FEBS Letters 483 (2000) 62–66 FEBS 24194

Increased synthesis and decreased stability of mitochondrial translation products in yeast as a result of loss of mitochondrial (NAD^+)-dependent isocitrate dehydrogenase

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Received 1 August 2000; accepted 16 September 2000

Edited by Horst Feldmann

Abstract We have previously demonstrated that the yeast Krebs cycle enzyme NAD^+-dependent isocitrate dehydrogenase (Idh) binds specifically and with high affinity to the 5′-untranslated leader sequences of mitochondrial mRNAs in vitro and have proposed a role for the enzyme in the regulation of mitochondrial translation [Elzinga, S.D.J. et al. (2000) Curr. Genet., in press]. Although our studies initially failed to reveal any consistent correlation between *idh* disruption and mitochondrial translational activity, it is now apparent that compensatory extragenic suppressor mutations readily accumulate in *idh* disruption strains thereby masking mutant behaviour. Now, pulse-chase protein labelling of isolated mitochondria from an Idh disruption mutant lacking suppressor mutations reveals a strong (2–3-fold) increase in the synthesis of mitochondrial translation products. 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. Enhanced degradation of translation products is also a feature of yeast mutants in which tethering/docking of mitochondrial mRNAs is disturbed. We therefore suggest that binding of Idh to mitochondrial mRNAs may suppress inappropriate translation of mitochondrial mRNAs.

Key words: RNA binding; Mitochondrial translation; Respiratory chain; *Saccharomyces cerevisiae*

1. Introduction

In the yeast *Saccharomyces cerevisiae*, seven components of the respiratory complexes are encoded by mitochondrial genes: subunits 1, 2 and 3 of cytochrome *c* oxidase (Cox1p, Cox2p and Cox3p; Complex IV), cytochrome *b* of the bc_1 complex (Cyt*b*; Complex III) and subunits 6, 8 and 9 of the F_0 portion of ATP synthase (Atp6p, Atp8p and Atp9p; Complex V). The mechanism of translation initiation and start site selection of the mRNAs coding for these subunits is obscure. The 5′-untranslated leader sequences are long (up to several hundred nucleotides), AU-rich and contain GC-rich clusters able to form stable secondary structures. In addition, these leaders can contain numerous AUG codons. Synthesis of all seven proteins depends on subunit-specific translational activators. Those required for Cox1p (Pet309p, Mss51p), Cox2p (Pet111p), Cox3p (Pet54p, Pet122p, Pet494p) and Cyt*b* (Cbs1p, Cbs2p) are the best characterised [2]. All but Mss51p depend for their action on the 5′-UTR of their target mRNA. In addition, Pet122p [3,4] has been shown to interact with the small ribosomal subunit. All translational activators studied so far are associated with the inner membrane, leading to the proposal [5] that a mRNA can be translated only after being tethered to the matrix-facing surface of the inner membrane. Tethering is proposed to facilitate the co-translational insertion of newly synthesised proteins into the membrane to allow assembly with nuclear encoded proteins. However, the question how synthesis of nuclear and mitochondrially encoded subunits is balanced is still open.

Although genetic evidence suggests that translational activators specifically recognise the 5′-UTR of their target mRNA, in no case has RNA-binding activity been demonstrated directly. In contrast, a search for RNA-binding proteins in yeast mitochondrial extracts led to the discovery that NAD^+-dependent isocitrate dehydrogenase (Idh) is an RNA-binding protein. Idh, one of the eight enzymes of the Krebs cycle, is an octamer composed of Idh1p and Idh2p (encoded by *IDH1* and *IDH2*, respectively) [6]. We have previously shown that Idh function is not limited to catalytic activity in the TCA cycle. Idh also binds specifically and with high affinity to the 5′-UTR of all mitochondrial mRNAs in vitro [7–9].

Here we show that cells disrupted for the *IDH* genes display a strong increase in mitochondrial translation activity. The newly synthesised products are also more rapidly degraded. Despite increased synthesis, steady-state levels of Cox1p, Cox2p and Cox3p and of Cyt*b* are reduced in the absence of Idh and idh^0 cells also display reduced steady-state levels of fully assembled Complexes III and IV. Surprisingly, levels of F_1F_0-ATP synthase (Complex V) were found not to be affected.

2. Materials and methods

2.1. Strains and growth conditions

The *S. cerevisiae* strain W303-1A (*ade2-1*; *his3-11,-15*; *leu2-3,-112*;

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Abbreviations: BNE, blue native electrophoresis; OXPHOS, oxidative phosphorylation

PII: S0014-5793(00)02086-X

ura3-1; *trp1-1*; *can1-100*; MAT**a**) was used. In this strain *IDH1* and *IDH2* have been disrupted using *LEU2* and *HIS3*, respectively [6,10].

2.2. Isolation of mitochondria

Cells were grown in rich medium containing 2% galactose and harvested at mid-log phase. Isolation of mitochondria and subsequent in vitro translation were carried out as described by McKee and Poyton with a few minor adjustments [11]: spheroplasts were lysed by Potter homogenisation and mitochondria were resuspended in 0.6 M mannitol to a final concentration of 3 mg protein per ml.

2.3. In organello pulse-chase labelling and immunoblotting

For efficient translation, isolated mitochondria were incubated in optimised protein-synthesising medium D in the presence of 8 μl/ml TRAN^{35}S-LABEL™ (1175 Ci/mmol; 10.5 mCi/ml; containing 70% L-(^{35}S)methionine and 15% L-(^{35}S)cysteine; ICN Biomedicals, Inc.). Labelling was allowed to continue for 30 min and 250 μl samples were taken at 10 min and 30 min. An excess of cold methionine (final concentration 0.2 M) was added after 30 min to start the chase. Samples were taken at 30 min and 60 min chase.

Samples were recovered by centrifugation for 2 min at 15 000 × *g* and the resulting pellet was prepared for SDS–PAGE in LSB (2% SDS; 5% β-mercaptoethanol; 5.8% glycerol; 62.5 mM Tris–HCl pH 6.8; 100 μg/ml bromphenol blue). Protein gels (both SDS–PAGE and blue native electrophoresis (BNE)) were blotted on MSI MicronSep nitrocellulose disc filters.

2.4. BNE and 2D gel electrophoresis

Analysis of intact mitochondrial oxidative phosphorylation (OXPHOS) complexes was done by BNE [12]. For these experiments a 6–16% polyacrylamide gradient was used.

For 2D electrophoretic analysis of pulse-chased mitochondria, mitochondria were solubilised by treatment with 2% lauryl-maltoside and the extracts separated in the first dimension by BNE as described above.

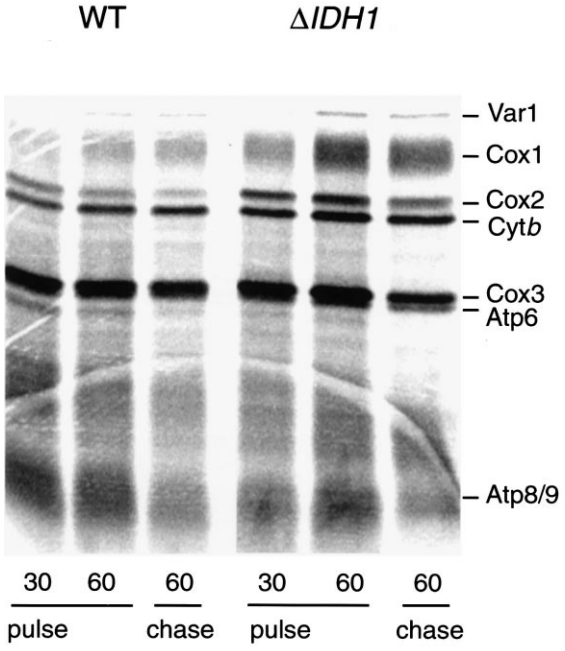

Fig. 1. Synthesis and turnover of mitochondrial encoded proteins in mitochondria isolated from a wild-type and an *idh1*^{0} strain. Mitochondrial translation products were labelled with ^{35}S-methionine during 60 min, followed by the addition of an excess (20 mM) of unlabelled methionine and a 60 min chase. Samples were taken after 30 and 60 min labelling and after 60 min chase and were analysed by SDS–PAGE and phosphoimaging (for details see Section 2). Newly synthesised proteins are indicated: the ribosomal protein Var1p, cytochrome *c* oxidase subunits Cox1p, Cox2p and Cox3p, Complex III subunit cytochrome *b* (Cyt*b*) and ATP synthase subunits Atp6p, Atp8p and Atp9p.

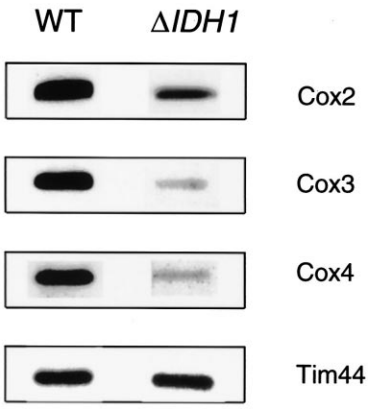

Fig. 2. Reduced steady-state levels of Cox2p, Cox3p and Cox4p in mitochondria from an *idh1*^{0} strain compared to wild-type. Mitochondrial proteins (15 μg) were separated by SDS–PAGE and blotted on membrane (Section 2). Cox2p, Cox3p and Cox4p were detected with monoclonal antibodies. As a control, the blot was incubated with an antibody against Tim44p, showing equal amounts in both strains.

3. Results

Despite the fact that Idh could readily be shown to bind specifically [7–9] and with high affinity (calculated K_d of 2–5 nM; M. Siep, unpublished results) to 5′-UTRs of all mitochondrial mRNAs in yeast, it turned out to be more difficult than we expected to assess the relevance of these findings in terms of mitochondrial translation.

Based on the observations that (1) mRNAs apparently lacking an Idh-binding site in their 5′-UTR are translatable [13], (2) our idh mutant (and that obtained from Dr. L. McAlister-Henn [6]) was capable of growth on glycerol-containing media, we concluded that Idh is not essential for translation and proposed a role as a translational repressor. For the extensively characterised *COX2* mRNA, such a role is also consistent with the finding that (1) Idh binds to a folded form of the 5′-UTR in which the initiator AUG is likely to be masked,

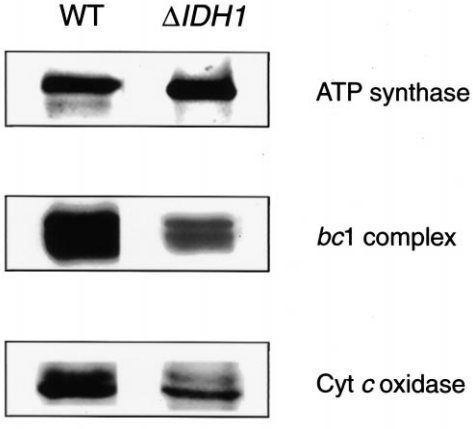

Fig. 3. Steady-state levels of respiratory complexes in mitochondria from wild-type and *idh1*^{0} cells. Mitochondria were solubilised in 2% lauryl-maltoside (Section 2) and electrophoresed in the presence of Serva Blue G on a 6–16% non-denaturing polyacrylamide gel as described by Schägger and von Jagow [12]. The separated proteins were analysed by immunoblotting with polyclonal antibodies against F1b, F1β of ATP synthase, Core II of bc_1 and cytochrome *c* oxidase.

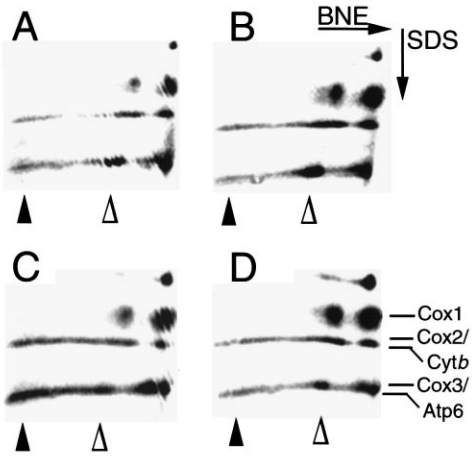

Fig. 4. Synthesis and assembly of mitochondrially encoded proteins in mitochondria isolated from a wild-type and an *idh1*^{0} strain. Mitochondrially encoded proteins of wild-type (panels A and B) and *idh*^{0} (panels C and D) were pulse labelled with ^{35}S-methionine for 60 min (panels A and C) and chased with an excess of unlabelled methionine (panels B and D). Proteins were analysed in a 2D BNE (first dimension)–SDS (second dimension) gel system (for details see Section 2). Individual subunits are indicated in the margin of panel D. The migration pattern of the cytochrome *c* oxidase complex in the first dimension is indicated by open triangles. Closed triangles indicate the high molecular weight position at which proteins accumulate (see Section 4).

and (2) an overlap of Idh- and Pet111p-binding sites within the 5′-UTR suggests that translational activation by the latter might be modulatable by Idh [14].

Apart from an initially intriguing observation of increased translational activity in strains disrupted for either or both Idh subunits [15], subsequent experimentation failed to reveal a consistent correlation between *idh* disruption and translational activity. Considering that our failure to establish such a correlation might be the consequence of complex and possibly compensatory effects of *idh* disruption on translation via changes in mitochondrial energy and redox balance in combination with loss of RNA binding, we turned our attention to the identification and construction of variant and mutant Idhs in which catalytic activity was retained, but RNA binding was reduced or lost [1]. In the course of this work, however, reports by McCammon and co-workers [10,16] provided new and alternative insights into our failure to demonstrate a clear effect on translational activity. Przybyla-Zawislak et al. show that strains disrupted for either *IDH1* or *IDH2* are in fact strongly impaired in respiratory growth. However, when such *idh*-disrupted strains are plated on glycerol-containing medium, accumulation of extragenic suppressor mutations eventually allows varying degrees of growth. We therefore re-examined our *idh* disruption strains for the presence of extragenic suppressor mutations by crossing the *idh2*^{0} haploid strain with a W303 wild-type haploid. After sporulation, 16 tetrads were dissected and analysed (see Table 1). The high numbers of tetrads showing an aberrant segregation pattern of the glycerol phenotype (4:0 and 3:1, 2 and 11 tetrads, respectively) clearly indicate the presence of an extragenic suppressor mutation. This mutation, when combined with the *idh2*^{0} allele, restores respiratory growth. The *HIS3* marker, which was used to disrupt the *IDH2* gene, shows the expected 2:2 segregation pattern in all tetrads.

We repeated our initial mitochondrial protein labelling experiments using *idh1*^{0} and *idh2*^{0} strains constructed in the McCammon laboratory [6,10]. Both mutants had been checked for the absence of extragenic suppressor mutations and both were unable to grow on non-fermentable carbon sources.

To assess the effect of loss of Idh on mitochondrial translation, we compared pulse labelling of newly synthesised proteins in mitochondria isolated from the wild-type and *idh1*^{0} strain (Fig. 1). Time samples were taken after 30 min and 60 min labelling and after 60 min chase in the presence of unlabelled methionine. Equal amounts of mitochondrial protein were separated by SDS–PAGE and then blotted to nitrocellulose filters. Labelled translation products were analysed by phosphoimager scanning and quantified with ImageQuant™.

The results presented in Fig. 1 show that in the absence of Idh1, the synthesis of ATP synthase subunit Atp6p, bc_1 subunit Cyt*b* and cytochrome *c* oxidase subunits Cox1p, Cox2p and Cox3p is strongly increased (Fig. 1; compare lanes 30/60 min pulse of W303 and *idh*^{0}). Quantitation shows that the increase is between 1.5-fold (for Cox3p) and 3-fold (for Cox1p) the wild-type level.

Strikingly, the increase of newly synthesised proteins in the *idh1*^{0} strain is accompanied by an enhanced turnover (Fig. 1; compare lanes 60 min chase of both strains). Quantitation of the radioactivity in the gel indicates that, during the time course of the chase, about 20% of the labelled protein is degraded in mitochondria from the wild-type cells compared to 50% in mitochondria from the *idh1*^{0} strain. The effect is most pronounced with Cox3p: 25% is degraded in wild-type mitochondria compared to 55% in the mitochondria lacking Idh. This increase in protein synthesis and enhanced turnover as observed in an idh1^0 strain is not observed when other genes coding for Krebs cycle enzymes like citrate synthase (*CIT1*) or malate dehydrogenase (*MDH1*) are disrupted (data not shown).

Since both synthesis and turnover of mitochondrial proteins are increased in mitochondria lacking Idh, we next investigated the effect on steady-state levels of cytochrome *c* oxidase subunits using immunodetection. The increased synthesis of the mitochondrially encoded subunits of cytochrome *c* oxidase in the absence of Idh is not reflected in the steady-state levels of the proteins (Fig. 2). Indeed, despite the 3-fold higher synthesis rate, Cox1p is undetectable, Cox3p is hardly detectable and Cox2p and Cyt*b* are reduced (Fig. 2; compare left and right panels; Cox1p and Cyt*b* data not shown). Interestingly, the level of the nuclear encoded Cox4p subunit is reduced to the same extent as Cox2p and Cox3p. In sharp contrast to these differences, levels of Tim44p (an inner membrane protein involved in mitochondrial protein import) appear to be the same in both strains.

Table 1
Tetrad analysis of a cross between a wild-type strain (W303) and an *idh2*^{0} strain (W303; idh2::*HIS3*)

Phenotype	Segregation		
	4:0	3:1	2:2
gly$^+$/gly$^-$	2	11	3
his$^+$/his$^-$	0	0	16

The effect on Cox4p implies that levels of fully assembled respiratory chain complexes should be reduced in the *idh1*^{0} strain. That this is indeed the case was confirmed by blue native gel electrophoretic separation of mitochondrial extracts. Three OXPHOS enzyme complexes contain mitochondrially encoded subunits: F_1F_0-ATP synthase (Complex V), cytochrome *c* oxidase (Complex IV) and the bc_1 complex (Complex III). To detect these complexes in a native gel, antibodies against ATP synthase subunit $F_1\beta$, subunit II of Complex III and cytochrome *c* oxidase (holoenzyme) were used (Fig. 3). As expected from the results shown (Fig. 2), cytochrome *c* oxidase and bc_1 levels are significantly reduced (by about 80%) in mitochondria lacking Idh. Surprisingly, and in contrast to the effect on respiratory enzymes, the level of ATP synthase appears not to be affected in the mutant strain.

From our results it is clear that despite increased mitochondrial translation in the absence of Idh1p, steady-state levels of the respiratory enzymes are low, suggesting impaired assembly either in addition to, or as a consequence of increased turnover. To investigate this further, newly synthesised proteins were subjected to 2D gel electrophoresis (Section 2), to allow the analysis of their assembly state. In the first dimension protein complexes are separated on a non-denaturing gel. In the second, denaturing dimension (SDS–PAGE), the individual subunits of these complexes are resolved. 2D BNE–SDS separations of pulse-chased wild-type and *idh1*^{0} mitochondria are shown in Fig. 4. In wild-type mitochondria (pulse: panel A, chase: panel B), newly synthesised Cox1p, Cox2p and Cox3p migrate at a number of discrete positions, which are likely to represent distinct intermediates in the assembly of cytochrome *c* oxidase [17,18]. After 60 min labelling, a substantial portion of newly synthesised Cox3p co-migrates in the first dimension with fully assembled cytochrome *c* oxidase and this portion continues to increase during the chase, indicating that even under in vitro conditions, in which only limited amounts of nuclear encoded subunits are available, assembly can still occur (Fig. 4A and B, open triangles). The absence of Idh1p results in enhanced Cox3p synthesis (as already shown in Fig. 1), but only a small fraction of the newly synthesised protein is assembled into cytochrome *c* oxidase (Fig. 4C, open triangle). This amount increases only slightly during the chase (Fig. 4D, open triangle). Unlike the situation in the wild-type cells, the bulk of the newly synthesised Cox3p visualised after 60 min of labelling accumulates at a position corresponding to an approximate molecular weight of 1 MDa (compare Fig. 4A and C, closed triangles). In contrast to normally assembled Cox3p, this material is rapidly degraded during the chase (Fig. 4D, closed triangle). Thus, despite high Cox3p synthesis, only a small fraction of the newly produced protein is assembled into Complex IV, a picture consistent with our observation of a reduced steady-state level of cytochrome *c* oxidase (Fig. 2).

Taken together, these results both support our initial hypothesis that Idh functions as a negative regulator of translation of mitochondrial mRNAs and suggest additional roles for RNA binding by Idh in terms of stabilisation of mitochondrial translation products and their subsequent assembly into functional respiratory complexes.

4. Discussion

Earlier work performed in our group identified the NAD^+-dependent Krebs cycle enzyme Idh as an RNA-binding protein. It was shown that Idh could bind specifically and with high affinity to the 5′-untranslated leader sequences of all mitochondrial mRNAs in vitro. Idh is an octameric enzyme consisting of two subunits, Idh1p and Idh2p. Disruption of the gene for either subunit leads to a loss of enzyme activity and RNA binding, indicating that the native enzyme is necessary for both activities. We have previously suggested that the enzyme may modulate translation, thereby in some way linking the need for Krebs cycle function and respiratory chain activity to the rate of mitochondrial biogenesis. However, apart from some initial results using strains disrupted for either Idh subunit which lent support to the view that the enzyme can act as a repressor of translation, we were in later experiments unable to reproducibly confirm a repressive effect on mitochondrial translation activity. More recent observations made by McCammon and co-workers now provide an explanation for this behaviour of Idh disruptants in terms of their ability to accumulate extragenic suppressor mutations. As shown by Przybyla-Zawislak et al. [16], disruptants of *IDH1* and/or *IDH2* are respiratory deficient, exhibiting strongly reduced growth on glycerol. Isolates of these strains readily accumulate colonies which display normal respiratory growth and which on subsequent genetic characterisation turn out to contain suppressor mutations in (primarily) *CIT1* and *MDH1* genes. Neither of these classes of suppressors restores Krebs cycle activity and the basis of their suppressive effect is not understood. Nevertheless, these findings prompted us to examine the possibility that our *Idh*^{0} strains may contain similar extragenic suppressor mutations. First, in contrast to Idh disruptant strains constructed by Przybyla-Zawislak et al. [16], both Idh disruptants constructed by us display growth on glycerol. Crossing of our *Idh*^{0} strain with a wild-type strain, followed by sporulation and tetrad dissection indeed showed the presence of an extragenic suppressor mutation.

Having identified the presence of an extragenic suppressor mutation in our *idh*^{0} strains, we repeated the pulse-chase labelling experiments of mitochondrial proteins with freshly constructed *idh*^{0} strains provided by the McCammon laboratory. The results of these experiments are reported here. As indicated in Fig. 1, the absence of Idh clearly results in an increase of translational activity in isolated mitochondria. The same effect was observed in experiments using cycloheximide-inhibited cells (data not shown). In both cases, synthesis is enhanced, but the newly produced proteins are degraded more rapidly. Additionally, in the absence of Idh1p, a large portion of newly synthesised Cox3p (and to a lesser extent Cox2p) is not assembled into cytochrome *c* oxidase. Instead, it is found in a high molecular weight complex (approximately 1 MDa) of unknown identity that is rapidly degraded. The identity of the protease(s) responsible for this degradation is at present unknown. However, membrane-bound members of the triple-A metalloprotease family, including Yme1p and Afg3p/Rca1p [19,20] are known to be responsible for the rapid turnover of non-assembled mitochondrial translation products and have been characterised as high molecular weight complexes. Although recently observed in a mutant putatively disturbed in mitochondrial translational fidelity control [21] generally increased mitochondrial translation activity is not a common feature of other yeast mutants affecting mitochondrial function. Pulse-chase labelling experiments of mitochondrial proteins are routinely performed in our laboratory, using

a wide variety of (mutant) strains [20,22,23]. A general increase in translation activity as seen in the *idh1*^{0} strain has not been seen. Additionally, disruptants of *CIT1* and *MDH1* show translation patterns indistinguishable from wild-type (data not shown).

The observation that steady-state levels of respiratory chain complexes are lower in the *idh*^{0} strain (see Figs. 2 and 3), correlates well with the enhanced degradation seen in Fig. 1. One could argue that conditions of enhanced production (and turnover) of mitochondrially encoded subunits would at least result in similar steady-state levels of respiratory chain complexes in wild-type and *idh*^{0} strains. Recently, studies have been described on chimeric mRNAs harbouring the *COX2* or the *COX3* coding region under transcriptional and translational control of the *VAR1* 5′-UTR [5]. As a protein of the mitoribosomal small subunit, it is thought that synthesis of Var1p does not depend on co-translational membrane insertion. In line with a membrane-tethering model for mitochondrial translation, both Cox2p and Cox3p were translated efficiently from the chimeric mRNAs, but their incorporation into active cytochrome *c* oxidase was found to be severely defective [5]. The increased turnover observed in Idh-deficient cells is reminiscent of that observed to take place when the mRNAs for Cox2p and Cox3p are prevented from membrane tethering/docking by the 5′-UTR replacement. Taking these data into account, we suggest that RNA binding by Idh may function to prevent translation of mRNAs in the mitochondrial matrix. As recently shown by Anderson et al. [24] and our lab [1], binding of RNA to Idh results in reduction of catalytic activity, but this effect can be reversed by AMP as allosteric effector of the enzyme [24]. This combination of functions in mitochondrial redox balance and translational control may therefore permit Idh to play a key role in regulating the rate of mitochondrial assembly to the need for mitochondrial function.

Acknowledgements: We thank M. Siep for sharing unpublished results. This work was supported in part by the Chemical Sciences section of the Netherlands Science Research Council (CW-NWO) and EU (Human Capital and Mobility Programme, contract No. CHRX-CT94-0520).

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