The role of yeast NAD+-isocitrate dehydrogenase in mitochondrial translation
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Chapter II

Yeast Mitochondrial NAD\(^+\)-dependent Isocitrate dehydrogenase is an RNA-binding Protein

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Abstract

We have previously described the characterization of an abundant mitochondrial protein (p40) that binds specifically to 5'-untranslated leaders of mitochondrial mRNAs in yeast. p40 consists of two polypeptides with Mr of 40 and 39 kDa. Limited sequence analysis of p40 identifies it as the Krebs cycle enzyme NAD$^+$-dependent isocitrate dehydrogenase (Idh). Both enzyme and RNA-binding activities are specifically lost in cells containing disruptions in either IDH1 or IDH2, the nuclear genes encoding the two subunits of the enzyme, thus conclusively identifying p40 as Idh and showing that both activities are dependent on the simultaneous presence of both subunits. Although we still must ascertain whether and how either function of Idh is regulated and whether the two functions are compatible or mutually exclusive, this combination of dehydrogenase activity and RNA-binding in a single protein may be part of a general regulatory circuit linking the need for mitochondrial function to mitochondrial biogenesis.

Introduction

Of all the steps in mitochondrial gene expression, probably least is known about the initiation of translation. Unlike their cell sap counterparts, for which cap recognition and leader scanning are important steps in initiation$^1$, most mRNAs in mammalian mitochondria lack leaders altogether$^2,3$, whilst in yeast, mitochondrial mRNAs are uncapped and most possess extremely long untranslated leader and trailer sequences of high average A+U content (>95%). These leader sequences are likely to be difficult for a ribosome to scan, since they often contain both short open reading frames and short G+C rich clusters capable of forming highly stable secondary structures. They are, nevertheless, important for translation. First, because mutations within them can affect translatability$^4$ and second, because parts of the leaders are absolutely required for interaction with nuclear-encoded activator proteins that govern translation of specific mRNAs$^5,6$.

We have previously described the characterization of a 40 kDa protein (p40) that binds specifically and with high affinity to the 5'-untranslated leaders of all major yeast mitochondrial mRNAs$^7,8$. p40 is nuclear-coded, but is distinct from the specific
translational activators characterized so far\(^9\). It is exclusively present in mitochondria, where it is predominantly matrix-localized and is so abundant (about 0.4% of total mitochondrial protein) that all mitochondrial mRNAs may be permanently complexed with the protein in vivo\(^9\).

We now report results of sequence and gene disruption analysis which conclusively show that p40 is identical to the mitochondrial NAD\(^+-\)dependent isocitrate dehydrogenase (Idh). On SDS-polyacrylamide gels p40 consists of two closely migrating bands of Mr 39 and 40 kDa, similarity between these being suggested by results of partial peptide mapping\(^7\). The two subunits of this Krebs cycle enzyme, encoded by the nuclear genes \(IDH1\) and \(IDH2\)\(^{10,11}\) also have apparent molecular weights of 39 and 40 kDa and display a high degree of sequence conservation both with each other and with the single subunit NADP\(^+-\)dependent isocitrate dehydrogenase of \(E.\ coli\). Both enzyme and RNA-binding activities are specifically lost in cells containing disruptions in either \(IDH1\) or \(IDH2\), confirming identity of p40 and Idh and showing that both activities are dependent on the simultaneous presence of both subunits. Idh is thus an additional member of a growing family of enzymes with novel RNA-binding activities. These include cytosolic aconitase (IRBP)\(^{12}\), thymidylate synthetase\(^{13}\) and glyceraldehyde-3-phosphate dehydrogenase\(^{14}\).

Materials and methods

Genetic manipulations and strains

\(IDH1\) and \(IDH2\) were cloned into pUC19*HincII, using PCR and gene specific-primers on chromosomal DNA from \(Saccharomyces\ cerevisiae\) (wild-type strain DL1\(^{15}\)). The \(IDH1\) gene was disrupted by replacing an EcoRV/HincII fragment of the coding region with the LEU2 gene. The \(IDH2\) gene was disrupted by insertion of the HIS3 gene using the BglII site in the coding region. These constructs were transformed into strains \(Saccharomyces\ cerevisiae\) W303-1Ba and W303-1Bα respectively (\(ade2-1;\ his3-11,15;\ leu2-3,112;\ ura3-1;\ trp1-1;\ can1-100\))\(^{16}\). Transformants were checked for proper integration by Southern blot analysis (data not shown).
Extrac tt Preparatio n
Wild-typ ee an d disrupte d strain s wer e grow n on lactate medium7. The preparation of p40 used for Edman degradation was purified as described7, except that gel filtration was performed on a Superose 6 (Pharmacia) column and an additional purification step involving chromatography on DEAE Sephacel (Pharmacia) was included. In this last step, p40 was bound to the column in buffer B7 and eluted by raising the salt concentration to 100 mM KCl. Protein concentrations were determined using the method of Bradford17.

RNA band-shift assays were routinely carried out using fractions obtained by salt elution of mitochondrial lysates from Heparin-Sepharose7. Fractions were checked by Western blotting for the presence of p40 prior to use.

In Vitro Transcription and Band-shift Assays
Labeled RNA was produced by run-off transcription from pCOX2Δ9*Rsa17, 8, encompassing the mitochondrial COX2 leader in the presence of [32P]UTP. This yielded transcripts containing the COX2 gene and entire 5'-leader from -65 until +50 with respect to the AUG codon.

Band-shift assays were performed as described previously7.

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 1mM DL-isocitrate18. Assays were performed at room temperature and initiated by the addition of 200 ng purified p40 or 2μg of Heparin-Sepharose fractions of mitochondrial lysate of wild-type, Δidh1, Δidh2 or Δidh1/Δidh2 strains. Mixed extracts were incubated 15 minutes at 30 °C prior to addition to the assay. Formation of NADH was measured spectrophotometrically at 340 nm.

Results

Peptide Sequence of p40 is Identical to Idh
p40 was purified as previously described7, with the inclusion of an additional purification step involving DEAE ion exchange chromatography. Consistent with
our previous observations, the preparation consisted of two polypeptides with apparent molecular weights of 39 and 40 kDa. Products of the digestion of p40 with Glu-C protease were separated by SDS-PAGE and subsequently blotted on PVDF membrane\textsuperscript{19, 20}. Selected peptides were then subjected to N-terminal sequence analysis by Edman degradation. One peptide yielded the sequence ATVKQPSIGXYTGPNPX, which matches perfectly residues 16 to 34 in the published sequence for IDH2, the gene for the smaller of the two subunits of the mitochondrial NAD\textsuperscript{+}-dependent isocitrate dehydrogenase\textsuperscript{10}. Subsequent assay revealed that preparations of p40 also possessed Idh activity (Table I).

\textbf{Disruption of IDH1 and IDH2}
In order to rule out the possibility that RNA-binding and dehydrogenase activities simply co-purify with each other, gene disruption studies were performed. Making use of published DNA sequence data\textsuperscript{10, 11}, IDH1 and IDH2 coding sequences were cloned by PCR amplification and the resulting clones were used to construct null mutations of each gene. A strain disrupted for both genes was kindly made available to us by Dr. Lee McAlister-Henn (University of Texas). All disruptants show apparently normal growth on glucose-containing media, grow slowly on media containing non-fermentable carbon-sources and show no growth on acetate-containing media\textsuperscript{10, 11}. In agreement with these authors, mitochondrial fractions from single and double disruption strains were shown to lack Idh activity (Table I).

\textbf{Table I. Specific NAD\textsuperscript{+}-dependent isocitrate dehydrogenase activity of purified p40 and mitochondrial fractions of wild-type and NAD\textsuperscript{+}-Idh disruptant strains.}

<table>
<thead>
<tr>
<th>Enzyme preparation</th>
<th>Amount added (\textmu g protein)</th>
<th>NADH formed (\textmu mol/min/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p40\textsuperscript{a}</td>
<td>0.2</td>
<td>38</td>
</tr>
<tr>
<td>wt\textsuperscript{b}</td>
<td>2.0</td>
<td>22</td>
</tr>
<tr>
<td>\textDelta idh1\textsuperscript{b}</td>
<td>2.0</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>\textDelta idh2\textsuperscript{b}</td>
<td>2.0</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>\textDelta idh1/\textDelta idh2\textsuperscript{b}</td>
<td>2.0</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>\textDelta idh1 + \textDelta idh2\textsuperscript{b}</td>
<td>2.0 + 2.0</td>
<td>&lt;0.1</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Purified p40
\textsuperscript{b}Mitochondrial Heparin-Sepharose fractions of strain indicated
Antibody Against p40 Reacts with Idh

Western blot analysis, using antibody specifically directed against p40, revealed the presence in total cell extracts of two bands, migrating with Mr 40 and 39 kDa in the wild-type strain, only one band (Idh1 or Idh2) in each of the single disruptants (Δidh2 or Δidh1, respectively) and no cross-reacting material in the double disruptant. The wild-type pattern was also observed with purified p40 (Fig. 1).

Fig. 1. Immunodetection of Idh with anti-p40 antiserum using Western blot analysis. Approximately 10μg of crude cell extracts of wild-type W303 (lane 2), Δidh1 (lane 3), Δidh2 (lane 4) and Δidh1/Δidh2 (lane 5) strains and purified p40 (1μg), were separated on a 15% SDS-polyacrylamide gel and blotted on nitrocellulose. The blot was incubated with a polyclonal antiserum raised against p40. Idh1 and Idh2 bands are indicated.

Both Idh-subunits are Required for RNA-binding

Partially purified mitochondrial protein fractions were tested in band-shift assays for their ability to form a specific complex with the 54-nt 5'-untranslated leader of the mRNA for the mitochondrial COX2. As shown in Fig. 2 (lanes 4 and 5), lack of either subunit results in loss of RNA-binding, strongly implying that this interaction, like dehydrogenase activity, requires heterodimer formation. That lack of RNA-binding is not simply due to the presence of RNase, or other inhibitory factors in the extracts was demonstrated by formation of the expected RNA-p40 complex after addition of a purified p40 preparation (lanes 6 and 7). Mixing of extracts from each of the single disruptant strains failed to regenerate either Idh or RNA-binding activities (table 1 and fig. 2 lane 8), possibly because the assay conditions used are inappropriate for efficient heterodimer formation.
Fig. 2. Band-shift analysis of the interaction of Idh with the COX2 mRNA leader. RNA was incubated as described without extract (lane 1), with 200 ng purified p40 (lane 2), or with 250 ng of Heparin-Sepharose fractions of the wild-type strain W303 (lane 3), Δidh1 (lane 4) or Δidh2 (lane 5). For lanes 6 and 7, 750 ng of purified p40 was preincubated for 15 minutes with 1 μg of the Δidh1 or Δidh2 extract, respectively, before addition of the RNA. Extracts from both disruptant strains were mixed with the RNA for lane 8.

Discussion

The RNA-binding property of yeast Idh is surprising and is the first reported case of a mitochondrial enzyme having such activity. Sequence comparisons show that Idh1 and Idh2 are members of a closely related family of proteins with NAD(P)+-dependent isocitrate dehydrogenase activity. The two proteins display 42% sequence identity with each other and 43% identity with the NADP+-dependent isocitrate dehydrogenase of *E. coli*.[10,11] Homology is uniformly spread along the sequence and primary sequence motifs characteristic of other RNA-binding proteins are absent. Preliminary results suggest that, unlike the recently reported RNA-binding activity
of the glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase\textsuperscript{14}, RNA-binding by Idh \textit{in vitro} is not noticeably influenced by addition of either substrate, cofactor, or allosteric effectors of the enzyme (data not shown). Catalytic and RNA-binding sites may therefore be distinct, but mutational analysis is necessary to verify this and to determine the exact nature of the relationship between the two activities. Additionally, in view of the strong evolutionary conservation of Idh, it is of interest to ask whether the enzyme from other sources has comparable RNA-binding activity.

What could be the advantage of combining two such disparate activities such as mRNA-binding and dehydrogenase in a single Krebs cycle enzyme? Despite quite extensive information on the RNA sequence and structure recognized by p40/Idh\textsuperscript{8}, it is unclear why such an enzyme should have a RNA-binding function. We have previously speculated that the protein, which at a level of some 25000 molecules per cell is in principle sufficient to complex all mitochondrial mRNAs, may act as a translational repressor in the mitochondrial matrix\textsuperscript{8}. Such a role is consistent with the observation that for several leaders, p40 binding leads to the exposure of sequences potentially capable of interacting with the initiator AUG p40 might regulate ribosome access to the start codon and thereby regulate the efficiency of translation\textsuperscript{8}. A role as translational repressor is also consistent with the finding that chimeric mRNAs apparently lacking p40 binding sites are still translatable\textsuperscript{21, 22}. The fact that Idh disruption strains are still capable of slow non-fermentable growth is also consistent with a non-essential role of p40 in translation or mRNA stability. It should be noted, however, that disruption strains display increased rates of rho\textsuperscript{-}cell production (data not shown), a property normally indicative of impaired translational activity in mitochondria\textsuperscript{23}.

Thus it is attractive to suggest that the combination of enzyme and RNA-binding activity in one enzyme forges a link between mitochondrial function and its biogenesis. It would be interesting to ascertain whether and how either function of Idh is regulated and whether the two functions are compatible or mutually exclusive. The iron response element binding protein (IRE-BP) is an RNA-binding protein that has recently been identified as the cytosolic aconitase in man\textsuperscript{12}. This protein's ability
to complex RNA may depend on post-translational modifications which reflect the metabolic state of the cell. Interestingly, other links between biogenesis and bioenergetics may also exist. As reported by Schulte et al.\textsuperscript{24} for Neurospora and by Braun et al.\textsuperscript{25} for potato mitochondria, subunits of the mitochondrial import-precurser protease are integral components of the bc1-complex in the respiratory chain, thus raising some interesting questions as to the relationship between protein import and the activity of this enzyme complex.

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