were prepared using a MAXIscript in vitro transcription kit (Ambion). Unincorporated nucleotides were removed by chromatography, product yields were calculated as described by the supplier, and ^{32}P-labeled transcripts were stored at

−20 °C overnight prior to use. For all experimental manipulations using RNA, nuclease-free water was used and all glassware and cuvettes were treated with RNase ZAP (Ambion).

Enzyme Expression and Purification. IDH was expressed in a Δ*IDH1*Δ*IDH2* yeast strain using a plasmid (p*IDH1*^{His}/*IDH2*; *11*) containing genes for the IDH2 subunit and for a histidine-tagged form of IDH1. Mitochondrial and cytosolic $NADP^+$-specific isocitrate dehydrogenases (IDP1 and IDP2) were expressed respectively in Δ*IDP1* and in Δ*IDP2* yeast strains (*32*, *33*) using multicopy plasmids containing genes with codons for five histidine residues at the 3′ ends of the coding regions.[2] Enzyme purifications using Ni^{2+}-nitrilotriacetic acid superflow resin (Ni^{2+}-NTA, Qiagen Inc.) were conducted as previously described (*11*). Fractions from the final eluate were assayed for activity, and those containing the highest levels of activity were pooled and stored on ice at 4 °C prior to analysis. Enzyme purities of >95% were confirmed by electrophoresis and staining with Coomassie blue. Concentrations of purified enzymes were determined using the method of Lowry et al. (*35*) with bovine serum albumin as the standard and by estimates of molar extinction coefficients using the method of Pace et al. (*36*).

Kinetic Analyses. The activities of the isocitrate dehydrogenase isozymes were measured as the isocitrate-dependent production of NADH or NADPH at A_{340nm} in assays containing approximately 5 μg of enzyme/mL. IDH assays contained 40 mM Tris-HCl (pH 7.4), 4 mM $MgCl_2$, 0.25 mM NAD^+, and 1 mM D-isocitrate. IDP1 and IDP2 assays contained 50 mM potassium phosphate (pH 7.75), 5 mM $MgCl_2$, 0.5 mM $NADP^+$, and 0.1 mM D-isocitrate. The concentrations of D-isocitrate (calculated as 50% of the total DL-isocitrate) and of NAD^+ were set at values giving 75−80% V_{max} to facilitate detection of allosteric effects (*5*). For assays with RNA, a 100 μL reaction volume was used, and an RNase inhibitor (ANTI-RNase, Ambion) was added at concentrations of 30 units/100 μL.

Gel Shift Assays. Mobility shift assays to analyze RNA/protein interactions were conducted essentially as described by Papadopoulou et al. (*22*). Binding was conducted in 30 μL of buffer A (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 3 mM $MgCl_2$, 50 mM NaCl, and 5% glycerol) with 0.05 ng of ^{32}P-labeled RNA and 25 ng of purified enzyme unless otherwise noted. Each assay contained 1 μg of *Escherichia coli* cellular RNA (Ambion) to reduce nonspecific binding and 0.3 μg ANTI-RNase. Samples were electrophoresed on 4% polyacrylamide gels. Results were visualized by autoradiography or analyzed using a Molecular Dynamics PhosphorImager.

Analytical Ultracentrifugation. Sedimentation velocity analyses were conducted with a Beckman Optima XL-I analytical ultracentrifuge equipped with UV absorbance optics. Runs for analysis of enzyme and/or RNA samples were performed simultaneously at 20 °C using an AN-50 rotor at 48000 rpm. Samples (420 μL) were made in buffer A, which also served as the reference buffer. Samples containing AMP (50 μM) and/or D-isocitrate (1.0 mM) were run with the reference buffer containing the same concentration of ligands. Runs with purified IDH alone (2.0 μM) were monitored using absorbance optics at 280 nm. Runs with RNA alone (0.3 μM) or with RNA plus varying concentrations of enzyme (0.15−1.2 μM) were monitored at 260 nm. The optimal concentrations of enzyme and RNA for the optical system were determined empirically. The sedimentation velocity data were analyzed by the method described by van Holde and Weischet (*37*) and interpreted as described by Carruthers et al. (*38*). Data from the bottom 5% of each boundary were excluded in analyses due to experimental noise in this region.

[2] V. Contreras, K. I. Minard, and L. McAlister-Henn, unpublished data.

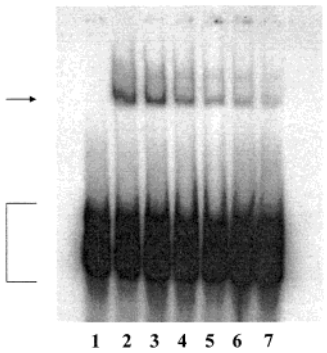

FIGURE 2: Gel mobility shift and competition assays of IDH/RNA interactions. The ^{32}P-labeled *COX2* transcript (0.05 ng) was preincubated prior to electrophoresis in the absence (lane 1) or presence (lanes 2−7) of purified IDH (25 ng) and in the presence of increasing concentrations of unlabeled transcript (5 ng in lane 3, 25 ng in lane 4, 50 ng in lane 5, 100 ng in lane 6, and 250 ng in lane 7). The arrow indicates the enzyme/RNA complex, and the bracket indicates free RNA.

RESULTS

Gel Mobility Shift Assays of the IDH/mRNA Interaction. AMP increases the apparent affinity of IDH for isocitrate (*2*, *37*), whereas a transcript containing the 5′-untranslated region of *COX2* mRNA has the opposite allosteric effect on IDH (*5*). Because of these reciprocal effects, and because AMP relieves inhibition of IDH activity by RNA, we wished to determine if the binding sites of IDH for RNA and for AMP are independent or overlapping. Our initial approach was to utilize gel mobility shift assays (*22*) to investigate IDH/RNA interactions. For these assays, the ^{32}P-labeled *COX2* RNA transcript was prepared by in vitro transcription, and IDH was affinity purified using Ni^{2+}-NTA affinity chromatography as described in Experimental Procedures. The RNA transcript and purified enzyme were preincubated prior to electrophoresis. As illustrated in Figure 2, a band representing the complex (indicated by an arrow) migrates significantly more slowly than the ^{32}P-labeled RNA alone (lane 1). The radioactivity associated with this band (lane 2) decreases with the addition of increasing concentrations of competing unlabeled *COX2* transcript (lanes 3−7). Although the conditions of the gel shift assay are quite different from the soluble kinetic assay, we note that competition is observed only when relative concentrations of unlabeled RNA exceed concentrations of holoenzyme (lanes 4−7). This is consistent with kinetic analyses demonstrating that greater than equimolar concentrations of RNA:holoenzyme are required for >50% inhibition of IDH activity (*5*).

To test a possible effect of AMP on formation or stability of the enzyme/RNA complex, ^{32}P-labeled transcript and

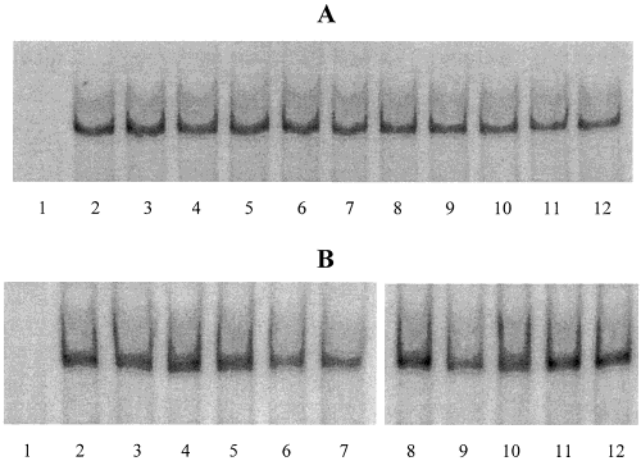

FIGURE 3: Effect of ligands on gel mobility shift assays. Panel A: the ^{32}P-labeled *COX2* transcript (0.05 ng) was preincubated in the absence (lane 1) or presence (lanes 2−12) of 25 ng of IDH and in the presence of increasing concentrations of AMP (1 μM in lane 3, 5 μM in lane 4, 50 μM in lane 5, 250 μM in lane 6, and 1 mM in lane 7) or of D-isocitrate (75 μM in lane 8, 375 μM in lane 9, 750 μM in lane 10, 1.25 mM in lane 11, and 1.5 mM in lane 12). Panel B: the ^{32}P-labeled *COX2* transcript (0.05 ng) was preincubated in the absence (lane 1) or presence (lanes 2−12) of 25 ng of IDH and in the presence of 50 μM AMP plus increasing concentrations of D-isocitrate (75 μM in lane 3, 375 μM in lane 4, 750 μM in lane 5, 1.25 mM in lane 6, and 1.5 mM in lane 7) or in the presence of 1 mM D-isocitrate plus increasing concentrations of AMP (1 μM in lane 8, 5 μM in lane 9, 50 μM in lane 10, 250 μM in lane 11, and 1 mM in lane 12).

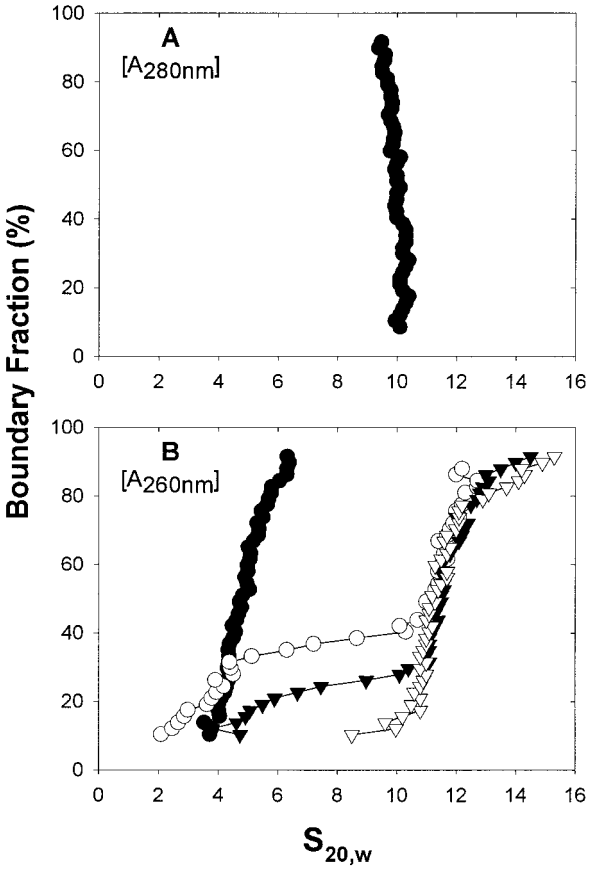

FIGURE 4: Sedimentation velocity analysis of IDH/RNA interactions. Panel A: The ultracentrifugation run was conducted with 0.2 μM IDH (●). Panel B: Ultracentrifugation runs with RNA alone or with RNA plus enzyme were conducted with 0.3 μM *COX2* transcript (●) or with 0.3 μM transcript plus 0.15 μM IDH (○), plus 0.3 μM IDH (▼), or plus 0.6 μM IDH (▽).

purified IDH were preincubated prior to electrophoresis in the absence (Figure 3A, lane 2) or in the presence of AMP concentrations ranging from 1 μM to 1 mM (lanes 3−7). No decrease in radioactivity associated with the enzyme/IDH complex was observed with any concentration of AMP. We also found no effect on levels of the complex upon preincubation with D-isocitrate in concentrations ranging from 75 μM to 1.5 mM (Figure 3A, lanes 8−12). Similar results were independently reported by Siep (*40*), who found that addition of AMP (or of NAD^+ or isocitrate) to concentrations as high as 15 mM has no effect on stability of the IDH/*COX2* complex in gel shift assays. Thus, addition of AMP in vast excess of amounts required to prevent inhibition of IDH activity by RNA appears to have no effect on RNA binding by IDH in these gel shift assays.

On the basis of our direct ligand-binding studies[3] and those reported by Kuehn et al. (*41*), the presence of isocitrate is a prerequisite for AMP binding by IDH. We therefore repeated gel shift assays in the presence of both ligands. As shown in Figure 3B, 50 μM AMP in preincubation mixtures containing concentrations of D-isocitrate of 75−750 μM (lanes 3−5) has little effect on the level of the IDH/RNA complex (lane 2). AMP with 1.25 or 1.50 mM isocitrate (lanes 6 and 7, respectively) produces an ∼25% reduction in levels of the complex, as estimated by densitometry of this and other gels. In reciprocal experiments, preincubation in the presence of 1 mM D-isocitrate and concentrations of AMP ranging from 1 μM to 1 mM (lanes 8−12) has little effect on levels of the IDH/RNA complex. The higher concentrations of isocitrate and AMP in these experiments exceed reported $S_{0.5}$ values by severalfold (*41*). Thus, these results would suggest that AMP, even in the presence of substrate, has little effect on the IDH/RNA complex. However, as described below, we obtain much more dramatic effects with these ligands using another experimental method, suggesting that the mobility shift assays may not accurately reflect ligand binding by IDH.

Ultracentrifugation Analyses of the IDH/RNA Complex. We also used sedimentation velocity centrifugation to analyze holoenzyme/ligand interactions under solution state conditions. Ultracentrifugation runs were performed as described in Experimental Procedures with the same buffer used for gel shift assays. Runs were monitored at A_{280nm} for samples containing only enzyme or at A_{260nm} for samples containing RNA or RNA plus enzyme. The resulting scans were analyzed by the method of van Holde and Weischet (*37*) to obtain the integral distribution of sedimentation coefficients, $G(s)$.

We first analyzed sedimentation characteristics of purified IDH. As illustrated in Figure 4A (closed circles), the vertical $G(s)$ plot indicates that purified IDH sediments as a homogeneous ∼10S species under these conditions. A sample of the same enzyme preparation examined by gel filtration HPLC elutes with an estimated molecular mass of ∼320000 Da, as expected for the octameric holoenzyme (data not shown). Similar sedimentation results were obtained using the purified enzyme in runs conducted simultaneously with all experiments described below.

[3] A.-P. Lin and L. McAlister-Henn, unpublished results.

In parallel runs, the *COX2* transcript exhibits a moderately heterogeneous sedimentation coefficient distribution (closed circles in Figures 4B). The distribution ranges from ~4S to ~6S, with an average of ~5S. Since samples of the same transcript preparations migrate with a uniform size during agarose gel electrophoresis (data not shown), the most likely explanation is that the heterogeneity is due to conformational differences in the sample. To test the effect of IDH on sedimentation of the RNA, we preincubated the same concentration of the *COX2* transcript with varying concentrations of enzyme to produce molar ratios ($r^{IDH:RNA}$) of holoenzyme:RNA of 0.5, 1, 2, or 4. For the $r^{IDH:RNA} = 0.5$ sample (open circles in Figure 4B), the sedimentation coefficient of ~35% of the RNA remains unchanged from that of free RNA. The remainder of the sample sediments at an average ~11.5 S, consistent with formation of an IDH/RNA complex. After incubation with an equimolar concentration of IDH holoenzyme ($r^{IDH:RNA} = 1$, closed triangles in Figure 4B), approximately 20% of the sample sediments as free RNA, 50−60% sediments at ~11.5 S, and the remainder sediments at increasingly higher *S* values. These results are consistent with incorporation of ~80% of the RNA into an enzyme/RNA complex(es). The presence of 11−16S material at the top of the plot suggests that the nature of the IDH/RNA interaction may be quite complex, presumably involving formation of a 1:1 IDH/RNA complex as well as higher order complexes. At higher IDH:RNA ratios (e.g., $r^{IDH:RNA} = 2$, indicated by open triangles in Figure 4B, or $r^{IDH:RNA} = 4$, data not shown), we find that all of the RNA sediments as a complex with IDH; i.e., ~70% of the population sediments at an average 11.5 S and the remainder at the higher *S* values.

These results establish that IDH and the *COX2* transcript form a stable complex under the solution state conditions used for ultracentrifugation, providing a useful experimental method for analyses of IDH holoenzyme structure and of the IDH/RNA interaction. We further used this method to examine the effect of ligands on holoenzyme structure, and on formation and stability of the RNA/IDH complex, by conducting ultracentrifugation runs similar to those illustrated in Figure 4 but in the presence of AMP and/or isocitrate. As illustrated in Figure 5A, we found that addition of 100 μM AMP (open circles) has no effect on holoenzyme sedimentation (closed circles). Similarly, addition of isocitrate (1 mM D-isocitrate, closed triangles) or of isocitrate plus AMP (1 mM and 50 μM, respectively, open triangles) produces no obvious effect on holoenzyme structure at this level of evaluation.

We proceeded to test the effect of addition of D-isocitrate, of AMP, or of both ligands on sedimentation patterns of the IDH/mRNA complex. These runs were conducted with $r^{IDH:RNA} = 0.5$ (Figure 5B) or with $r^{IDH:RNA} = 1.0$ (Figure 5C). In control runs, the COX2 transcript alone again sedimented at an average ~5 S (closed circles). In runs with RNA plus enzyme, ~30% of the RNA in the $r^{IDH:RNA} = 0.5$ sample (Figure 5B, open circles) and ~10% of the RNA in the $r^{IDH:RNA} = 1.0$ sample (Figure 5C, open circles) sediment as free RNA with the remainder in both samples sedimenting in an apparent complex with IDH.[4] For both the $r^{IDH:RNA} = 0.5$ and the $r^{IDH:RNA} = 1.0$ samples, the presence of 50 μM AMP (closed triangles in panels B and C of Figure 5, respectively) or of 1 mM D-isocitrate (open triangles in panels B and C of Figure 5, respectively) has little effect on sedimentation characteristics of the RNA. Each ligand produces a shift of <10% of the RNA from the bound to free populations. For the $r^{IDH:RNA} = 1.0$ sample, the presence of isocitrate or AMP may slightly retard formation of higher order IDH/RNA complexes, although this requires further investigation.

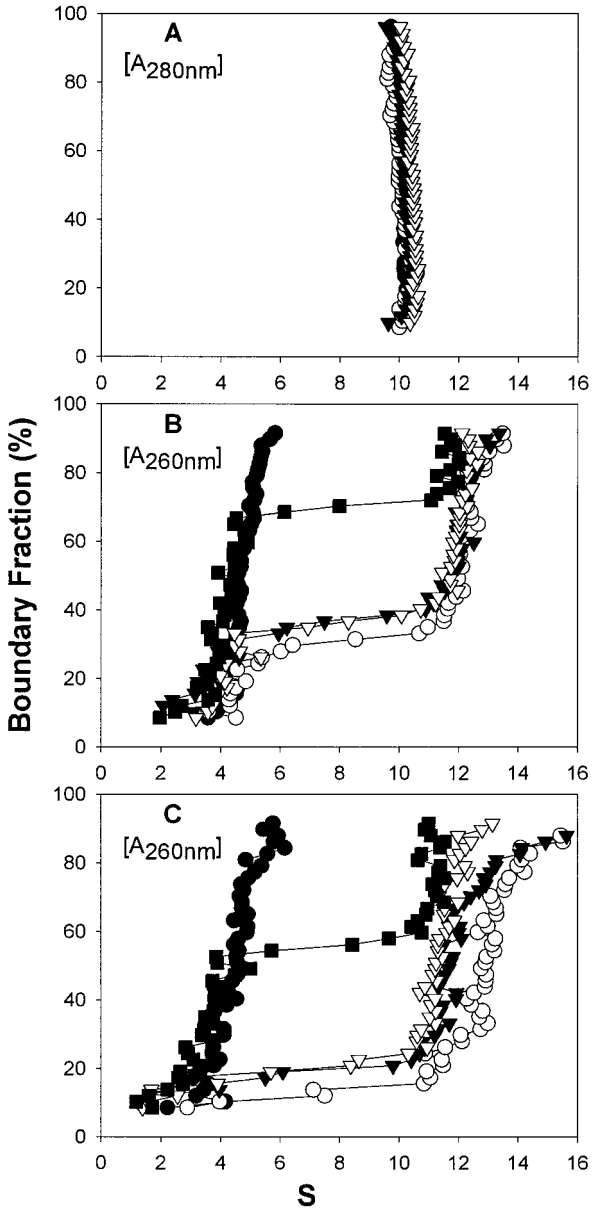

FIGURE 5: Effect of ligands on sedimentation of the IDH/RNA complex. Panel A: Ultracentrifugation runs with IDH (0.2 μM) were conducted in the absence of ligand (●), in the presence of 100 μM AMP (○), in the presence of 1.0 mM D-isocitrate (▼), or in the presence of 1.0 mM D-isocitrate plus 50 μM AMP (▽). Panels B and C: Ultracentrifugation runs with RNA (0.3 μM *COX2* transcript) and IDH were conducted at $r^{IDH:RNA} = 0.5$ (panel B) or at $r^{IDH:RNA} = 1.0$ (panel C). Samples contained RNA alone (●), RNA and IDH (○), or RNA and IDH plus 50 μM AMP (▼), plus 1.0 mM D-isocitrate (▽), or plus 50 μM AMP and 1.0 mM D-isocitrate (■).

In striking contrast, the presence of both ligands (50 μM AMP and 1 mM D-isocitrate) has a dramatic effect upon

[4] The relative ratios of free:bound RNA for these samples differ slightly from those with similar $r^{IDH:RNA}$ ratios presented in Figure 4B, illustrating experimental variation in the method due to use of different preparations of RNA transcript and purified enzyme.

sedimentation of the RNA (closed squares in Figure 5B,C). Approximately 65% of the RNA in the $r^{IDH:RNA} = 0.5$ sample, and approximately 50% of the RNA in the $r^{IDH:RNA} = 1.0$ sample, sediments as free RNA in the presence of AMP plus isocitrate.

These data have several implications. First, the presence of AMP alone (or of isocitrate alone) appears to have relatively minor effects on either IDH holoenzyme structure or upon formation or stability of the IDH/RNA complex. Second, the combination of allosteric activator and substrate does not appear to affect holoenzyme structure at this level but does significantly reduce interaction of IDH with the *COX2* transcript. This difference between effects on IDH/RNA interaction obtained with AMP alone versus with AMP plus isocitrate presumably reflects the dependence of activator binding upon the presence of substrate (*41*). Below, we discuss implications of these results regarding competitive binding of RNA and AMP.

Specificity of Enzyme/RNA Interactions. Interactions of IDH with different types of RNA have been extensively investigated to demonstrate the relative specificity for the 5′-untranslated regions of mitochondrial mRNAs in both binding and inhibition of enzyme activity (*3*, *5*). However, data on specificity with respect to the type of enzyme capable of binding to these mRNA regions have not been published. Therefore, we decided to investigate potential interactions of other isocitrate dehydrogenases with yeast mitochondrial mRNAs.

In initial experiments, we compared binding of the *COX2* transcript by IDH and by yeast mitochondrial $NADP^+$-specific isocitrate dehydrogenase (IDP1). Other than organellar localization, IDP1 shares few characteristics with IDH. IDP1 is a homodimer (subunit molecular mass = 46400 Da), is not allosterically regulated, has a different cofactor specificity, and does not function in the tricarboxylic acid cycle (*32*, *42*). For these experiments, a histidine-tagged form of IDP1 was affinity purified from yeast extracts and found to be catalytically active (specific activity = 60 units/mg). To examine relative affinity of IDP1 for RNA, gel shift assays were performed with purified IDP1 and with increasing concentrations of the ^{32}P-labeled *COX2* transcript. As illustrated in Figure 6, IDP1 does appear to form a complex with the *COX2* transcript (lanes 7−9). The IDP1/RNA complex migrates faster than the IDH/RNA complex (lanes 2−5) as might be expected for a complex formed with a lower molecular weight holoenzyme. In these experiments, we used a 1:4 dilution of labeled:unlabeled *COX2* transcript to compare any differences in saturable binding. On the basis of the amount of radioactivity in the bands representing enzyme/RNA complexes (Figure 6 inset), IDP1 appears to be saturated at lower concentrations of RNA than is IDH.

Since IDP1 binds the *COX2* transcript, we further investigated binding specificity using another yeast isocitrate dehydrogenase, IDP2, and an additional mitochondrial mRNA transcript from the 5′-untranslated region of *ATP9* prepared as previously described (*5*). IDP2 is cytosolic $NADP^+$-specific isocitrate dehydrogenase. It is a homodimer of similar size and shares >70% amino acid sequence identity with IDP1 (*33*). The *ATP9* transcript has been shown to bind IDH in gel shift assays (*3*) and to inhibit IDH enzymatic activity (*5*). For these experiments, a histidine-tagged form of IDP2 was prepared, and the purified enzyme

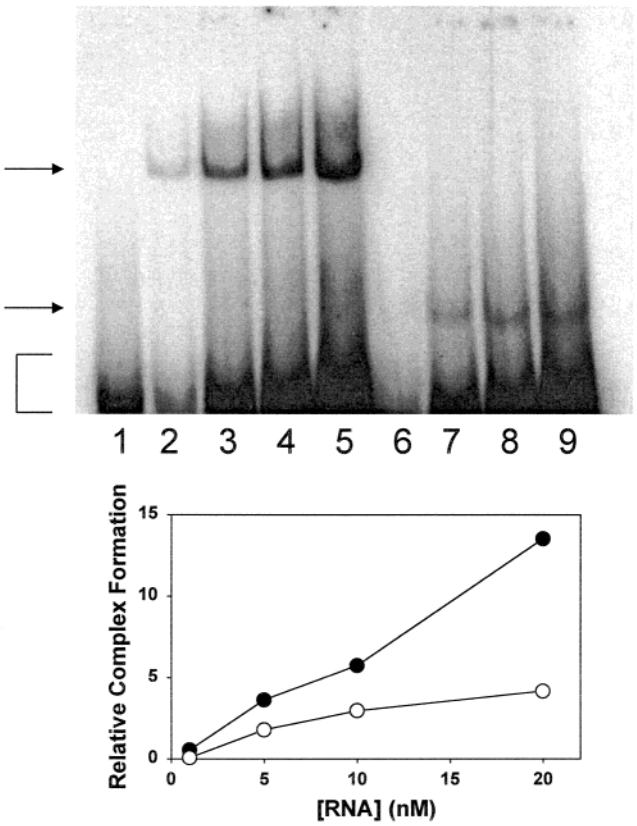

FIGURE 6: Mobility shift assays of IDH and IDP1 with *COX2* RNA. Electrophoresis was conducted with 25 ng of purified IDH (lanes 2−5) or with 25 ng of purified IDP1 (lanes 6−9) plus increasing concentrations of the *COX2* transcript (a 1:4 dilution of ^{32}P-labeled:unlabeled transcript) (1 ng in lanes 2 and 6, 5 ng in lanes 3 and 7, 10 ng in lanes 4 and 8, and 20 ng in lanes 5 and 9). Lane 1 contained 3 ng of the transcript. Arrows indicate enzyme/RNA complexes, and the bracket indicates free RNA. The inset compares relative radioactivity in arbitrary units measured using a phosphorimager for the IDH/RNA complexes in lanes 2−5 (●) and for the IDP1/RNA complexes in lanes 6−9 (○).

was found to be catalytically active (specific activity = 20 units/mg). Gel binding assays were conducted with a constant concentration of ^{32}P-labeled RNAs and increasing concentrations of enzymes (Figure 7). Complexes with similar electrophoretic mobilities are observed for IDH with the *COX2* or with the *ATP9* transcript (panels A and B, respectively, lanes 2−4). Faster migrating complexes are observed for IDP1 with both the *COX2* and *ATP9* transcripts (panels A and B, respectively, lanes 5−7). On the basis of radioactivity associated with the bands, more of the *COX2* transcript appears to be bound by IDH than by equivalent concentrations of IDP1 as noted above. However, similar amounts of the ^{32}P-labeled *ATP9* transcript are bound by both enzymes. Under these conditions, no enzyme/RNA complex is observed following incubation and electrophoresis of IDP2 with either the *COX2* or *ATP9* transcript (panels A and B, respectively, lanes 8−10). Thus, despite similarity in primary and quaternary structures, yeast mitochondrial IDP1 and cytosolic IDP2 appear to have different capacities for binding mitochondrial mRNA transcripts.

The possibility that binding of a mitochondrial mRNA might inhibit IDP1 activity was investigated by conducting enzyme assays in the presence of increasing concentrations of the *COX2* transcript. As illustrated in Figure 8, no effect on activity is observed at RNA concentrations ranging from

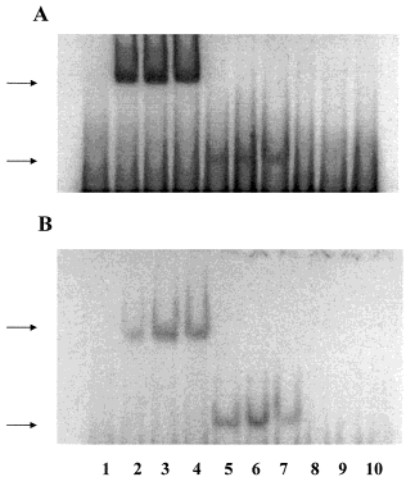

FIGURE 7: Mobility shift assays of IDH, IDP1, and IDP2 with *COX2* and *ATP9* RNAs. Electrophoresis was conducted with samples containing 0.05 ng of ^{32}P-labeled *COX2* transcript (panel A) or 0.05 ng of ^{32}P-labeled *ATP9* transcript (panel B) and containing either no enzyme (lanes 1) or increasing concentrations of IDH (25 ng in lanes 2, 50 ng in lanes 3, or 100 ng in lanes 4), of IDP1 (25 ng in lanes 5, 50 ng in lanes 6, or 100 ng in lanes 7), or of IDP2 (25 ng in lanes 8, 50 ng in lanes 9, or 100 ng in lanes 10). Arrows indicate enzyme/RNA complexes.

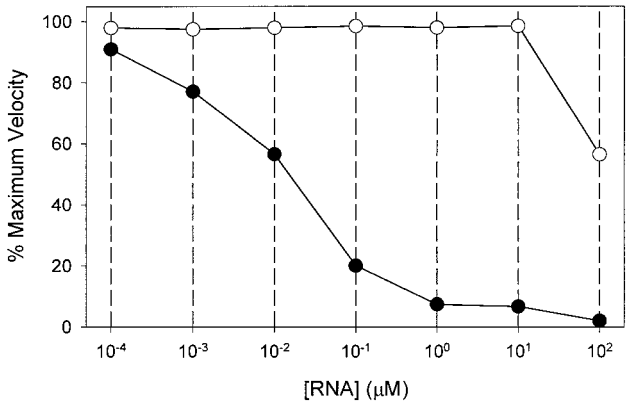

FIGURE 8: Effect of RNA on IDP1 enzyme activity. The activity of affinity-purified IDP1 (○) was measured in the presence of increasing concentrations of the *COX2* transcript and is compared with data for IDH (●; *5*). Values represent the average of two independent experimental determinations and are presented as the percent of activity measured in the absence of RNA.

1 nM to 10 μM. A 40% reduction of activity is obtained with 100 μM RNA, a concentration 4 orders of magnitude greater than that required for a similar inhibition of IDH activity. Thus, in contrast with IDH, the interaction of IDP1 with the *COX2* transcript appears to have little effect on IDP1 activity.

DISCUSSION

The RNA-binding properties of yeast NAD^+-specific isocitrate dehydrogenase were originally described by Papadopoulou et al. (*22*), who identified an abundant mitochondrial protein that binds to the 5′-untranslated region of *COX2* mRNA. Elzinga et al. (*16*) later established the identity of this protein as IDH. Regions for IDH binding within the 5′-untranslated regions of all eight major yeast mitochondrial were identified, and finer mapping studies implicated relatively conserved sequences that may form a base-paired helical structure to direct binding of the enzyme to intervening nonconserved single-stranded regions (*3*). The relevance of this enzyme/RNA interaction in vivo is suggested by recent studies of mitochondrial translation in *IDH* gene disruption mutants. In strains lacking IDH, the overall rates of mitochondrial translation were found to increase, suggesting that IDH binding normally represses translation (*4*). Importantly, the proteins that were produced at increased rates in these mitochondria were found to be unstable. It was therefore suggested that IDH may bind the mRNAs as they are produced in the matrix and prevent premature translation until contact is made with translational activators in the inner mitochondrial membrane, thus ensuring that the hydrophobic translation products are made in close proximity of their cellular destination.

We have focused on how the mRNA/IDH interaction affects the enzyme as a catalytic component and an allosteric regulator of the tricarboxylic acid cycle. We initially found that mRNA transcripts containing the IDH-binding site are potent allosteric inhibitors of IDH activity and that the allosteric activator AMP can prevent this inhibition (*5*). These results suggested that these ligands might competitively regulate activity. In the current report, we have initiated studies to determine if binding of mRNA and of AMP is mutually exclusive, i.e., if binding sites for these ligands might be the same or overlapping. Results of ultracentrifugation studies provide evidence that AMP does, in fact, reduce IDH/RNA interactions but only if isocitrate is also included in the sedimentation velocity run. Similar conditions were previously reported to be necessary for measuring AMP binding by IDH. No binding of AMP by IDH was detected in the absence of isocitrate, but in the presence of 2 mM D-isocitrate, an $S_{0.5}$ value for AMP of 8 μM was obtained (*42*). Thus, we believe that the absence of effects of AMP alone on sedimentation characteristics of the IDH/RNA complex is simply due to the inability of IDH to bind AMP in the absence of substrate.

In contrast to results from ultracentrifugation studies, we observe much less of an effect of combined ligands on the IDH/RNA complex using gel mobility shift assays. This is probably due to fundamental differences in the experimental methods. For example, AMP and isocitrate are present with macromolecules in the buffer during sedimentation runs, whereas ligands present in a sample for electrophoresis are not included in gel or running buffers. This could impact results depending on relative affinities and kinetics for binding of different ligands, factors which are not well-defined at this point. We are examining these possibilities and exploring alternative methods for analysis of the IDH/RNA complex.

The results of sedimentation velocity ultracentrifugation experiments indicate that AMP (plus isocitrate) significantly increases the amount of free RNA in samples with $r^{IDH:RNA} = 0.5$ or 1.0 (Figure 5). This method thus allows assessment of effects on RNA/enzyme interactions at the near equimolar concentrations that produce significant inhibition (~50%) of IDH activity in activity assays (*5*). However, due to optical limitations with the ultracentrifuge, it is necessary to use significantly higher total concentrations (10−20-fold) of both RNA and enzyme than are used in kinetic assays and, due to the same limitations, it is impossible to proportionally increase the relative concentration of AMP used in kinetic assays for ultracentrifugation analysis. Despite the lower

relative concentration of ligand, the significant effect of AMP (plus isocitrate) on sedimentation of the IDH/RNA complex suggests that the allosteric activator can protect IDH from inhibition by RNA by reducing the interaction of IDH with RNA.

Our results indirectly suggest that IDH in a complex with RNA is inactive and that RNA binding, and thus activity, can be significantly affected by binding of AMP (in the presence of isocitrate). The reverse effect of mRNA on binding of AMP has not yet been experimentally addressed. It is also unclear at this point whether the binding sites for these two allosteric effectors overlap, since the effect of AMP on the IDH/RNA complex could also be an indirect allosteric effect. The current model for AMP activation (*11*, *13*, *42*) suggests that isocitrate binding at the cooperative binding site of IDH is a prerequisite for AMP binding. The binding of AMP (at two sites per octamer) then allosterically increases affinity for isocitrate binding at the catalytic site. Allosteric inhibition by RNA is likely to occur by a very different mechanism, since current studies indicate that the presence of isocitrate in gel shift assays or in ultracentrifugation experiments is unnecessary for IDH binding of RNA. Thus, RNA may preferentially bind to a less active conformational state of the enzyme. If this is the case, then the allosteric activator AMP (plus isocitrate) could either compete directly with RNA for binding or compete indirectly by shifting the equilibrium toward a more active conformational state. We are in the process of investigating these possibilities using mutant forms of IDH with defects in allosteric responses to AMP.

There are several significant implications from these conclusions. First, on the basis of measurements made by others (*29*, *43*), we previously calculated that the molar ratio of mitochondrial mRNA:IDH holoenzyme in vivo may range from 1:1 to 1:4. On the basis of our ultracentrifugation data (Figure 4) and a reported K_d value for the *COX2* transcript of 3 nM (*41*), this would suggest that most of the mRNA molecules in vivo would be bound by enzyme. This is consistent with the model of de Jong et al. (*4*), who suggest that mitochondrial mRNAs would bind IDH unless they are being actively translated. A correlate is that a significant proportion of mitochondrial IDH could be bound to mRNA. Second, the proportion of IDH bound to RNA, and presumably inactive, would largely depend on mitochondrial concentrations of AMP (and of isocitrate). We have previously shown that inhibition produced by concentrations of RNA transcripts as high as 1.0 μM can be relieved by the presence of $\geq$25 μM AMP (*5*). Thus, while association with RNA may modulate IDH activity, levels of AMP may be the primary determinant of activity in vivo. Estimates of cellular concentrations of AMP in yeast range from 50 to 300 μM (*30*, *31*), and a cellular concentration of 500 μM for isocitrate has been reported (20), but levels and variations in mitochondrial concentrations of these metabolites are unknown. Finally, since AMP modulates IDH activity and apparently IDH association with RNA, mitochondrial levels of AMP may be a primary determinant both for catalytic function in the tricarboxylic acid cycle and for rates of translation of mitochondrial mRNAs.

In other experiments to test the specificity of enzyme binding to the 5′-untranslated regions of mitochondrial mRNAs, we found that mitochondrial $NADP^+$-specific isocitrate dehydrogenase (IDP1), but not its cytosolic counterpart (IDP2), binds transcripts containing these regions of *COX2* and *ATP9* mRNAs. Since the *COX2* transcript does not significantly affect IDP1 activity, we assume that this binding is largely nonspecific. IDP1 and IDP2 isozymes are quite similar in size, sequence, and quaternary structure. However, the purified enzymes differ dramatically in measured p*I* values, 8.5 for IDP1 and 5.5 for IDP2 (*41*). This property may be relevant to RNA binding or to our inability to detect such binding for IDP2 under the electrophoretic conditions used in these experiments.

The classic example of a metabolic enzyme with a crucial RNA-binding function is cytosolic aconitase or iron regulatory protein 1 (*44*). However, other NAD(P)-binding enzymes, including catalase (*45*) and glyceraldehyde-3-phosphate dehydrogenase (46), have been shown to be RNA-binding proteins. For the latter enzymes, it has been proposed that the dinucleotide-binding domain comprised of the classical Rossmann fold motif (*47*) has evolved for binding RNA (*48*). In several instances, binding of RNA and binding of cofactor have been demonstrated to be competitive (*45*, *46*, *48*). However, it is not clear that these observations can be extrapolated to IDH. Crystallographic analyses of several related enzymes (*8*, *9*) have indicated that the NAD^+-binding sites of decarboxylating dehydrogenases are quite distinct in that they lack the classical Rossmann fold for nucleotide (cofactor) binding. There is also experimental evidence that RNA- and NAD^+-binding sites in IDH are distinct. For example, high concentrations of NAD^+ do not appear to affect formation of the IDH/RNA complex in gel shift assays (*40*). In addition, Elzinga (*49*) used site-directed mutagenesis to identify IDH1 Lys-171, and potentially Tyr-173, as necessary for RNA binding by IDH but not for enzyme activity. Since activity depends on cofactor binding, this result implies that the RNA-binding site is unlikely to correlate with the NAD^+-binding site. We have also found that inhibition of IDH activity by high concentrations of RNA ($\geq$1 μM) is not prevented by the presence of 1 mM concentrations of NAD^+. However, we have noted and are further investigating differences in the degree of inhibition obtained at lower concentrations of RNA and NAD^+.

Grivell (*21*) has suggested that the respiratory-deficient phenotype associated with IDH gene disruption strains may correlate with effects on mitochondrial translation due to the absence of IDH as an RNA-binding protein. However, we recently found a close correlation between this phenotype and expression of mutant forms of IDH with residue substitutions in the predicted isocitrate-binding site that significantly reduces catalytic activity but not mitochondrial levels of the enzyme (*13*). Thus, it may be instructive to analyze the RNA-binding properties of these mutant forms of IDH and of mutant forms with defects in NAD^+ binding to determine if there are potential correlative effects on translation. Also, mutant forms of IDH with demonstrated defects in RNA binding, including those with substitutions in the IDH1 Lys-171 residue (*49*), should be very informative in kinetic analyses to determine the basis for RNA inhibition of catalytic activity.

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