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In vitro RNA editing in plant mitochondria does not require added energy

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Abstract **RNA editing in flowering plant mitochondria is investigated by in vitro assays. These cauliflower mitochondrial lysates require added NTP or dNTP. We have now resolved the reason for this requirement to be the inhibition of the RNA binding activity of the glutamate dehydrogenases (GDH). Both GDH1 and GDH2 were identified in RNA–protein cross-links. The inhibition of in vitro RNA editing by GDH is confirmed by the ability of the GDH-specific herbicide phosphinothricin to substitute for NTP. NADH and NADPH, but not NAD or NADP, can also replace NTP, suggesting that the NAD(P)H-binding-pocket configuration of the GDH contacts the RNA. RNA editing in plant mitochondria is thus intrinsically independent of added energy in the form of NTP.**

Keywords: RNA editing; Plant mitochondria; Glutamate dehydrogenase; RNA-binding; In vitro RNA editing; Cauliflower

1. Introduction

In plants RNA molecules are in both organelles altered by RNA editing. In plastids of flowering plants about 35 and in mitochondria about 400 selected cytosines are changed to uridines. The direct biochemical effect of RNA editing in these plants is thus a site-specific deamination. Despite years of investigation neither the reaction mechanism nor the enzymes involved have been identified. Several clues on – or rather conditions of – the biochemistry of the reaction have emerged mostly from in vitro analyses of plastid and mitochondrial lysates, respectively. The first in vitro assays of plant mitochondrial lysates suggested that the sugar-phosphate bonds of the affected RNA molecule are not disrupted in the polynucleotide chain [1]. This observation excludes insertional editing which would excise and exchange either the nucleotide or the base. Thus either direct deamination or transamination are the most likely mechanisms. The first reaction would per se require no added energy, while for the latter, the transamination reaction, additional molecules of higher molecular energy would most likely be involved.

For mitochondrial lysates a strict requirement for added ATP has been observed [2]. Similarly in plastids additional ATP has been reported to be required in most lysates [3–5], while in some residual activity can be seen without any added ATP [6,7]. Surprisingly the added ATP can be substituted to full effect by some or all of the other NTPs and even dNTPs [2,6,7]. While all of these molecules are virtually interchangable in mitochondrial lysates, they vary in their effect in plastid extracts [6,7]. In both organelles at least one of the dNTPs is as effective as ATP, suggesting that one of the few enzymes accepting either triphosphate is involved. One group of such enzymes is a class of RNA helicases and their participation has consequently been proposed [2].

We have now investigated the requirement for nucleotide triphosphates in detail and come to the surprising conclusion that this dependence is at least in mitochondria almost entirely explained by the behaviour of the enzyme glutamate dehydrogenase (GDH).

2. Materials and methods

2.1. Preparation of mitochondrial extracts

Cauliflower mitochondria were purified by differential centrifugation steps and a Percoll gradient as described [2,8]. Isolated mitochondria were lysed, the lysate was cleared and the supernatant was recovered and dialyzed as detailed previously [2,8].

2.2. RNA substrates

DNA clones were constructed in an adapted pBluescript SK^+ to allow run-off transcription of the editing substrate RNA as described [2,8]. Substrate RNAs containing vector sequences at the 5′- and 3′-ends of the mitochondrial insert sequences were synthesized from the T7 RNA polymerase promoter in the linearized template DNA. The bordering bacterial sequences were used for specific amplification of the substrate RNAs by RT-PCR after the in vitro assay [9,10]. The ^{32}P-labelled RNA templates for the gel shift assays were obtained by direct incorporation of labelled ATP into the RNA during run-off transcription from the DNA template.

2.3. In vitro RNA editing reactions

The in vitro RNA editing reactions were performed as described [8,11]. After incubation, substrate sequences were amplified by RT-PCR, the upstream primer being labelled with the Cy5 fluorophor. RNA editing activity was detected by mismatch analysis employing the TDG enzyme activity (thymine DNA glycosylase, Trevigen). The TDG treated fragments were separated and the Cy5 fluorescence was scanned and displayed using an ALF express DNA sequencer (GE Healthcare).

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2.4. RNA binding proteins

Proteins in the mitochondrial lysate binding to RNA were analysed by incubating 1 pmol RNA template for 30 min in the reaction mixture. In the template RNA every uridine was labeled with ^{32}P by in vitro transcription without adding cold UTP. The proteins in contact with the RNA were irradiated with UV light for 20 min (Stratalinker 1800, stratagene). This step was followed by digestion of the RNA with RNase A at 37 °C for 30 min. Protein samples were dissolved in buffer and separated by SDS–PAGE on a 10% gel. Labelled proteins were visualized with a Bioimaging Analyzer BAS-3000 (Fuji Photo Film Co).

2.5. Protein identification

RNA editing templates were labelled with biotin (biomers GmbH) and 500 pmol were incubated with 100 μl of mitochondrial lysate in a total volume of 200 μl under standard in vitro RNA editing conditions in the presence of 1 mg total yeast RNA [2]. The proteins in contact with the RNA were cross-linked by UV irradiation for 20′ (UV Stratalinker 1800, Stratagene). The biotin-labelled RNA–protein complexes were bound to streptavidine sepharose™ High performance (GE healthcare). Unbound proteins were washed off by six rinses in spin columns with 400 μl wash buffer each (30 mM HEPES-KOH pH 7.7, 3 mM magnesium acetate, 45 mM potassium acetate, 30 mM ammonium acetate, 10% glycerol). The sepharose beads were treated with RNase A to release the RNA-bound proteins, which were collected with wash buffer. Proteins with their bound residual RNA fragments were analysed by PAGE and/or were (directly) identified by liquid chromatography coupled (LC) electrospray ionization (ESI) tandem mass spectrometry (MSMS). Proteins cross-linked to RNA were digested within the gel using trypsine according to Shevchenko et al. [12]. For digestion of proteins in solution after extraction from the beads, the samples are denatured in 4 M urea and 2 M thiourea, diluted to a final concentration of $\leqslant$1 M urea and incubated with 2 μg trypsine overnight at room temperature. Peptides are desalted and separated by nanoLC equipped with a pre-column working in backflush and directly analyzed by ESI MSMS in a Q-ToF (Q-ToF ultima, Waters) or a hybrid triple quadrupole/linear iontrap mass spectrometer (4000 QTrap, ABI) under standard conditions. Fragment spectra of peptides are searched against the NCBInr database using Mascot as search engine. Matches with resulting protein identification were usually obtained against the *Arabidopsis thaliana* sequences, which is phylogenetically closely related to the here analysed cauliflower (*Brassica oleracea*). Since different species are compared, similarities/differences in the respective orthologous protein sequences additionally influence the generated scores.

3. Results and discussion

3.1. NTP as well as dNTP support the in vitro RNA editing reaction

The strict requirement for added NTP in the in vitro RNA editing reaction is documented in Fig. 1. No RNA editing product with uridine in the relevant position of RNA editing is detectable if no NTP is added. Addition of ATP at the optimal concentration of 15 mM yields about 2–5% C to U converted nucleotide, the amount depending upon the lysate, the source of the mitochondria and the template used. The positive effect of ATP can be fully recovered by substitution with CTP, shown as example here (Fig. 1). Any of the NTPs or dNTPs can replace ATP and will yield comparable editing rates [2]. Since in plastid as in mitochondrial in vitro systems NTPs and dNTPs can substitute for ATP, analogous factors may be responsible for the in vitro ATP requirement in both organelles.

3.2. Influence of the added NTP on template RNA–protein interactions

To investigate the protein moieties affected by the added NTP/dNTP in their ability to contact and bind to the RNA

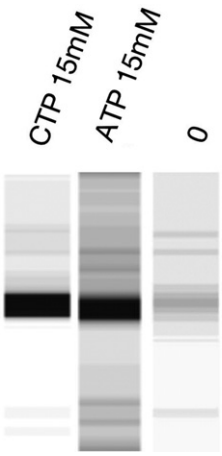

Fig. 1. In vitro RNA editing of an *atp4* mRNA template requires the addition of ATP or CTP. Similar to the in vitro editing of an *atp9* RNA [2], dNTP-nucleotides can substitute for the NTP in this reaction (data not shown).

substrate molecules, we performed gel shift experiments with radioactively labelled RNA editing substrates (data not shown). Comparisons of the protein patterns cross-linked with the template RNA in the presence or absence of ATP on PAGE-gels and by more sensitive mass spectroscopy (MS)-analyses did not reveal any qualitative differences. The overall quantity of the proteins labelled with the cross-linked RNA, however, changes dramatically with the presence or absence of NTP: Without added ATP the same proteins are labelled much stronger (Fig. 2). This suggests that addition of ATP disturbs all protein–RNA interactions non-specifically.

3.3. Identification of proteins bound by the RNA editing template

Since the gel shift and UV cross-linking experiments (Fig. 2) did not yield detectable proteins differentially affected by NTP in their affinity to the RNA editing template, we employed a more sensitive affinity purification scheme to identify respective polypeptides. This procedure achieved an enrichment of the proteins bound to the editing template RNA (Fig. 3). The Coomasie-stained SDS–PAGE patterns of proteins retained by the RNA template in the presence or absence of ATP, however, again showed only little qualitative difference and no clear candidate protein could be assigned by its ATP-dependent affinity to the template in this gel analysis (data not shown).

Because the ATP-dependence of the in vitro reaction may not manifest in abundant specific RNA–protein interactions and the PAGE analysis may not be sensitive enough, we proceeded to analyse the set of proteins interacting with the RNA directly by the much more sensitive MS without prior gel separation. The proteins bound by the RNA editing template were hydrolyzed and peptides were identified by liquid chromatography (LC) coupled tandem MS (MSMS) analysis. To identify the proteins, the obtained fragment spectra were searched against all entries in the NCBInr database. Since cauliflower and *A. thaliana*, which latter is completely sequenced, are closely related and the protein sequences of many household enzymes are to a large extent identical, we should be able to identify cauliflower proteins through their