Regulators of mitochondrial translation in Saccharomyces cerevisiae

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Chapter II

Characterization of the RNA-binding properties of (NAD\(^+\))-linked isocitrate dehydrogenase (Idh) from *Saccharomyces cerevisiae*

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Submitted for publication.
The Krebs cycle enzyme (NAD\(^+\))-dependent isocitrate dehydrogenase (Idh) in the yeast *Saccharomyces cerevisiae* binds to the 5'-untranslated region (UTR) of all mitochondrial mRNAs (Elzinga *et al.*, 1993). Recently we obtained evidence that RNA-binding by Idh inhibits translation of mitochondrial mRNAs (De Jong *et al.*, 2000). For further understanding of the physiological significance of RNA-binding, detailed information about the nature of this interaction is needed. Here, we report that Idh binds the COX2 mRNA leader with high affinity (K\(_d\) = 3 nM), and that isocitrate, NAD and AMP do not affect RNA-binding. In addition, we searched for a mutant of Idh that is unable to bind RNA but still contains normal enzyme activity, in relation to Krebs cycle function. Since no obvious RNA-binding regions are present in either Idh1p or Idh2p, we analyzed the RNA-binding capacity of Idh in other yeast species. We utilized the observation that Idh isolated from *Kluyveromyces lactis* has a strongly reduced affinity for mitochondrial mRNA leaders (Elzinga *et al.*, 2000). Expression of *K. lactis* IDH genes in *S. cerevisiae* idh-null strains resulted in the formation of heterologous Idh complexes displaying reduced RNA-binding activity, confirming that both Idh1p and Idh2p from *S. cerevisiae* are involved in RNA-binding. Mutational analysis of Idh1p from *S. cerevisiae*, based on alignment with the deduced amino acid sequence from *K. lactis*, resulted in Idh mutants exhibiting strongly reduced RNA-binding activity while enzymic activity appeared unaltered.

**Introduction**

In search of an RNA-binding factor involved in regulation of mitochondrial translation in *S. cerevisiae*, we demonstrated that the Krebs cycle enzyme (NAD\(^+\))-dependent isocitrate dehydrogenase (Idh) binds specifically to the 5'-untranslated region (UTR) of all mitochondrial mRNAs (Dekker *et al.*, 1992; Elzinga *et al.*, 1993). Idh consists of a heterodimer formed between related subunits encoded by the nuclear *IDH1* and *IDH2* genes. Neither subunit contains recognizable RNA-binding motifs, yet both are required for RNA-binding activity (Elzinga *et al.*, 1993). Pulse-chase protein labelling studies in isolated mitochondria from an Idh disruption mutant reveal a strong (2-3 fold) increase in the synthesis of mitochondrial translation products, as compared to wild-type. Strikingly, the newly synthesised proteins are more short-lived than in mitochondria from wild-type cells, their degradation occurring with a 2-3 fold reduced half-life (de Jong *et al.*, 2000). Enhanced degradation of translation products
is also a feature of yeast mutants in which tethering/docking of mitochondrial mRNAs is disturbed (Sanchirico et al., 1998). We have therefore suggested that binding of Idh to mitochondrial mRNAs may suppress inappropriate translation of mitochondrial mRNAs (de Jong et al., 2000). In addition, we and others recently observed that in vitro Idh enzymic activity is strongly inhibited by mitochondrial mRNA (Anderson et al., 2000; Elzinga et al., 2000). This inhibitory effect could be counteracted by the addition of the allosteric effector AMP (Anderson et al., 2000). Taken together, these data strongly suggest a regulatory link between the need for Krebs cycle function and mitochondrial gene expression.

To study the exact role of RNA-binding by Idh, it is imperative to obtain Idh mutants that are disturbed in RNA binding whilst enzymic activity remains unaltered. Since no obvious RNA-binding region could be identified in the sequence of either Idh1p or Idh2p, we have recently cloned the IDH genes of the closely related yeast Kluyveromyces lactis to identify residues involved in RNA-binding. Idh from K. lactis was shown to have a very low affinity for COX2 mRNAs of S. cerevisiae and also for the COX2 mRNA from K. lactis itself (Elzinga et al., 2000). Sequencing and subsequent alignment of the deduced amino acid sequences of the genes encoding Idh from S. cerevisiae and those from K. lactis revealed a very high overall sequence identity (79.9% and 86.2 % for Idh1p and Idh2p, respectively). Moreover, residues involved in substrate and co-factor binding as well as regions surrounding these amino acids were conserved in both genes. Analysis of the RNA-binding capacity of heterologous complexes consisting of one subunit of S. cerevisiae Idh and the other subunit of K. lactis Idh, indicated that both Idh1p and Idh2p are involved in RNA-binding. Alignment of the S. cerevisiae and K. lactis IDH genes and close examination of a deduced 3D-model for S. cerevisiae Idh1p, identified candidate residues capable of influencing RNA-binding. Mutation of two of these residues indeed resulted in a drastically decreased affinity for mRNA leaders.

Material & Methods

Strains.

As wild-type S. cerevisiae strain W303 was used (Rothstein, 1983), the wild-type K. lactis strain was CBS2359 (source: CBS, The Netherlands). A strain disrupted for both IDH genes (idh-null) was kindly made available to us by Dr. Lee McAlister-Henn (Cupp and McAlister-Henn, 1992). All strains are summarized in Table I.
Extract preparation.
All strains were grown on lactate medium. Wild-type, heterologous and mutant Idh complexes were purified by salt elution from Heparin-Sepharose, followed by DEAE-cellulose column chromatography as described previously (Papadopoulou et al., 1990). Mitochondrial lysate fractions of the disruptant strain were obtained by salt elution from Heparin-Sepharose as described for the purification of Idh (Papadopoulou et al., 1990).

In vitro transcription and band-shift assays.
Labeled RNA was produced by run-off transcription from pCOX2Δ9*Rsa1, encompassing the mitochondrial COX2 leader in the presence of \([^{32}P]\)-UTP. This yielded transcripts containing the entire COX2 5'-leader from -65 until +50 with respect to the AUG codon (Dekker et al., 1992). Band-shift assays were performed as described previously (Papadopoulou et al., 1990). Binding reactions were incubated with Heparin-Sepharose elution fractions, isocitrate, NAD, AMP or heparin for 15 min. at 30°C at the indicated concentrations, prior to electrophoresis.

Enzyme assay.
NAD⁺-Idh activity was measured in 1 ml 100 mM Tris-acetate (pH 7.2), 1 mM MnCl₂, 0.5 mM AMP, 0.5 mM NAD⁺ and 1 mM DL-isocitrate (Robinson et al., 1987). Assays were performed at room temperature and were initiated by the addition of isocitrate. Prior to addition of the substrate, purified Idh was incubated with RNA for 15 min. at 30°C, as in the band-shift assays. Formation of NADH was measured spectrophotometrically at 340 nm.

Binding affinity measurement.
The binding affinity of Idh for COX2 RNA was estimated by mobility-shift assays in which binding of a fixed amount of protein was assayed in the presence of increasing amounts of labeled COX2 RNA. To calculate the dissociation constant (K_d), free and bound RNA were quantitated by PhosphorImager analysis and the amount of bound RNA was plotted against the ratio bound RNA:free RNA (Scatchard analysis).

Constructs.
The IDH genes from S. cerevisiae and K. lactis were cloned in the multicopy vector YEp352, under control of their own promoter. The construct bearing both IDH1 and IDH2 from S. cerevisiae (YEp352::Sc/IDH1/IDH2) was generously provided by Dr. Lee McAlister-
Henn. The construct expressing only S. cerevisiae IDH1 was derived from YEp352::SclDH1/IDH2, by subcloning an IDH1 containing XbaI fragment into an empty YEp352 vector. Religation of the XbaI-digested YEp352::SclDH1/IDH2 construct resulted in a construct expressing only S. cerevisiae IDH2 (YEp352::IDH2). The genes and flanking sequences of both K. lactis Idh subunits were also cloned into YEp352, as described earlier (Elzinga et al., 2000).

**Mutagenesis of S. cerevisiae IDH1.**

To mutate S. cerevisiae Idhlp, site-directed mutagenesis was performed based on the “megaprimer” method (Sarkar and Sommer, 1990). Three oligonucleotide primers and two rounds of PCR were used with the S. cerevisiae IDH1 gene as template. The first round of PCR was performed using primer I (5'-GGTCACCGTTCACTAAACGTT-3') and a degenerate oligonucleotide with three weak sites (5'-CTTCTGTGTWTWCTWWGGCGAAGTC-3', W is A or T), the underlined codons contain degenerate sites which result in the mutagenesis of residues Lys-182 and Tyr-184. This resulted in the generation of eight different PCR fragments. These fragments were used as a new primer in a second round of PCR along with another flanking primer II (5'-CCACGTTAATGTTGAAC-3'). A KpnI-EcoRI fragment of the final PCR-product was cloned into pBluescript. Sequence analysis identified two mutants: single mutant K182L and double mutant K182L/Y184N. The KpnI-EcoRI fragment from YEp352::Sc/DH7 was replaced by the KpnI-EcoRI mutant fragments, resulting in single mutant YEp352::Scidh1K182L or double mutant YEp352::Scidh1K182L/Y184N. An XbaI fragment containing S. cerevisiae IDH2 was subcloned into YEp352::Scidh1K182L, resulting in YEp352::Scidh1K182L/IDH2).

**Miscellaneous.**

All DNA manipulations were done according to standard techniques as described by Sambrook (Sambrook et al., 1989). Yeast was transformed with plasmid DNA by the one-step method as described by Chen (Chen et al., 1992). Sequencing was performed according to the dideoxy-method of Sanger (Sanger et al., 1977). Protein samples were separated by SDS-PAGE according to the method of Laemmli (Laemmli et al. 1970).
Table I: Strains used in this study

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<th>Strain::plasmid</th>
<th>Genotype</th>
<th>Reference</th>
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<td>Δidh1</td>
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<td>Elzinga et al., 1993</td>
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Results

Idh binds COX2 RNA with high affinity.

We have previously established that Idh binds specifically to all mitochondrial mRNA leaders (Dekker et al., 1992; Elzinga et al., 1993). To further investigate the characteristics of this RNA-binding we set out to measure the dissociation constant ($K_d$) of Idh for the COX2 mRNA leader by titration of a fixed amount of enzyme with increasing amounts of RNA. As is shown in Fig. 1A, up to 30 minutes incubation time of the binding reactions leads to increased complex formation; longer incubation times did not increase the amount of complex. Since 30 minutes were sufficient to obtain maximum complex formation, this incubation time was used in further experiments. Subsequently we incubated increasing amounts of RNA with 20 ng Idh (Fig. 1B). Quantitation of free and bound radioactivity and analysis of the resulting Scatchard plots showed that Idh binds COX2 RNA with a $K_d = 3$ nM (the average from three separate experiments). This value is in the same range as measured for the MS2 coat protein-RNA interaction (LeCuyer et al., 1996), which is generally regarded as an extremely stable interaction.

Regulation of the RNA-binding activity of Idh

It has been shown for other RNA-binding proteins that the RNA-binding activity can be inhibited by co-factors or allosteric ligands (Chu et al., 1993; Nagy and Rigby, 1995; Clerch et al., 1996; Bringaud et al., 1997). We therefore investigated whether isocitrate, $\text{NAD}^+$ or AMP influence the RNA-binding of Idh (Figure 2). These compounds were added to the
binding reactions in high molar excess (final concentrations indicated in Fig. 2; the Idh and RNA concentrations in these experiments are in the nM range). The effect on RNA-binding was assayed by mobility-shift analysis. As is shown in Figure 2, none of the added compounds inhibits RNA-binding. Isocitrate does not affect complex formation even up to a concentration of 150 mM (Fig. 2, panel A). NAD\(^+\) and AMP did not affect RNA-binding up to a concentration of 15 mM (Fig. 2, panel A). An effect on complex formation can be observed when still higher concentrations of NAD\(^+\) and AMP are used (Fig. 2). This effect may well be artefactual, since these concentrations affect the mobility of free RNA as well.

Since addition of up to 15 mM (which is 5.10\(^6\)-fold the \(K_d\)) does not affect RNA-binding, we conclude that the RNA-binding of Idh is not regulated by either isocitrate or NAD\(^+\) and AMP.

**Figure 1**

**Figure 1: Measurement of the RNA-binding affinity of Idh.**

A: 2 fmol of COX2 RNA was incubated with 100 ng of purified *S. cerevisiae* Idh. The binding reactions were carried out at 30°C as indicated (lanes 2-8). Band-shift assay was performed as described in Material & Methods. As a control the RNA was incubated without Idh (lane 1). B: Increasing amounts of COX2 RNA were incubated for 30 min. at 30 °C with 20 ng of Idh. For Scatchard analysis, the amounts of free and bound RNA were determined by PhosphorImager analysis of the dried band-shift gel.
Figure 2: Characterization of RNA-binding.

A: COX2 RNA was incubated without (lane 1) and with 100 ng of purified Idh (lanes 2-14). For lanes 3-14 isocitrate, NAD$^+$ or AMP were added to the binding reactions in the final concentrations shown in the figure (in mM). B: Heparin was added to the binding reactions in the final concentrations shown in the figure (in nM). Lanes 1 and 2 contained the same controls as in panel A.

The oligosaccharide heparin was found to inhibit RNA-binding. Mobility-shift assays showed that addition of 60 nM of heparin starts to inhibit RNA-binding of Idh; at a concentration of 600 nM heparin, complex formation is virtually absent (Fig. 2B). This concentration of heparin is only 20 to 200-fold higher than the $K_d$ value of Idh for COX2 RNA, suggesting that competition of RNA-binding by heparin might involve part of the same binding site. Heparin sensitivity might be an indication that positively charged domains of Idh interact with the negatively charged heparin (see Discussion).

Both Idh subunits contribute to RNA-binding

Computer analysis of the amino acid sequence of Idh1p and Idh2p failed to reveal any obvious similarities with characterized RNA-binding regions identified in other RNA-binding proteins. We therefore searched for a naturally occurring variant of Idh that does not bind RNA. We found that Idh from the closely related yeast *Kluyveromyces lactis* displays hardly any detectable RNA-binding activity (Elzinga et al., 2000).
We have previously shown that RNA-binding of Idh is dependent on the simultaneous presence of both Idh subunits (Elzinga et al., 1993). We do not know whether this dependence is due to a direct contribution to binding, or a mere need for heteromer formation (the holoenzyme is an octamer containing 4 copies of Idh1 and Idh2 each). To investigate this, we first expressed the K. lactis IDH genes in a S. cerevisiae strain lacking both IDH genes. Expression of both K. lactis genes was shown to occur by growing the transformants on medium containing acetate as a carbon source (Figure 3), since strains disrupted for one or both of the IDH genes are unable to grow on this carbon source (a phenotype that is shared by other Krebs cycle mutants; Cupp and McAlister-Henn, 1992). No growth on acetate is observed when an empty control plasmid was introduced into the idh-null strain (Fig. 3). This suggests that the K. lactis IDH genes are not only expressed in S. cerevisiae but also that the products are imported into the mitochondria and that the Idh complex is sufficiently enzymically active to support growth under these conditions.

Figure 3: Complementation of the Ac' phenotype. Growth analysis of an idh-null strain transformed with YEp352, YEp352::ScIDH1ScIDH2, and YEp352::KiIDH1KiIDH2. S. cerevisiae strain W303 was used as positive control. First transformed cells were plated on minimal medium supplemented with amino acids, to select for the presence of the plasmid (left panel). Cells containing the desired plasmid were grown on medium containing acetate as non-fermentable carbon source (right panel).
Subsequently we created strains harboring heterologous Idh complexes. *K. lactis IDH1* was expressed in a *S. cerevisiae idh1-null* strain and *K. lactis IDH2* in a *S. cerevisiae idh2-null* strain. These heterologous Idh complexes were purified by Heparin-Sepharose and DEAE-cellulose column chromatography and tested for enzymic- and RNA-binding ability. During isolation the enzyme activity was determined (Table II). Both heterologous complexes displayed enzyme activity in *S. cerevisiae*, confirming the complementation of the Ac" phenotype (Fig. 3). This means that both *K. lactis* subunits are sufficiently expressed in *S. cerevisiae* and are able to form heterologous Idh complexes with their *S. cerevisiae* counterparts. The specific activity of both heterologous complexes is substantially lower than the specific activity of the wild-type *S. cerevisiae* Idh and also of the *K. lactis* Idh complex expressed in *S. cerevisiae* (Table II). This indicates that although the heterologous complexes are formed, these are less enzymically active, maybe due to less efficient subunit interaction.

| Table II: Specific NAD+-ldh enzyme activity (Units/mg) (U) and yield of activity (%) |
|---------------------------------|---------------------------------|---------------------------------|
| Fraction from                  | Sc idh1-null::KI IDH1 Act (U) / yield (%) | Sc idh2-null:: KI IDH2 Act (U) / yield (%) | Sc idh1/idh2-null::KI IDH1/IDH2 Act (U) / yield (%) |
| Mitochondrial lysate           | 2.3 / 100                         | 0.7 / 100                        | 6.5 / 100 |
| Heparine-Sepharose             | 4.5 / 59                          | 2.1 / 89                         | 11.8 / 57 |
| DEAE-Cellulose                 | 7.9 / 26                          | 3.4 / 21                         | 32.9 / 23 |

Isolated Idh complexes were analyzed by SDS-PAGE and visualized by silver staining (Fig. 4A). Western blots of these SDS-PAGE gels, were incubated with a polyclonal antibody against Idh from *S. cerevisiae*. This antibody also recognized the *K. lactis* subunits expressed in *S. cerevisiae* (Fig. 4B; lane 2). Klldh1p and Scldh2p are almost indistinguishable on Western blot (Fig. 4B, lane 3), which correlates well with their calculated mass (39 kDa). Scldh1p and Klldh2p can be separated into two bands of about 40 kDa, also in agreement with the calculated mass of the respective proteins (Fig. 4B, lane 4; Elzinga et al., 2000). The Idh subunits isolated from a wild-type *K. lactis* strain (Fig. 4B, lane 5) are indistinguishable from the *K. lactis* Idh subunits expressed in (and purified from) *S. cerevisiae* (Fig. 4B, lane 2).
Figure 4: Purification of homologous and heterologous Idh complexes.
A: Silver-stained SDS-PAGE of purified (DEAE cellulose chromatography) Idh complexes. 150 ng fraction wild type Idh from S. cerevisiae and K. lactis was loaded in lanes 1 and 5, respectively. 250 ng was loaded of the purified heterologous complexes Kl Idh1/Kl Idh2 (lane 2), Kl Idh1/Sc Idh2 (lane 3) and Sc Idh1/Kl Idh2 (lane 4). B: Western blot of panel A. The Idh subunits were detected with a polyclonal antibody raised against S. cerevisiae Idh.

Figure 5: RNA-binding of heterologous Idh complexes. Mobility-shift assay using labeled COX2 leader RNA from S. cerevisiae. RNA was incubated with DEAE-fractions containing S. cerevisiae Idh (lane 2), DEAE-elution fractions from a idh-null strain (lane 3), Idh consisting of Kl Idh1/Sc Idh2 (lane 4) or Sc Idh1/ Kl Idh2 (lane 5) and K.lactis Idh (lane 6). As a control the RNA was incubated without Idh (lane 1).
Subsequently, we analyzed the heterologous complexes in mobility-shift assays to obtain information about the contribution of either or both Idh subunits in RNA-binding. As target RNA we used radioactively labelled *S. cerevisiae* COX2 leader RNA. Partially purified DEAE-fractions were used in each reaction (Fig. 5). The homologous *K. lactis* Idh expressed in *S. cerevisiae* shows strongly reduced RNA-binding compared to *S. cerevisiae* Idh (Fig. 5, compare lanes 2 and 6). This was expected since we have shown previously that Idh isolated from *K. lactis* showed very low RNA-binding activity (Elzinga et al., 2000). Both heterologous complexes, however, could bind COX2 RNA (Fig. 5, lanes 4 and 5), but showed decreased affinity for the target RNA as compared to wild type Idh from *S. cerevisiae* (Fig. 5, lane 2). These data either suggest that both subunits equally contribute to RNA-binding, or that an ‘active’ conformation (heterodimer or octamer, as for the Idh enzymic activity) is necessary for RNA-binding.

**Generating a mutant Idh by site-directed mutagenesis**

Amino acid sequence comparison of *S. cerevisiae* and *K. lactis* Idh1 and Idh2 highlighted several conserved regions (Elzinga et al., 2000). These regions were superimposed on a predicted 3D structure of Idh from *S. cerevisiae*, obtained from SWISS-MODEL (Peitsch et al., 1995). This program was used to model *S. cerevisiae* Idh in analogy with the crystal structures of a number of related proteins, including NADP⁺-Idh from *E. coli* (Thorsness and Koshland, 1987), which has 43% similarity and 32% identity with Idhlp. We observed a putatively interesting region that forms a wide cleft, that divides Idhlp in a large and a small domain (Fig. 6). This cleft appears wider in the *S. cerevisiae* Idh1 subunit than in the *K. lactis* Idh1 subunit. A second observation was that three aromatic residues (Phe-176, Phe-180 and Tyr-184) protrude into the cleft of *S. cerevisiae* Idh1p (Fig. 7). This region is markedly different in the *K. lactis* sequence: Tyr-184 is replaced by an Asparagine and Lys-182 is replaced by a Leucine residue. Remarkably, these differences are the only changes within a large conserved stretch of amino acids. We therefore chose to change the Lys-182 and the Tyr-184 ("KKY") of *S. cerevisiae* into Leu and Asn ("LKN") respectively, according to the *K. lactis* Idh1p sequence using the “megaprimer” mutational PCR method (Sarkar and Sommer, 1990) (Fig. 7).
Figure 6: Putative 3D model of *S. cerevisiae* Idh1. Ribbon diagram of *S. cerevisiae* Idh1p was drawn with MOLSCRIPT, based on Idh sequence alignments and crystallographic data obtained for *E. coli* NADP-Idh (Peitsch *et al.*, 1995).

```
L^N

Sc_Idh1 170-   T E R I A R F A F D F A K K Y N R K S V T - 1 9 0
Kl_Idh1     S E R I A R F A F D F A L K N R K S V C

Sc_Idh2    S E R V I R Y A F E Y A R A I G R P R V I
            **************  *  *****
Kl_Idh2    S E R V I R Y A F E Y A R A V D R S K V L
```

Figure 7: Comparison of *S. cerevisiae* and *K. lactis* Idh1p. Alignment of residues 170 to 190 (coordinates taken from *S. cerevisiae* Idh1) of Idh1p and Idh2p from *S. cerevisiae* with Idh1p and Idh2p from *K. lactis*. Identical residues are indicated with an asterisk, similar residues with a vertical line. Underlined residues protrude into the cleft; shadowed residues have been mutated to Leucine (Lys-182) or Asparagine (Tyr-184) as indicated.
Two mutated Idh1 subunits were obtained (the single mutant Idh1LKN; see Methods) and these were expressed in a S. cerevisiae idh1-null mutant. Both mutants were able to complement the Ac− phenotype of the idh1-null strain, denoting that both mutated Idh1 subunits were able to interact with wild-type Idh2p to form an enzymically active Idh complex. Following isolation of the Idh complexes, the mutated Idh complexes showed normal enzyme activity under standard conditions, indicating that the mutations did not interfere with the active site of the enzyme or with substrate or co-factor binding (data not shown). RNA-binding of partially purified Idh complexes, containing either the single or the double mutated Idh1p, was determined in mobility-shift assays. The Idh mutants form complexes with COX2 RNA that differ in mobility, indicating that the RNA-binding affinity of Idh has changed (Fig. 8, lanes 4 and 5). Quantitation of the amount of bound RNA by PhosphorImager analysis, showed that the mutated Idh complexes had 80% lower affinity for RNA than wild type Idh.

Figure 8: RNA-binding of wild-type and mutated Idh complexes. Mobility-shift assay using labelled COX2 leader RNA and Idh from S. cerevisiae. RNA was incubated with DEAE-fractions containing wild-type S. cerevisiae Idh (lane 2; 100 ng), wild-type commercially available Idh (lane 3; 50 ng), single mutant IdhLKN (lane 4; 250 ng) and double mutant IdhLKN (lane 5; 250 ng). As a control the RNA was incubated without Idh (lane 1).
NAD$^+$-dependent isocitrate dehydrogenase (Idh) is an allosterically regulated enzyme of the Krebs cycle. This enzyme also specifically binds yeast mitochondrial mRNA leaders (Elzinga et al., 1993), and negatively influences translation of these mitochondrial mRNAs (De Jong et al., 2000). In this paper, we show that NAD$^+$-Idh binds COX2 RNA with a $K_d$ value of 3 nM. Based on earlier work, we know that Idh binds the other mitochondrial mRNAs with comparable affinity (Dekker et al., 1992). This $K_d$ value compares well to other known protein-RNA interactions for which a biological function has been shown (like for instance the extremely stable MS2 coat protein-RNA interaction; LeCuyer et al., 1996).

This, together with the abundance of Idh relative to mitochondrial mRNA molecules (see also Dekker et al., 1992; Anderson et al., 2000), strongly suggests that RNA-binding of Idh is likely to occur in vivo and is biologically functional.

In the TCA-cycle Idh catalyzes the oxidative decarboxylation of isocitrate with NAD$^+$ as cofactor and AMP as allosteric effector. The fact that these compounds did not affect RNA-binding when added in concentrations 5.10$^5$-fold the $K_d$ value indicates that the RNA-binding site is different from the catalytic site and apparently insensitive to the allosteric changes these molecules (might) induce.

Several other enzymes also bind RNA. These include aconitase (Roualt et al., 1988; 1990), glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Singh and Green, 1993), thymidylate synthase (TS; Chu et al., 1993), enoyl-CoA hydratase (Nakagawa et al., 1995), MnSOD (Fester and Schuster, 1995), catalase (Clerch et al., 1996) and glutamate dehydrogenase (GDH; Preiss et al., 1997; Bringaud et al., 1997). None of these proteins contain recognizable RNA-recognition motifs (like for instance the RNP1/2 motifs or RGG-box; Mattaj, 1993). All these enzymes, including Idh, do however contain a dinucleotide binding domain (Rossmann fold, Rossmann et al., 1975). RNA-binding of some of these enzymes (GAPDH, GDH, catalase, TS) could be competed by NAD(H), NADP(H) or ATP, suggesting that the Rossmann-fold might serve as an RNA-binding domain, and indicating that the enzymic function and RNA-binding are mutually exclusive (Chu et al., 1993; Nagy and Rigby, 1995; Clerch et al., 1996; Bringaud et al., 1997). These observations led to the hypothesis that the RNA-binding domain present in these enzymes evolved from a dinucleotide binding site, or vice versa (Hentze, 1994).

Our data clearly indicate that the Rossmann-fold of Idh is not involved in RNA interaction, since even high concentrations of NAD$^+$ did not affect the capacity of Idh to bind RNA. This
implies that in addition to the two types of RNA-binding proteins thus far described (RNA recognition motifs or Rossmann-fold), there is a third class of RNA-binding proteins with Idh being the first member of this group.

In mobility-shift assays, heparin is often used to eliminate nonspecific RNA-protein interactions. In this light the observed sensitivity of Idh RNA-binding to heparin is unexpected but not unprecedented (Ladomery and Sommerville, 1994; Freire and Pages, 1995). In these studies heparin sensitivity was explained as an indication for the involvement of electrostatic interactions and of positively charged domains (arginine clusters). Idh band-shift assays are routinely performed in the presence of 50-100 mM NaCl; high salt concentrations (250-500 mM) only slightly decrease complex formation (data not shown). This may indicate that the Idh-RNA interaction is not exclusively an electrostatic interaction.

In previous studies we tried to determine the function of the RNA-binding activity of Idh using strains disrupted for either one or both of the Idh subunits (Elzinga et al., 2000). However, disruption of these genes causes loss of Krebs cycle activity, with complex effects on mitochondrial energy balance and on translation. Therefore, to investigate the role of Idh in translational regulation, we searched for a mutant that was impaired in RNA-binding but that still contains enzyme activity. A natural RNA-binding mutant of Idh was discovered in Kluyveromycyes lactis. NAD⁺-dependent Idh isolated from this strain had very low affinity for either its own COX2 mRNA leader or for the mRNA leaders from S. cerevisiae (Elzinga et al., 2000). Apparently, K. lactis Idh lacks an RNA-binding region.

The K. lactis IDH genes were expressed in a S. cerevisiae strain disrupted for both IDH genes. Complementation of the Ac⁻ phenotype of the S. cerevisiae disruptant strain and presence of NAD⁺-dependent Idh activity in the mitochondrial fractions, indicated that the K. lactis IDH genes are expressed and imported at a level sufficient to satisfy growth requirements. Expression of heterologous Idh complexes (KIIDH1/SclDH2 and ScIDH1/KIIDH2) also showed complementation of the Ac⁻ phenotype. Protein purification and SDS-PAGE/Western analysis indeed showed that the heterologous subunits are expressed and mitochondrially localized (Fig. 4). However, the specific activity of these heterologous Idh complexes was substantially lower compared to homologous Idh complexes (both from S. cerevisiae and from K. lactis). This indicates that subunit interaction and communication, necessary for normal enzyme activity, is far from optimal. In mobility-shift assays, both heterologous complexes showed decreased affinity for RNA
as compared to wild type Idh from *S. cerevisiae*. From these data we were unable to assign a more dominant role for either Idh subunit in RNA binding.

The deduced amino acid sequences of both Idh1 subunits share 78.5% identical residues. Alignment of *S. cerevisiae* Idh1p and *K. lactis* Idh1p identified a region potentially involved in RNA-binding (amino acids 170 to 190), containing some non-conserved residues. From a 3D-structure based on *E. coli* NADP⁺-Idh (Thorsness and Koshland, 1987), we identified a cleft region, dividing the subunit in a large and a small domain. Three aromatic residues of the potential RNA-binding region were notably sticking in the cleft: two Phenylalanine residues (at 176 and 180) and a Tyrosine (at 184). The Phenylalanine residues are conserved in *K. lactis* Idh1p, but the Tyrosine is not conserved. Except for Tyr-184 and Lys-182, this region is highly conserved between *S. cerevisiae* Idh1p and *K. lactis* Idh1p. Lys-182 of *S. cerevisiae* Idh1p is also different from the residue at the same position in *S. cerevisiae* and *K. lactis* Idh2p. To test the involvement in RNA-binding of this region, we decided to mutate residues 182 and 184 from Lysine and Tyrosine to Leucine and Asparagine, respectively. We obtained a mutant containing the K182L mutation and a mutant containing both the K182L and the Y184N mutation. Both mutated Idh1 subunits were able to restore the Ac⁻ phenotype and the enzyme activity of a *S. cerevisiae* idh1-null strain (both in yield as in specific activity), indicating that the mutation did not have an obvious effect on enzyme activity of Idh. However, mobility-shift assays clearly showed that both mutations had a drastic effect on RNA-binding, resulting in a reduction of RNA binding with 80% (Fig. 9). The observed lower affinity for RNA shows that residues in the cleft region are important for RNA-binding. The question arises whether these amino acids are directly involved in RNA-binding. The *IDH1* homologue of *Schizosaccharomyces pombe* might provide a clue to the answer to this question. We have reported before that Idh from *S. pombe* is able to bind RNA with an affinity comparable to *S. cerevisiae* Idh (Elzinga et al., 2000). Recently, as a result of the *S. pombe* sequencing project, a sequence with 57.6% identity with *S. cerevisiae* Idh1p was found in the *S. pombe* database. This sequence is assumed to encode the Idh1 subunit of *S. pombe* NAD⁺-Idh. However, the amino acids in the potential RNA-binding region are conserved between *K. lactis* and *S. pombe*. Idh1p of *S. pombe* contains a Leucine residue at position 182 and an Asparagine residue at position 184, like *K. lactis* Idh1p. When this observation is taken into account, it is unlikely that the mutated Lysine and Tyrosine residues are directly involved in RNA-binding and suggest that other determinants (like the width of the cleft) are likely to play a role.
We hypothesized that the combination of dehydrogenase and RNA-binding activities in a single protein may form a regulatory link between the need for mitochondrial function and rate of biogenesis. Recently we reported that pulse-labeling experiments show increased translation of mitochondrial translation products in an idh-null strain, indicating that RNA-binding of Idh effects the level of synthesis of respiratory chain subunits in vivo (De Jong et al., 2000). The data we present here add to the biological role of Idh in S. cerevisiae. Having created a mutant of Idh which is disturbed in RNA binding but wild-type in enzymic activity provides us with an imported tool to further study the biological role of RNA-binding and determination of the novel way this protein is able to bind RNA.