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Analysis of Interactions with Mitochondrial mRNA Using Mutant Forms of Yeast NAD+-Specific Isocitrate Dehydrogenase†

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Abstract

Yeast NAD^+-specific isocitrate dehydrogenase (IDH) is an allosterically regulated tricarboxylic acid cycle enzyme that has been shown to bind specifically and with high affinity to 5'-untranslated regions of yeast mitochondrial mRNAs. The absence of IDH has been shown to result in reduced expression of mitochondrial translation products, leading to the suggestion that this macromolecular interaction may contribute to regulating rates of translation. The interaction with mitochondrial mRNAs also produces a dramatic inhibition of IDH catalytic activity that is specifically alleviated by AMP, the primary allosteric activator of IDH. Using mutant forms of IDH with defined catalytic or regulatory kinetic defects, we found that residue changes altering ligand binding in the catalytic site reduce the inhibitory effect of a transcript from the mitochondrial *COX2* mRNA. In contrast, residue changes altering binding of allosteric regulators do not prevent inhibition by the *COX2* RNA transcript but do prevent alleviation of inhibition by AMP. Results obtained using surface plasmon resonance methods suggest that the mRNA transcript may bind at the active site of IDH. Also, the presence of AMP has little effect on overall affinity but renders the binding of mRNA ineffective in catalytic inhibition of IDH. Finally, by expressing mutant forms of IDH *in vivo*, we determined that detrimental effects on levels of mitochondrial translation products correlate with a substantial reduction in catalytic activity. However, concomitant loss of IDH and of citrate synthase eliminates these effects, suggesting that any role of IDH in mitochondrial translation is indirect.

The reaction catalyzed by mitochondrial NAD^+-specific isocitrate dehydrogenase (IDH) is considered to be a rate limiting step in the tricarboxylic acid (TCA) cycle. The reaction is essentially irreversible under physiological conditions (1), and IDH is subject to multiple allosteric controls. The primary allosteric activator is ADP for the mammalian enzyme (2) or AMP for the *Saccharomyces cerevisiae* enzyme (3), while ATP is a negative allosteric regulator. These observations led to a hypothesis that rates of respiration are controlled at the level of IDH (4). These allosterically regulated eukaryotic IDHs are structurally complex. Yeast IDH is an octameric enzyme composed of four IDH1 and four IDH2 subunits (5). The IDH1 and IDH2 subunits are 42% identical in sequence (6,7). Mutagenesis of analogous residues in these subunits has demonstrated that the catalytic isocitrate/Mg^{2+}-binding site is primarily contained in IDH2 and shares structural similarity with the regulatory cooperative isocitrate-binding site primarily contained in IDH1 (8-10). Similarly, the cofactor NAD^+-binding site in IDH2 shares structural similarity with the allosteric AMP-binding site in IDH1 (11,12).

In addition to allosteric regulation of energy metabolism, yeast IDH has also been found to be an mRNA-binding protein (13). This was revealed in a search for proteins that bind yeast mitochondrial mRNAs to identify possible regulators of translation (14), a major point for

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control of mitochondrial gene expression. IDH was shown to bind near conserved secondary structures in the 5'-untranslated regions (UTRs) of all eight major mRNAs encoded by mitochondrial genes in yeast (15). De Jong *et al*. (16) analyzed the importance of this interaction *in vivo*. They found that while the rate of mitochondrial translation was increased in a yeast strain lacking IDH, the newly synthesized proteins were rapidly degraded, resulting in an overall reduction in their steady-state levels. Since these mitochondrially-encoded proteins are primarily hydrophobic subunits of electron transport complexes, De Jong *et al*. (16) suggested that IDH binding may normally prevent premature translation in the matrix until the mRNAs are in close proximity of known translational activators in the inner mitochondrial membrane (17,18). Therefore, IDH was proposed to act as a transient negative regulator of translation to ensure that hydrophobic subunits of respiratory complexes are synthesized only in close proximity of their cellular membrane destination.

We have elucidated another important aspect of the IDH/mRNA interaction (19). We found that addition of short RNA transcripts containing yeast mitochondrial UTRs dramatically inhibits IDH activity. Inhibition was observed with the *COX2* and *ATP9* UTRs but not with addition of other unrelated RNAs (e.g. tRNAs) of similar size. The inhibition of IDH by mitochondrial mRNA transcripts is due to a decrease in the apparent affinity of IDH for isocitrate (19). Remarkably, all inhibitory effects of the mRNA are eliminated by the presence of the allosteric activator AMP. This relief from inhibition was shown to be specific for AMP, with little effect resulting from addition of ADP, ATP, or GMP. Thus, allosteric inhibition of IDH appears to be specific for mitochondrial mRNA transcripts containing IDH-binding sites, and alleviation of this inhibition correlates with the property of allosteric activation by AMP. Importantly, these kinetic effects were obtained with relative concentrations of enzyme, mRNA, isocitrate, and AMP that are similar to reported cellular levels of these molecules (15,20,21), suggesting that these interactions may be physiologically relevant. In particular, this macromolecular interaction could provide a mechanism for coordinate regulation of rates of flux through oxidative metabolic pathways and rates of synthesis of mitochondrially-encoded proteins.

We previously used analytical ultracentrifugation techniques to examine the interaction of IDH and mitochondrial mRNAs (22). Complex formation was observed with near equimolar concentrations of the *COX2* mRNA UTR transcript and IDH holoenzyme. This macromolecular interaction was not affected by the presence of isocitrate or of AMP alone; however, the combination of isocitrate and AMP produced a moderate increase in free mRNA. This requirement for both ligands is explained by the fact that binding of the allosteric activator requires isocitrate (10). To extend these studies, we have employed mutant forms of IDH with well characterized defects that alter specific kinetic and ligand-binding properties to determine structural properties important for catalytic inhibition and for binding of a mitochondrial mRNA transcript. Effects of expression of these mutant enzymes on mitochondrial translation were also examined.

EXPERIMENTAL PROCEDURES

Yeast Strains

The parental yeast strain was MMY011 (*MATα ade2−1 can1−100 his3−11,15 leu2−3,112 trp1−1, ura3−1*; 23). Mutant strains (designated *idh1Δidh2Δ, mdh1Δ, cit1Δ, fum1Δ, sdh2Δ*, or *idh1Δidh2Δcit1Δ*) containing disruptions in genes encoding TCA cycle enzymes (IDH, malate dehydrogenase, citrate synthase, fumarase, succinate dehydrogenase, or IDH and citrate synthase, respectively) were derived from this strain as previously described (24,25).

Purification of IDH

For affinity purification, histidine-tagged wild-type and mutant forms of IDH were expressed in the *idh1Δidh2Δ* strain using a multicopy pRS426 plasmid carrying both *IDH1* and *IDH2* coding regions with authentic promoters as previously described (10,12). The *IDH1* gene contained a penta-histidine codon tag at the 3' end of the coding region and, for mutant enzymes, either the $IDH1^{His}$ or the *IDH2* gene carried a specific codon replacement. For purification of mutant IDH enzymes, transformant strains were pregrown overnight in 25 ml YNB medium (0.17% yeast nitrogen base, 0.5% ammonium sulfate, pH 6.5) with 2% glucose containing 20 μg/ml of appropriate supplements to satisfy auxotrophic requirements and lacking uracil to select for plasmid retention. The precultures were diluted into 500 ml YP medium (1% yeast extract, 2% Bacto-peptone) with 2% ethanol as the carbon source, and cells were harvested by centrifugation at A_{600nm} = 1.6−1.9. Cell pellets were lysed, and enzymes were purified using Ni^{2+}-nitrilotriacetic acid superflow resin (Ni^{2+}-NTA, Qiagen Inc.) as previously described (11). Purified enzymes were dialyzed overnight at 4°C in buffer A (10 mM Tris-HCl pH 7.4, 1.0 mM EDTA, 3 mM $MgCl_2$, 50 mM NaCl, and 5% glycerol). The dialysates were centrifuged at 14,000 rpm in a microcentrifuge for 30 min at 4°C to remove any precipitates. Purities of >95% were verified by denaturing gel electrophoresis and staining with Coomassie blue. Concentrations of purified enzymes were determined by molar extinction coefficients estimated using the method of Pace *et al.* (26).

Preparation of RNAs

A DNA template containing sequences encoding the 5'-untranslated region (UTR) of the yeast mitochondrial *COX2* mRNA was generated by polymerase chain reaction (PCR) as previously described (19). The template was used for *in vitro* transcription with a T7 MEGAshortscript kit (Ambion) following instructions provided by the supplier. Unincorporated nucleotides were removed from the transcription product using a ProbeQuant G-50 Micro Column (Amersham Pharmacia Biotech). The purity and integrity of the RNA transcript were assessed using agarose gel electrophoresis prior to storage in aliquots at −70°C. Control yeast tRNA (Sigma) was suspended in nuclease-free water and subjected to similar gel filtration and analysis. Nuclease-free water was used for all experimental manipulations involving RNA, and all glassware was pretreated with RNase ZAP (Ambion).

Kinetic Analyses

Activities of affinity-purified IDH enzymes were measured as NADH production at A_{340nm} in 100 μl assays containing 40 mM Tris-HCl (pH 7.4), 4 mM $MgCl_2$, 0.25 mM NAD^+, 30 units RNase inhibitor (ANTI-RNase, Ambion), and concentrations of the *COX2* mRNA UTR transcript ranging from 0−10 μM. Reactions were initiated by addition of DL-isocitrate. The concentration of D-isocitrate (calculated as 50% of the total isocitrate) used with each wild-type or mutant form of IDH was that producing a velocity of ~85% of the measured V_{max} value. This substrate concentration produces measurable velocities and permits detection of allosteric effects (9,19).

Surface Plasmon Resonance Analyses

Surface plasmon resonance (SPR) analyses were performed on a Biacore 3000 instrument at the Center for Macromolecular Interactions at the University of Texas Health Science Center at San Antonio. Proteins were coupled to research grade CM5 sensor chips (Biacore) using amine coupling chemistry (27) to obtain surface densities of 10,000−12,000 response units (RUs), with one RU equaling a change in concentration on the surface sensor of ~1 pg/mm^2. The proteins used were affinity-purified wild-type or mutant forms of IDH, or bovine immunoglobulin (reagent grade, Sigma) to assess non-specific association. In runs conducted at 25°C, the *COX2* UTR transcript or yeast tRNA in concentrations ranging from 0 to 10 μM

in buffer A was injected over the sensor surface. In some cases, the injection buffer also contained 2 mM DL-isocitrate, 50 μM AMP, 0.5 mM NAD^+, or 2 mM DL-isocitrate plus 50 μM AMP. Surfaces were regenerated using 10 sec injections of 400 mM NaCl. Duplicate experiments conducted within 48 h indicated no significant loss in interactions with the regenerated sensor chips. SPR data were analyzed using BIAevaluation® software (Biacore).

Analysis of Mitochondrial Translation Products

To analyze effects of expression *in vivo*, mutant forms of IDH were expressed in the *idh1Δidh2Δ* strain using single copy pRS316 plasmids containing both *IDH1* and *IDH2* genes with a codon replacement in one of the subunit genes as previously described (8,9,11,28). Wild-type levels of expression of the mutant IDH enzymes were verified by Western blot analysis conducted as previously described (5).

For analyses of mitochondrial translation products, transformant strains were precultured on minimal YNB medium with 2% glucose, then inoculated to A_{600nm} = 0.2 into rich YP medium containing 2% galactose as the carbon source. Cells were harvested at A_{600nm} = 2.0. Prior to harvesting cells, a cocktail of inhibitors (0.5 mg/ml chloramphenicol, 1.0 mg/ml cycloheximide, and 2.6 mg/ml sodium azide) was added to the cultures to stop respiration and protein synthesis. Harvested cells were lysed using glass beads with a buffer containing 8 M urea, 100 mM NaH_2PO_4, 10 mM Tris-HCl, and NaOH to a final pH of 8.0. The cleared lysates were used for protein determinations by the Bradford procedure (29) with bovine serum albumin as the standard. 30 μg samples of total cellular protein were electrophoresed on 10% polyacrylamide/sodium dodecyl sulfate gels for immunoblot analyses conducted with antisera (Molecular Probes, Inc.) against yeast mitochondrial Cox2p (1:1000 dilution), Cox3p (1:2000 dilution), or porin (1:2000 dilution). A secondary anti-mouse antiserum (Amersham Biosciences, Inc.) used at a dilution of 1:5000 was employed for chemiluminescent detection. Densitometry was conducted using ImageQuant software (Molecular Dynamics).

Construction and Kinetic Analysis of an $IDH1^{K171L}$ Mutant Enzyme

The multicopy pRS426 plasmid carrying both $IDH1^{His}$ and *IDH2* coding regions was used for mutagenesis to replace the Lys-171 codon of *IDH1* with a codon for leucine. Mutagenesis was conducted using the Stratagene Quick Change Site Directed Mutagenesis Kit, and the alteration in the *IDH1* coding sequence was verified by nucleotide sequence analysis. The $IDH1^{K171L}$ mutant enzyme was purified by affinity chromatography as described above for kinetic and RNA-binding analyses. Kinetic properties of the enzyme were assessed as previously described (9). The $IDH1^{K171L}$ gene was transferred to generate pRS316$IDH1^{K171L}$/*IDH2* for expression of the mutant enzyme at normal levels *in vivo*.

RESULTS

Effects of Residue Substitutions on RNA Inhibition of IDH Activity

The dramatic effect of a mitochondrial mRNA UTR on IDH activity is illustrated in Figure 1A (•). In this example, addition of increasing concentrations of a 130-nucleotide transcript containing the UTR of the yeast mitochondrial *COX2* mRNA inhibits IDH, with a 50% reduction in activity observed with ~40 nM mRNA. This inhibition is eliminated by the presence of 100 μM AMP (Figure 1A, ○) and by concentrations of AMP as low as 25 μM (data not shown).

To dissect the inhibitory effect of mitochondrial mRNA on IDH activity, we performed assays with mutant forms of IDH with well defined kinetic and ligand-binding defects. As summarized in Table 1, these enzymes included previously described pairs of IDH1 and IDH2 mutant enzymes containing substitutions in analogous residues that produce, respectively, regulatory

or catalytic defects. An alanine replacement for a critical conserved serine residue (designated S→A) in the isocitrate-binding site of each subunit essentially eliminates isocitrate binding by each type of subunit (10). Kinetically, this replacement (S92A) in IDH1 produces a loss of cooperativity with respect to isocitrate and a loss of allosteric activation by AMP, while the enzyme containing the analogous replacement (S98A) in IDH2 retains regulatory properties but exhibits a dramatic 160-fold decrease in apparent V_{max} (8,9). In addition, based on modeling studies, the putative isocitrate-binding sites of IDH1 and IDH2 differ in four of nine residue positions. Reciprocal replacement of the four residues in the IDH1 isocitrate-binding sites with those found in the IDH2 site, and of the four residues in the IDH2 site with those found in IDH1, produces mutant enzymes (designated Quad) with kinetic properties similar to those observed for the respective S→A mutant enzymes (9,28,Table 1). However, the Quad mutant enzymes differ from the S→A mutant enzymes in that they retain the capacity for binding isocitrate at the altered sites (10). The other pairs of mutant enzymes contain residue substitutions (designated DI→AA or H/R→A) that alter analogous nucleotide-binding sites for NAD^+ in IDH2 and for AMP in IDH1 and thus, respectively, produce primarily catalytic or regulatory kinetic defects (11,12, Table 1).

These mutant forms of IDH were purified and used in enzyme assays to determine if catalytic or regulatory defects altered the extent of inhibition of IDH by the *COX2* UTR transcript. These assays were straightforward for IDH1 mutant enzymes ($IDH1^{S→A}$, $IDH1^{Quad}$, $IDH1^{DI→AA}$, and $IDH1^{R→A}$) with primary defects in regulatory properties, since these enzymes retain substantial catalytic activity. As shown in Figure 1B (•), all four regulatory mutant enzymes were inhibited by addition of the mRNA transcript, although the concentrations of RNA producing a 50% reduction in velocity varied substantially, from ~28 nM for the $IDH1^{DI→AA}$ enzyme to ~3.0 μM for the $IDH1^{R→A}$ enzyme. Thus, alterations in cooperative isocitrate-binding or in the AMP-binding sites of IDH1 do not eliminate inhibition by the RNA transcript. Importantly, the presence of 100 μM AMP in these assays had little effect on inhibition by the *COX2* transcript (Figure 1B, ○). This result was not unexpected for mutant enzymes ($IDH1^{DI→AA}$ and $IDH1^{R→A}$) with defects in the AMP-binding site. Also, our previous demonstration that binding of AMP and allosteric activation are dependent on concomitant binding of isocitrate by IDH1 (10) explains the absence of an AMP effect on RNA inhibition of the mutant enzymes ($IDH1^{S→A}$ and $IDH1^{Quad}$) with defects in the IDH1 isocitrate-binding site. Thus, these results clearly emphasize the importance of AMP binding and activation in alleviating inhibition by the RNA transcript.

We similarly examined effects of addition of the *COX2* RNA transcript on catalytically defective IDH enzymes (Table 1). For three of these mutant enzymes, $IDH2^{S→A}$, $IDH2^{Quad}$, and $IDH2^{DI→AA}$, residual activities were very low. However, we did not detect any reduction in these activities with RNA concentrations as high as 10 μM (data not shown). For the $IDH2^{H→A}$ enzyme, which retains substantial catalytic activity (Table 1), a modest ~25% reduction was observed with RNA concentrations ranging from 10^{-3} μM to 10 μM (Figure 1C); this reduction in activity was alleviated by the presence of AMP.

These results suggest that the inhibitory effect of the *COX2* transcript on IDH activity requires intact isocitrate-Mg^{2+}/NAD^+ catalytic sites in IDH2 but does not require intact isocitrate/AMP regulatory sites in IDH1. The latter are essential, however, for the AMP-mediated elimination of inhibition.

Effects of Residue Substitutions on RNA Binding

We previously examined interactions of wild-type IDH and the *COX2* transcript using analytical ultracentrifugation, and determined that a macromolecular complex can be observed at near equimolar concentrations of enzyme and transcript (22). For the current studies, we developed surface plasmon resonance techniques to allow real-time analyses and to permit

comparison of relative affinities of various mutant forms of IDH for the *COX2* UTR transcript. We tested different affinity-based methods for coupling either the RNA or protein to sensor chips, but found these chips to be unstable during regeneration. We therefore chose to use purified wild-type or mutant forms of IDH covalently immobilized on carboxymethyldextran chips with the *COX2* UTR transcript added to the buffer passed over the sensor chips. The protein sensor chips were stable with storage, and that the *COX2* UTR transcript could be stably recovered in buffers following individual runs was verified by electrophoresis.

Representative experimental results from SPR analyses of wild-type IDH interactions with RNAs are illustrated in Figure 2. Binding to immobilized IDH is compared for similar concentrations of the *COX2* UTR transcript (Figure 2A), measured in this case in the presence of AMP and isocitrate, and of yeast tRNA (Figure 2B). An apparent K_d value of 27 nM was calculated for the *COX2* transcript (Figure 2C), whereas binding of the tRNA was much lower and not saturable under similar experimental conditions. In other controls, no binding of either RNA was observed to immobilized bovine immunoglobulin (data not shown).

Combined data for the wild-type enzyme from several different preparations of enzyme and of the *COX2* UTR transcript gave an average apparent K_d of ~15 nM in the absence of any other ligand of the enzyme. This value was not significantly affected when the RNA injection buffer also contained individual ligands (2 mM DL-isocitrate, 50 μM AMP, or 0.5 mM NAD^+). When both isocitrate and AMP were included in the injection buffer, the apparent K_d value for the wild-type enzyme increased slightly to ~26 nM (Figure 3A). Similar results previously obtained with analytical ultracentrifugation showed that the presence of AMP plus isocitrate resulted in an approximate two-fold increase in the concentration of free relative to bound transcript (22). Thus, it appears that the *COX2* transcript primarily remains bound to the 'activated' enzyme in the presence of isocitrate and AMP. However, since kinetic analyses suggest that there is no inhibition of IDH activity by the transcript in the presence of AMP (Figure 1A), the nature of this ineffective binding is different from that producing catalytic inhibition in the absence of activator.

Similar surface plasmon resonance analyses were conducted using immobilized mutant forms of IDH (Figure 3). In the absence of any IDH ligand, the apparent K_d values for the *COX2* transcript were comparable for the wild-type and for all mutant enzymes. Again, we saw no significant effect upon addition of single ligands (isocitrate, AMP, or NAD^+) to the injection buffer (data not shown). For the regulatory mutant enzymes ($IDH1^{S\rightarrow A}$, $IDH1^{Quad}$, $IDH1^{DI\rightarrow AA}$, or $IDH1^{R\rightarrow A}$), there was also little effect of addition of isocitrate plus AMP (Figure 3B). These results are compatible with kinetic analyses (Figure 1) indicating that these enzymes are catalytically inhibited by the RNA transcript. However, since these enzymes are refractive to allosteric activation (11,12), RNA binding remains effective, i.e. inhibitory, in the presence of AMP.

For the catalytically deficient mutant enzymes, the *COX2* UTR transcript was apparently bound efficiently in the absence of other ligands (Figure 3C). However, as described above, this binding had little effect on the residual catalytic activity of these enzymes, suggesting there may be some alteration in the 'effective' RNA-binding site. In addition, two of the catalytic mutant enzymes, $IDH2^{S\rightarrow A}$ and $IDH2^{H\rightarrow A}$, demonstrated substantial increases, 12-fold and 24-fold, respectively, in apparent K_d values measured in the presence of AMP and isocitrate (Figure 3C). Interestingly, the $IDH2^{Quad}$ mutant enzyme exhibited no such increase. This catalytically defective enzyme retains the capacity for binding isocitrate, whereas the $IDH2^{S\rightarrow A}$ enzyme does not (10). Since the $IDH2^{H\rightarrow A}$ enzyme is deficient in NAD^+ binding (12), these results thus suggest that residue changes that reduce either substrate or cofactor binding in the catalytic site make the enzyme refractory to inhibition by RNA and also dramatically reduce RNA binding in the presence of AMP plus isocitrate. Thus, allosteric

activation may expose changes in the RNA-binding site, decreasing affinity for the UTR transcript. [Note: The other catalytically defective mutant enzyme, $IDH2^{DI\rightarrow AA}$, is unstable during purification (12), precluding similar analysis by SPR.]

Analyses of Mitochondrial Translation Products

To examine effects of IDH on levels of mitochondrial translation products, yeast strains were grown on YP medium with galactose as the carbon source, a condition that is non-repressing for mitochondrial functions and is permissive for growth of IDH mutants. Western blot analyses of whole cell protein extracts were performed using antisera specific for two mitochondrially-encoded subunits of cytochrome *c* oxidase, Cox2p and Cox3p. Densitometry was used to compare immunochemical levels of these proteins with that for porin, a nuclearly-encoded outer membrane protein. As shown in Figure 4A, loss of IDH due to disruption of *IDH1* and *IDH2* genes produced a five-fold decrease in levels of Cox2p and a ten-fold decrease in levels of Cox3p [analyses of similar samples in Figure 5A and B illustrate experimental variation in these values]. This reduction in levels of mitochondrial translation products due to loss of IDH was previously shown by De Jong *et al.* (16) to be due to an increase in rates of degradation presumably because of premature translation in the matrix.

To determine the importance of catalytic and regulatory properties of IDH, we similarly examined effects on mitochondrial translation products due to expression of mutant forms of IDH. The mutant enzymes were expressed in the *idh1Δidh2Δ* strain using a single copy, centromere-based plasmid carrying one mutated subunit gene and the other unaltered subunit gene. Normal levels of expression of the mutant forms of IDH were verified by Western blot analysis (data not shown).

Effects on mitochondrial translation products due to expression of related pairs of IDH1 and IDH2 mutant enzymes are illustrated in Figure 4B. In each pair, expression of the IDH1 mutant enzymes ($IDH1^{S\rightarrow A}$, $IDH1^{Quad}$, $IDH1^{DI\rightarrow AA}$, or $IDH1^{R\rightarrow A}$) with regulatory kinetic defects was found to have no deleterious effect on levels of the mitochondrial translation products. In fact, relative levels of Cox2p were generally slightly elevated and levels of Cox3p were equivalent to levels in the parental strain. In contrast, expression of IDH2 mutant enzymes ($IDH2^{S\rightarrow A}$, $IDH2^{Quad}$, or $IDH2^{DI\rightarrow AA}$) with significant defects in catalytic activity resulted in decreased expression of Cox2p and Cox3p, although to a lesser extent than that produced by loss of IDH. Expression of the $IDH2^{H\rightarrow A}$ mutant enzyme, which exhibits a relatively modest reduction in apparent V_{max} (12, Table 1), produced no reduction in levels of mitochondrial translation products.

Collectively, these results suggest that reduction in levels of mitochondrial translation products correlates closely with decreased IDH activity. Alterations in allosteric regulatory properties of the enzyme have little effect on translation under these conditions.

Effects of Co-disruption of CIT1

De Jong *et al*. (16) reported that loss of malate dehydrogenase (Mdh1p), another TCA cycle enzyme, had no effect on levels of mitochondrial translation products. We confirmed this using an *mdh1Δ* disruption mutant (Figure 5A), and similarly found little effect on translation product levels in mutant strains containing disruptions of several other genes (*SDH2* encoding the iron-sulfur protein subunit of succinate dehydrogenase, *FUM1* encoding fumarase, and *CIT1* encoding citrate synthase) for yeast TCA cycle enzymes. While these data suggest that the detrimental effect on mitochondrial translation products is specific to IDH defects, we reasoned that this effect might be due to other factors resulting from a specific block at this point in the TCA cycle. Also, we and others have previously reported (21,25,30) that many of the phenotypes associated with loss of IDH are eliminated or moderated by concomitant loss of

mitochondrial citrate synthase (Cit1p). Therefore, we examined effects on mitochondrial translation products in a strain (*Δidh1Δidh2Δcit1*) lacking both IDH and Cit1p. As shown in Figure 5B, the relative level of Cox2p in this strain was essentially equivalent to that in the parental strain, and the relative level of Cox3p exceeded that in the parental strain. Thus, loss of Cit1p appears to eliminate the detrimental effects of loss of IDH on mitochondrial translation.

Since there is no detrimental effect on mitochondrial translation products in the *Δidh1Δidh2Δcit1* strain, the reduction in levels of these proteins observed with loss of IDH alone (or with catalytic dysfunction of IDH) is apparently not due simply to the absence of (or changes in) a physical interaction between IDH and mitochondrial mRNAs.

Effects of a Residue Change in the RNA-binding Site

Finally, we have extended these analyses to examine a mutant enzyme containing a substitution (K171L) in the putative RNA-binding site of IDH (31). Based on structural modeling of IDH with the known structure of *Escherichia coli* $NADP^+$-specific isocitrate dehydrogenase (32), Lys-171 was predicted to lie in a cleft on the surface of IDH. The K171L substitution was reported by Elzinga (31) to reduce formation of a *COX2* UTR transcript/IDH complex in gel shift assays. We constructed plasmids for expression of the $IDH1^{K171L}$ mutant enzyme *in vivo* and for affinity purification. Kinetic characterization of the purified mutant enzyme indicated that the K171L substitution had little effect on catalytic or allosteric regulatory properties of IDH (summarized in Table 1).

We examined the effects of RNA on activity of the $IDH1^{K\rightarrow L}$ enzyme. As shown in Figure 1D, the *COX2* UTR transcript had little effect on activity of this mutant enzyme, producing a moderate ~20% reduction in activity at the highest concentration of RNA. The presence of AMP did alleviate this slight reduction in activity. Thus, in addition to competent catalytic sites in IDH2, binding of the RNA by residues not involved in catalysis is apparently important for inhibition of IDH.

Results of SPR analyses with the $IDH1^{K\rightarrow L}$ mutant enzyme (Figure 3D) showed that the enzyme binds the *COX2* UTR transcript in the absence of other ligands with an apparent K_d similar to that of the wild-type enzyme. However, since the $IDH1^{K\rightarrow L}$ enzyme is refractory to inhibition by the transcript, some feature of the effective RNA-binding site is presumably different. An ~10-fold increase in the apparent K_d for this enzyme was measured in the presence of isocitrate plus AMP (Figure 3D), suggesting, as observed for the catalytic mutant enzymes, that the activated conformation of IDH exposes this change in the RNA-binding site, and this change reduces the affinity of the enzyme for the RNA transcript.

Finally, using a single-copy pRS316*IDH1*^{K171L}*/IDH2* plasmid, we examined effects of expression of the $IDH1^{K171L}$ mutant enzyme on levels of mitochondrial translation products. As shown in Figure 4C, expression of this enzyme did not result in reduced levels of Cox2p or Cox3p. This result is consistent with analyses of other mutant enzymes described above, in that detrimental effects on levels of these proteins were observed only with expression of IDH mutant enzymes with significant catalytic dysfunctions. It also suggests that defects reducing the interaction of IDH with mitochondrial mRNA UTRs have no effect on mitochondrial translation, consistent with our conclusion that such interactions do not directly control this process.

DISCUSSION

A reduction in levels of mitochondrial translation products in a yeast strain lacking the TCA cycle enzyme IDH was previously reported by De Jong *et al*. (16). Based on this and other

observations including increased rates of mitochondrial translation and of protein turnover in this mutant strain, they proposed that IDH normally binds the mitochondrial mRNA UTRs and inhibits their translation until contact is made with mRNA-specific translational activators in the inner mitochondrial membrane. In this report, we also observed similar reductions in levels of mitochondrial translation products with expression of mutant forms of IDH with substantial catalytic defects, i.e. with apparent V_{max} values 150-fold or lower than that of the wild-type enzyme. This indicates that the deleterious effects on mitochondrial translation are due to catalytic dysfunction of IDH, not simply to its physical absence. In addition, we have found these alterations in mitochondrial protein levels due to the loss of IDH are eliminated by co-disruption of the *CIT1* gene encoding citrate synthase. Thus, effects on mitochondrial translation or protein stability are apparently independent of any direct interaction between IDH and the mitochondrial mRNA UTRs. Instead, it appears that loss or catalytic dysfunction of IDH indirectly affects mitochondrial translation, and these effects are suppressed by concomitant loss of Cit1p.

Several other phenotypes associated with loss of IDH have been reported to be moderated by loss of Cit1p. For example, IDH disruption mutants, as well as strains expressing several of the catalytically defective forms of IDH used in this study, exhibit increased frequencies of generation of petite colonies lacking functional mitochondrial DNA (9). These frequencies are reduced to levels similar to that exhibited by the parental strain by concomitant loss of Cit1p (30). In addition, the slow growth of IDH gene disruption mutants on glycerol as a carbon source is suppressed by loss or inactivation of Cit1p (21). Finally, in a global microarray analysis of gene expression in TCA cycle gene disruption mutants, a set of 20 genes that exhibits elevated expression in IDH mutants but depressed expression in a Cit1p mutant was found to exhibit intermediate levels of expression in the double mutant (25). Thus, one possible explanation for these observations and for our current findings related to mitochondrial translation is that a deleterious protein(s) may be expressed to high levels in IDH mutants, and this expression is reduced by defects in Cit1p. Alternatively, some metabolite (e.g. citrate, aconitate, or isocitrate) may accumulate to detrimental levels in mutants lacking IDH or expressing a catalytically inactive enzyme, and this accumulation may be alleviated by loss of Cit1p. We are currently examining these possibilities.

The substantial inhibitory effects of mitochondrial UTR transcripts on IDH activity and the specificity of this macromolecular interaction demonstrated by us and others (15,19,31) suggest that the interaction may modulate IDH activity *in vivo*. Also, the low apparent K_d value for the interaction between IDH and the *COX2* UTR transcript, along with estimates of near-equimolar organellar concentrations of IDH holoenzyme and total mitochondrial mRNA (15,20), suggests substantial complex formation is physiologically feasible. Thus, as illustrated in Figure 6, mitochondrial IDH may exist in an equilibrium between free and mRNA-bound states. The free form of IDH is active and can be allosterically activated (i.e. affinity for isocitrate is increased) by an elevation in AMP levels. Our current and previous results (19,22) suggest that the mRNA-bound form of IDH is inactive in the absence of AMP, and that elevated levels of AMP (plus isocitrate) alleviate this inhibition but have little effect on overall affinity of the enzyme for RNA. Thus, we predict that activation by AMP allosterically alters the macromolecular interaction to permit catalysis but not dissociation (Figure 6), with the RNA binding being either effective (i.e., producing inhibition) in the absence of AMP or ineffective in the presence of AMP. Overall, the interaction with mRNA could provide a regulatory mechanism to reduce IDH activity below levels exhibited by the free enzyme and, consequently, reduce TCA cycle flux under conditions of energy sufficiency, i.e. when mitochondrial levels of AMP are relatively low.

Several lines of evidence suggest that the *COX2* UTR transcript may bind at or near the active site of IDH. The transcript reduces activity of the wild-type enzyme by decreasing apparent

affinity for isocitrate (19) and for NAD^+.[2] We also observe little reduction in activity of catalytically defective mutant forms of IDH, suggesting that residue changes in the active site reduce effective binding of the mRNA. In addition, residue changes in the active site in IDH2 that alter binding of isocitrate/Mg^{2+} or NAD^+ (10,12) produce a decrease in apparent affinity for the RNA in the activated state, i.e. in the presence of AMP plus isocitrate (Figure 3). Consistent with this interpretation, the residue changes in the catalytically defective $IDH2^{Quad}$ enzyme do not alter the apparent affinity for RNA in the activated state, presumably because isocitrate binding is conserved in this mutant enzyme (12). Thus, intact ligand-binding active sites appear to be important both for suppression of catalysis by the UTR transcript and for retention of an ineffective association with RNA in the activated state.

Results obtained with the $IDH1^{K171L}$ mutant enzyme suggest residues outside the active sites in IDH are also important for effective inhibitory interaction with the *COX2* UTR transcript. Elzinga (31) previously reported that this mutant enzyme fails to bind the same transcript in gel shift assays. Using different buffer conditions and our SPR method, we found that the $IDH1^{K171L}$ enzyme does bind the *COX2* transcript in the absence of AMP (Figure 3D) but is not inhibited by high concentrations of transcript (Figure 1D), implying that the effective RNA-binding site is altered in this mutant enzyme. An alteration in the macromolecular interaction is also substantiated by the increase in apparent K_d measured in the presence of AMP (Figure 3D). It is not surprising that the interaction may involve multiple residues in IDH, since the UTRs of mitochondrial mRNAs are quite large. In fact, the *COX2* UTR transcript used in these studies, which has a M_r approximately equivalent to that of an IDH subunit, represents the shortest of the UTRs in yeast mitochondrial mRNAs (17,18). Expression of the $IDH1^{K171L}$ mutant enzyme has no obvious detrimental effect on mitochondrial translation, a finding also previously communicated by L. A. Grivell in a review by Contamine and Picard (33). This suggests, as discussed above, that the direct interaction of IDH with mitochondrial mRNA UTRs does not regulate rates of translation.

Our results clearly demonstrate that allosteric activation of IDH by AMP can eliminate inhibition by mitochondrial UTR transcripts (19, Figure 1A). Mutant enzymes with defects in the cooperative isocitrate-binding site or in the allosteric AMP-binding site primarily contained in IDH1 are inhibited by the presence of the *COX2* transcript, but inhibition is not relieved by the presence of AMP plus isocitrate (Figure 1B). While the concentration of *COX2* UTR transcript producing a 50% reduction in activity varies substantially within this group of mutant enzymes, these enzymes exhibit little difference in apparent affinity for the RNA transcript in the presence or absence of AMP plus isocitrate (Figure 3B), consistent with the idea that RNA binding is insufficient for inactivation. These observations also suggest that, while normal transmission of allosteric regulatory signals to the active site does not appear to be essential for binding of the mRNA transcript, it does control whether this binding is effective or ineffective in inhibition of catalysis.

It remains to be determined the extent that interactions with mitochondrial mRNA UTRs control IDH activity *in vivo*. The conditions used in this study were permissive for growth of strains lacking IDH or of strains expressing mutant forms of IDH. It will be instructive to examine other steady-state growth conditions that are less permissive for growth of strains expressing catalytically defective enzymes (30) and transition-state conditions that we have found to impact growth of strains expressing allosterically defective enzymes.[3] Future studies will also examine effects of altering mitochondrial levels of mRNA UTR transcripts.

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

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Abbreviations

IDH, NAD^+-specific isocitrate dehydrogenase; UTR, untranslated region; SPR, surface plasmon resonance.

REFERENCES

1. Barnes LD, McGuire JJ, Atkinson DE. Yeast diphosphopyridine nucleotide specific isocitrate dehydrogenase. Regulation of activity and unidirectional catalysis. Biochemistry 1972;11:4322–4329. [PubMed: 4342903]
2. Chen RF, Plaut GWE. Activation and inhibition of DPN-linked isocitrate dehydrogenase of heart by certain nucleotides. Biochemistry 1963;2:1023–1032. [PubMed: 14087354]
3. Kornberg A, Pricer WE. Di- and triphosphopyridine nucleotide isocitric dehydrogenases in yeast. J. Biol. Chem 1951;189:123–136. [PubMed: 14832224]
4. Hathaway JA, Atkinson DE. The effect of adenylic acid on yeast nicotinamide adenine dinucleotide isocitrate dehydrogenase, a possible metabolic control mechanism. J. Biol. Chem 1963;238:2875–2881. [PubMed: 14063317]
5. Keys DA, McAlister-Henn L. Subunit structure, expression, and function of NAD(H) specific isocitrate dehydrogenase in *Saccharomyces cerevisiae*. J. Bacteriol 1990;172:4280–4287. [PubMed: 2198251]
6. Cupp JR, McAlister-Henn L. NAD^+-dependent isocitrate dehydrogenase: cloning, nucleotide sequence, and disruption of the *IDH2* gene from *Saccharomyces cerevisiae*. J. Biol. Chem 1991;266:22199–22205. [PubMed: 1939242]
7. Cupp JR, McAlister-Henn L. Cloning and characterization of the gene encoding the IDH1 subunit of NAD^+-dependent isocitrate dehydrogenase from *Saccharomyces cerevisiae*. J. Biol. Chem 1992;267:16417–16423. [PubMed: 1644826]
8. Cupp JR, McAlister-Henn L. Kinetic analysis of NAD^+-isocitrate dehydrogenase: the contribution of IDH1 and IDH2 subunits to regulation and catalysis. Biochemistry 1993;32:9323–9328. [PubMed: 8369302]
9. Lin A-P, McCammon MT, McAlister-Henn L. Kinetic and physiological consequences of alterations in homologous isocitrate-binding sites of yeast NAD^+-specific isocitrate dehydrogenase. Biochemistry 2001;40:14291–14301. [PubMed: 11714283]
10. Lin A-P, McAlister-Henn L. Isocitrate binding at two functionally distinct sites in yeast NAD^+-specific isocitrate dehydrogenase. J. Biol. Chem 2002;277:22475–22483. [PubMed: 11953438]
11. Zhao W-Z, McAlister-Henn L. Affinity purification and kinetic analysis of mutant forms of yeast NAD^+-specific isocitrate dehydrogenase. J. Biol. Chem 1997;272:21811–21877. [PubMed: 9268311]
12. Lin A-P, McAlister-Henn L. Homologous binding sites in yeast isocitrate dehydrogenase for cofactor (NAD^+) and allosteric activator (AMP). J. Biol. Chem 2003;278:12864–12872. [PubMed: 12562755]
13. Elzinga SD, Bednarz AL, van Oosterum K, Dekker PJ, Grivell LA. Yeast mitochondrial NAD(+)-dependent isocitrate dehydrogenase as an RNA-binding protein. Nucleic Acids Res 1993;21:5328–5331. [PubMed: 7505425]
14. Papadopoulou B, Dekker P, Blom J, Grivell LA. A 40 kd protein binds specifically to the 5′-untranslated regions of yeast mitochondrial mRNAs. EMBO J 1990;9:4135–4143. [PubMed: 1701144]
15. Dekker PJT, Stuurman J, van Oosterum K, Grivell LA. Determinants for binding of a 40 kDa protein to the leaders of yeast mitochondrial mRNAs. Nucleic Acids Res 1992;21:2647–2655. [PubMed: 1377379]

16. De Jong L, Elzinga SD, McCammon MT, Grivell LA, van der Spek H. Increased synthesis and decreased stability of mitochondrial translation products in yeast as a result of loss of mitochondrial (NAD(+))-dependent isocitrate dehydrogenase. FEBS Lett 2000;483:62–66. [PubMed: 11033357]

17. Costanzo MC, Fox TD. Control of mitochondrial gene expression in *Saccharomyces cerevisiae*. Ann. Rev. Genet 1990;24:91–113. [PubMed: 2088182]

18. Dieckmann CL, Staples RR. Regulation of mitochondrial gene expression in *Saccharomyces cerevisiae*. Int. Rev. Cytol 1994;152:145–181. [PubMed: 8206703]

19. Anderson SL, Minard KI, McAlister-Henn. Allosteric inhibition of NAD^+-specific isocitrate dehydrogenase by a mitochondrial mRNA. Biochemistry 2000;39:5623–5629. [PubMed: 10801312]

20. Mueller DM, Getz GS. Steady state analysis of mitochondrial RNA after growth of yeast *Saccharomyces cerevisiae* under catabolite repression and derepression. J. Biol. Chem 1986;261:11816–11822. [PubMed: 2427512]

21. Gadde DM, McCammon MT. Mutations in the *IDH2* gene encoding the catalytic subunit of the yeast NAD^+-dependent isocitrate dehydrogenase can be suppressed by mutations in the *CIT1* gene encoding citrate synthase and other genes of oxidative metabolism. Arch. Biochem. Biophys 1997;344:139–149. [PubMed: 9244391]

22. Anderson SL, Schirf V, McAlister-Henn L. Effect of AMP on mRNA binding by yeast isocitrate dehydrogenase. Biochemistry 2002;41:7065–7073. [PubMed: 12033940]

23. McCammon MT, Veenhuis M, Trapp SB, Goodman JM. Association of glyoxylate and beta-oxidation enzymes with peroxisomes of *Saccharomyces cerevisiae*. J. Bacteriol 1990;172:5816–5827. [PubMed: 2211514]

24. Przybyla-Zawislak B, Gadde DM, Ducharme K, McCammon MT. Genetic and biochemical interactions involving tricarboxylic acid cycle (TCA) functions using a collection of mutants defective in all TCA cycle genes. Genetics 1999;152:153–166. [PubMed: 10224250]

25. McCammon MT, Epstein CB, Przybyla-Zawislak B, McAlister-Henn L, Butow RA. Global transcription analysis of Krebs tricarboxylic acid cycle mutants reveals an alternating pattern of gene expression and effects on hypoxic and oxidative genes. Mol. Biol. Cell 2003;14:958–972. [PubMed: 12631716]

26. Pace CN, Vajdos F, Fee L, Grimsley G, Gray T. How to measure and predict the molar absorption coefficient of a protein. Protein Sci 1995;4:2411–2423. [PubMed: 8563639]

27. Johnsson B, Lofas S, Lindquist G. Immobilization of proteins to a carboxymethyldextran-modified gold surface for biospecific interaction analysis in surface plasmon resonance sensors. Anal. Biochem 1991;198:268–277. [PubMed: 1724720]

28. Panisko, EA. Ph.D. Dissertation. University of Texas Health Science Center at San Antonio; 2000. Subunit interactions of *Saccharomyces cerevisiae* NAD^+- dependent isocitrate dehydrogenase.

29. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem 1976;72:248–254. [PubMed: 942051]

30. McCammon MT, McAlister-Henn L. Multiple cellular consequences of isocitrate dehydrogenase isozyme dysfunction. Arch. Biochem. Biophys 2003;419:222–233. [PubMed: 14592466]

31. Elzinga, E. Ph.D. Dissertation. Universiteit van Amsterdam; 2001. The role of yeast NAD^+-isocitrate dehydrogenase in mitochondrial translation.

32. Stoddard BL, Dean A, Koshland DE. Structure of isocitrate dehydrogenase with isocitrate, nicotinamide adenine dinucleotide phosphate, and calcium at 2.5-Å resolution: a pseudo-Michaelis ternary complex. Biochemistry 1993;32:9310–9316. [PubMed: 8369300]

33. Contamine V, Picard M. Maintenance and integrity of the mitochondrial genome: a plethora of nuclear genes in the budding yeast. Microbiol. Mol. Biol. Rev 2000;64:281–315. [PubMed: 10839818]

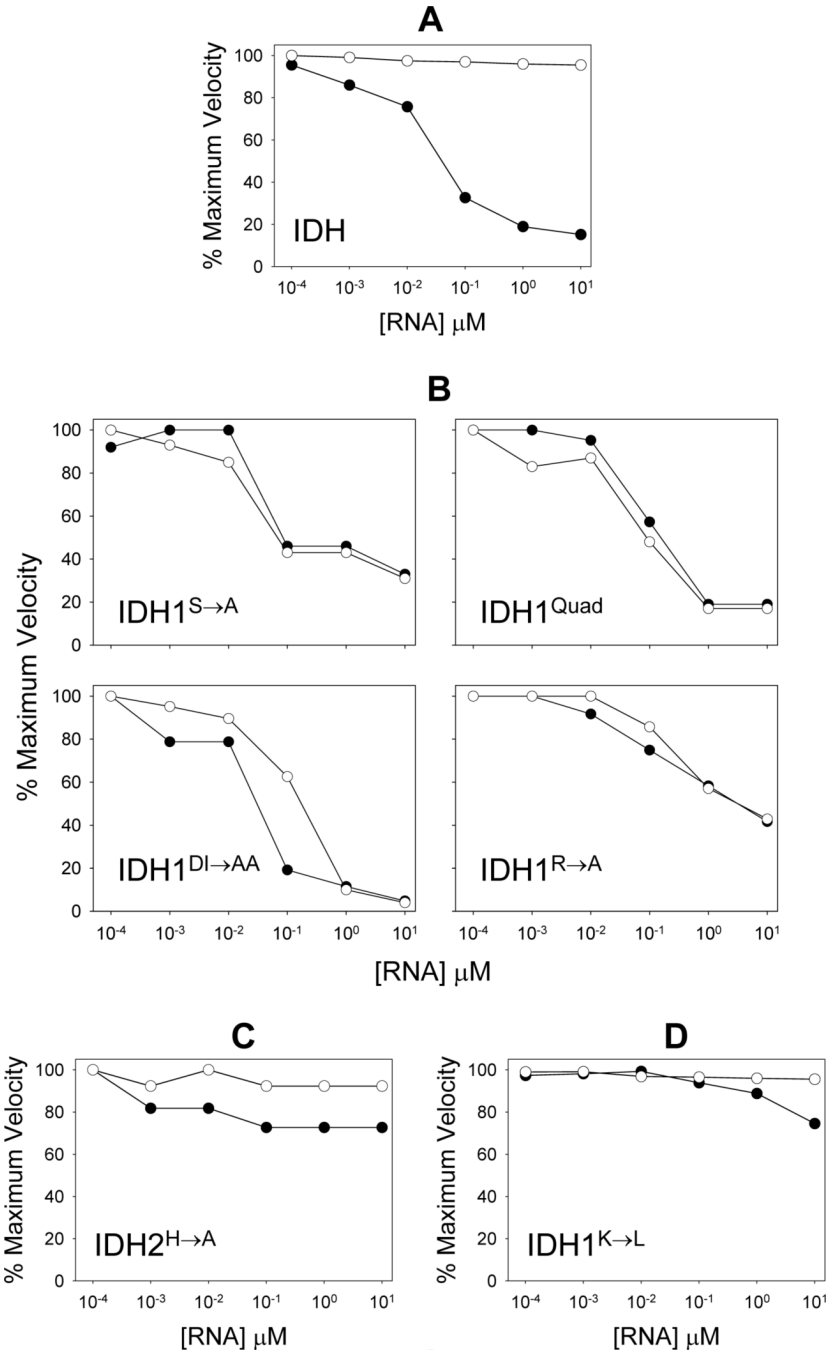

FIGURE 1.

Effect of the *COX2* mRNA UTR on IDH activity. IDH assays were conducted as described under Experimental Procedures using affinity-purified wild-type IDH (A) or affinity-purified mutant enzymes (B, C, and D) as indicated. Increasing concentrations of the *COX2* UTR transcript were added to the enzymatic assays conducted in the absence (•) or presence (○) of 100 μM AMP. Values for velocity represent the average of two or three independent determinations and are presented as percent of the activity measured in the absence of RNA.

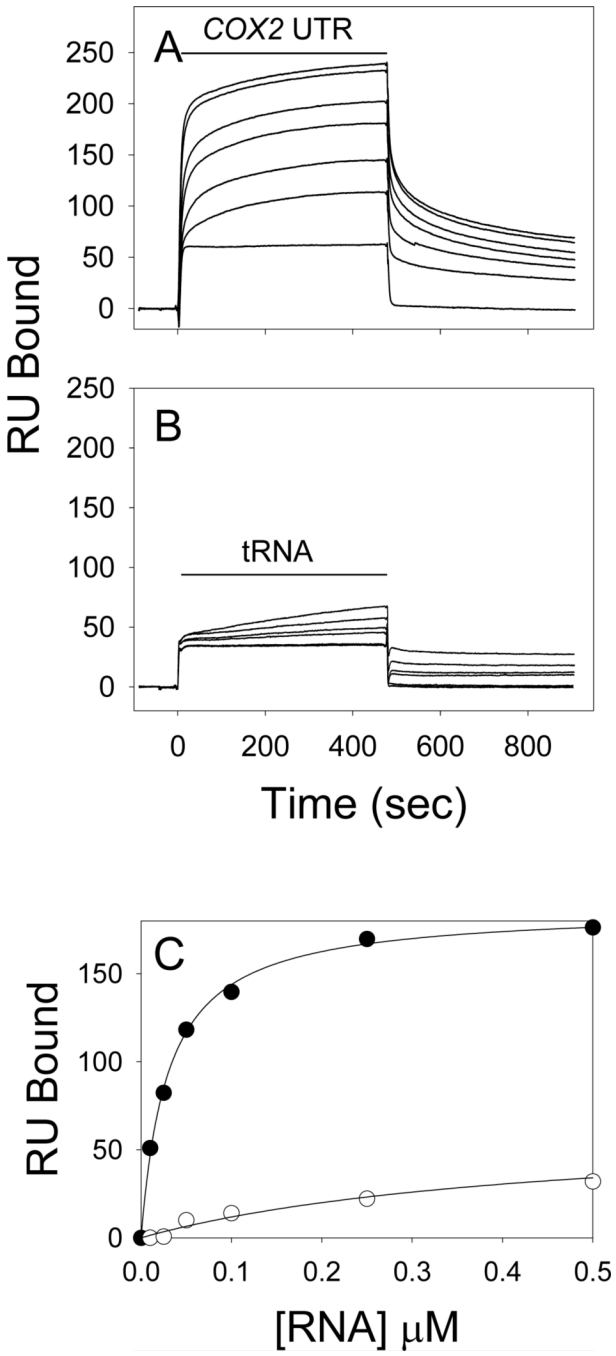

FIGURE 2.

SPR analysis of the interactions of IDH with RNAs. Interactions of wild-type IDH coupled to sensor chips with buffer concentrations of *COX2* mRNA UTR (A) or of yeast tRNA (B) ranging from 0 μM to 0.5 μM were analyzed using SPR. Runs in A were conducted in the presence of 2 mM DL-isocitrate and 50 μM AMP. Times of RNA injections are indicated by bars. The lowest trace in each experiment was recorded in the absence of RNA and reflects differences in refractive index caused by introducing buffer alone. This response was subtracted from responses recorded in the presence of RNA, and the maximal response at the stop of each injection was plotted (C) as a function of the concentration of *COX2* UTR RNA (•) or of tRNA

(○). The data were fit to rectangular hyperbolic equations, and the concentration of *COX2* mRNA producing half maximal binding was taken as the apparent K_d.

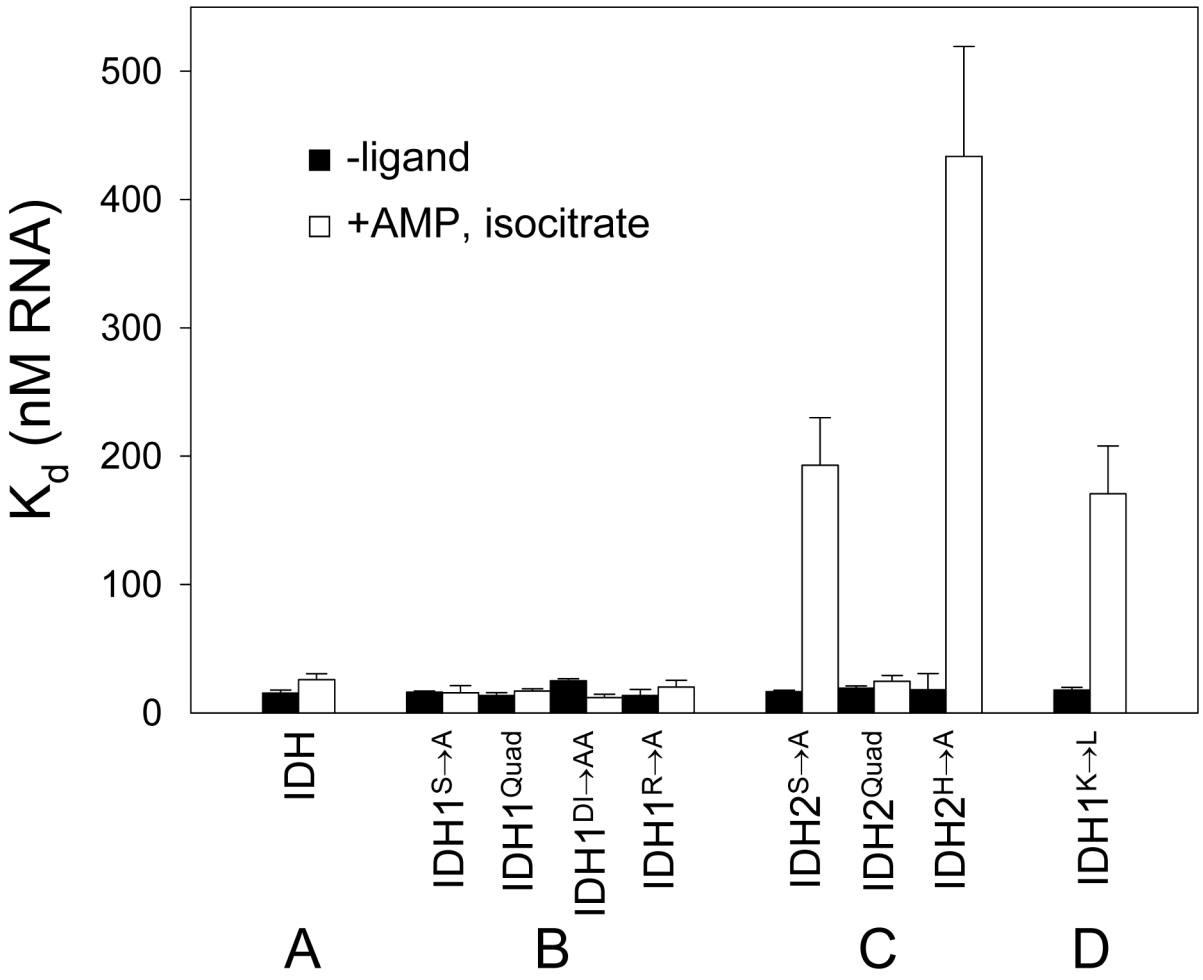

FIGURE 3.

Apparent affinities of wild-type and mutant forms of IDH for the *COX2* mRNA transcript. Data from SPR measurements conducted with buffer containing no ligand (black bars) or containing 2 mM DL-isocitrate and 50 μM AMP (white bars) were used to calculate apparent K_d values of immobilized forms of IDH for the *COX2* UTR transcript as described under Experimental Procedures and in the legend for Figure 2. Values were obtained from duplicate samples in two or three independent experiments.

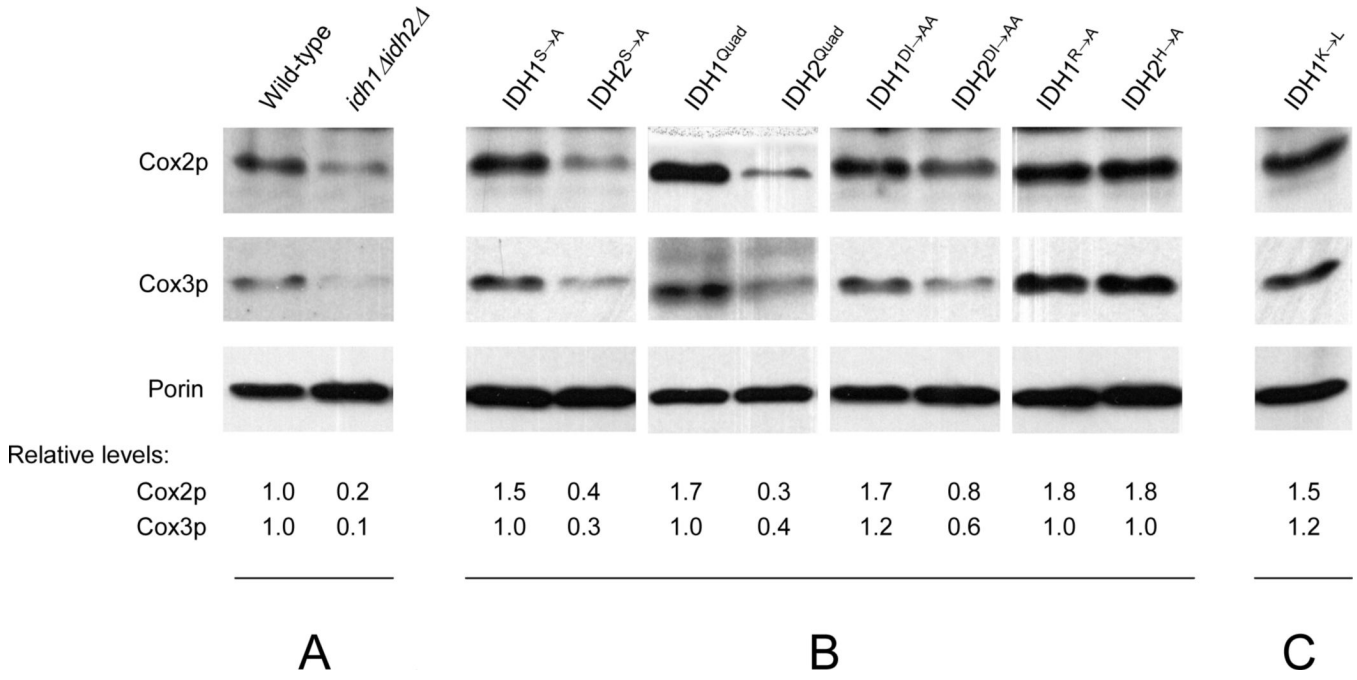

FIGURE 4.

Effects of loss or of expression of mutant forms of IDH on levels of mitochondrial translation products. Total cellular proteins (30 μg samples) from the parental and *Δidh1Δidh2* strains (A), or from the *Δidh1Δidh2* strain transformed with plasmids to express the indicated mutant forms of IDH (B and C), were electrophoresed and used for Western blot analysis with antisera specific for Cox2p, Cox3p, or porin. Densitometry was used to compare relative levels of Cox2p or Cox3p to porin, and these values were expressed relative to those observed for the parental wild-type strain (set at 1.0).

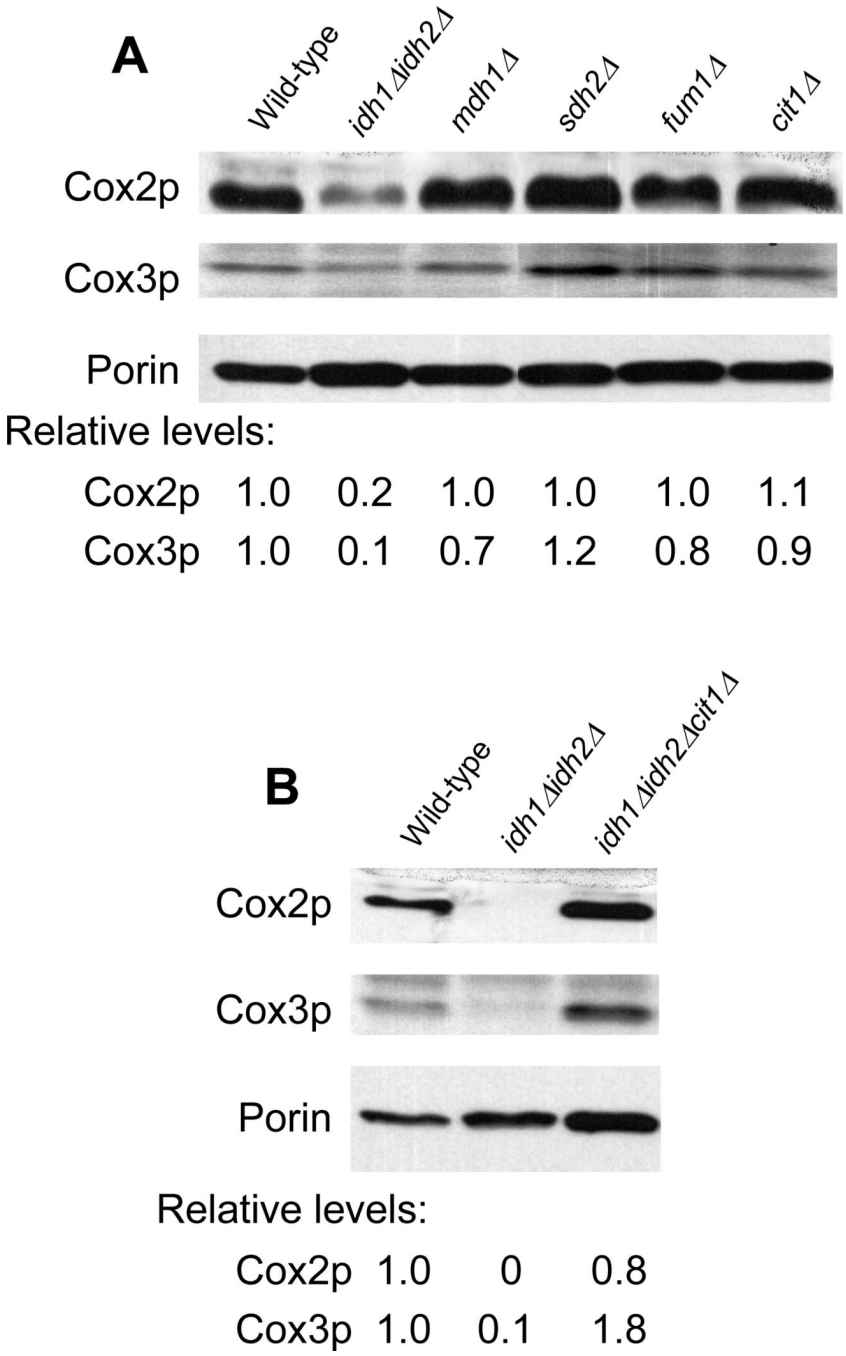

FIGURE 5.

Effects of loss of TCA cycle enzymes on levels of mitochondrial translation products. Total cellular proteins (30 μg samples) from the parental strain and from strains containing disruptions in the indicated genes encoding individual TCA cycle enzymes (A) were electrophoresed and used for Western blot analysis with antisera specific for Cox2p, Cox3p, or porin. Similar analyses were conducted with the parental strain and with mutant strains lacking either IDH or both IDH and Cit1p (B). Densitometry was used to compare relative levels of Cox2p or Cox3p to porin, and these values were expressed relative to those observed for the parental wild-type strain (set at 1.0).

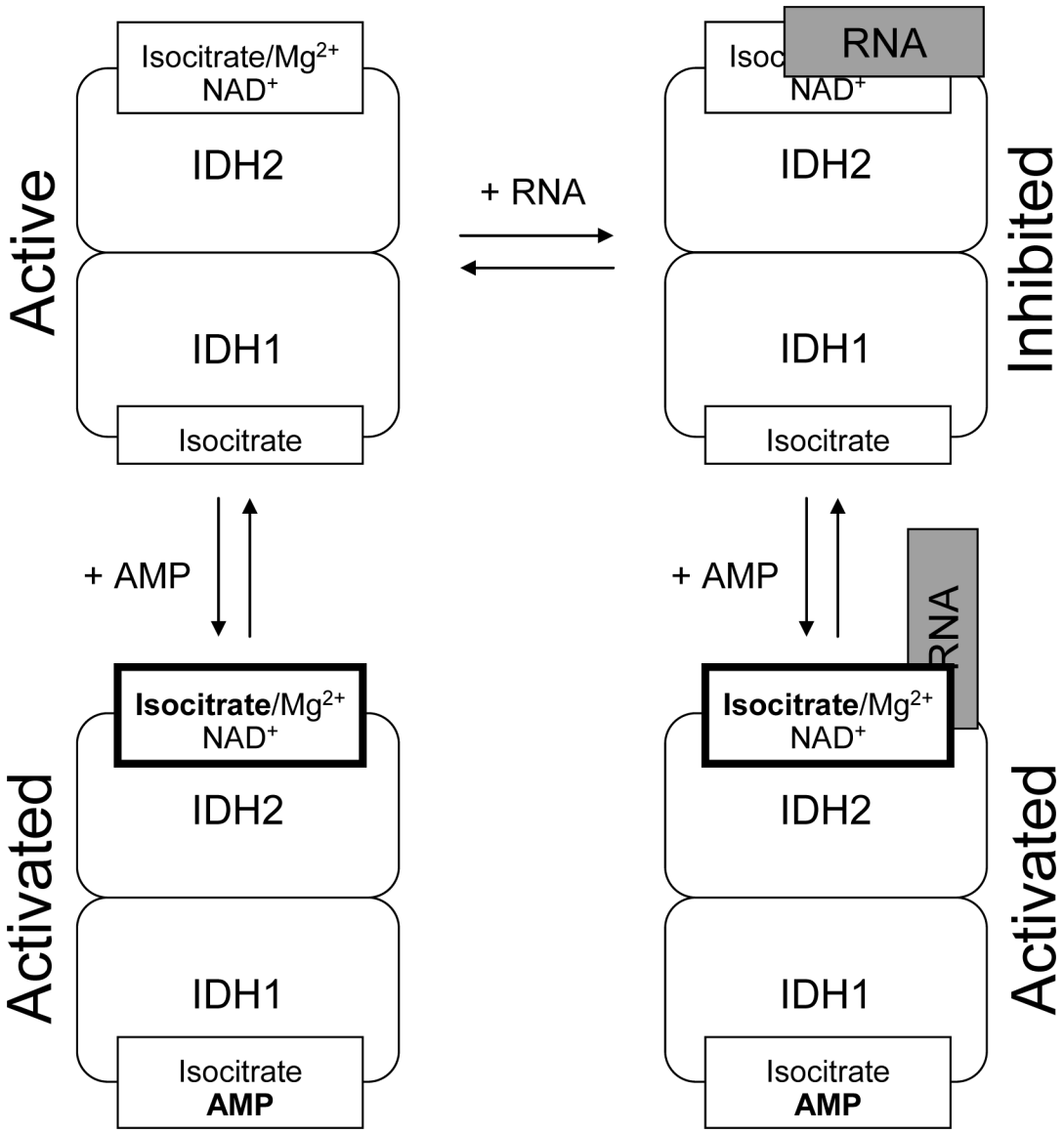

FIGURE 6.

Model for the interaction of IDH with a mitochondrial UTR transcript. For simplicity, IDH is represented as a dimer of IDH1 and IDH2 subunits. The equilibrium between free active IDH and inhibited IDH is determined by binding of mitochondrial mRNA UTRs (RNA). The presence of AMP (and isocitrate binding to IDH1) produces activated forms (indicated by bold boxes in IDH2 active sites) of both the free enzyme and the RNA-bound enzyme. Based on current results, the effective (inhibited) RNA-binding state is represented as an occlusion of the active site in IDH2 by the RNA, whereas the ineffective (activated) RNA-binding state is represented by displacement of the RNA from the active site.

Table 1

Properties of Mutant IDH Enzymes

designation	mutant enzyme	major defect	V_{max}[a]	cooperativity (isocitrate)[a]	AMP activation[a]
regulatory mutant enzymes:					
$IDH1^{S\rightarrow A}$	$IDH1^{S92A}$/IDH2	cooperative binding of isocitrate (9,10)	↓14×	–	–
$IDH1^{Quad}$	$IDH1^{A108R,F136Y,T241D,N245D}$/IDH2	″ (9,10)	↓17×	–	–
$IDH1^{DI\rightarrow AA}$	$IDH1^{D279A,I280A}$/IDH2	allosteric binding of AMP (11,12)	↓2×	+	–
$IDH1^{R\rightarrow A}$	$IDH1^{R274A}$/IDH2	″ (12)	↓8×	–	–
catalytic mutant enzymes:					
$IDH2^{S\rightarrow A}$	$IDH1/IDH2^{S98A}$	catalytic binding of isocitrate (9,10)	↓160×	+	+
$IDH2^{Quad}$	$IDH1/IDH2^{R114A,Y142F,D248T,D252N}$	″ (9,10)	↓150×	+	+
$IDH2^{DI\rightarrow AA}$	$IDH1/IDH2^{D286A,I287A}$	catalytic binding of NAD^+ (11,12)	↓300×	+	+
$IDH2^{H\rightarrow A}$	$IDH1/IDH2^{H281A}$	″ (12)	↓9×	+	+
RNA-binding mutant enzyme:					
$IDH1^{K\rightarrow L}$	$IDH1^{K171L}$/IDH2	RNA binding (31)	NR[b]	+	+

[a] Kinetic parameters previously reported for wild-type IDH (9,12) include: V_{max} = ~30 units/mg, $S_{0.5}$ values for D-isocitrate of ~0.5 mM in the absence of AMP and of ~0.1 mM in the presence of 100 μM AMP, Hill coefficients of 3.5−4.0 with respect to isocitrate, and an $S_{0.5}$ value of ~0.2 mM for NAD^+.

[b] NR = no significant reduction relative to wild-type IDH. Kinetic parameters measured in this study for the $IDH1^{K\rightarrow L}$ mutant enzyme include: V_{max} = ~20 units/mg, $S_{0.5}$ values for D-isocitrate of ~0.6 mM in the absence of AMP and of ~0.2 mM in the presence of 100 μM AMP, Hill coefficients of 3.2−4.5 with respect to isocitrate, and an $S_{0.5}$ value of ~0.2 mM for NAD^+.