BUDAPEST UNIVERSITY OF TECHNOLOGY AND ECONOMICS
FACULTY OF CHEMICAL TECHNOLOGY AND BIOTECHNOLOGY
GEORGE OLAH DOCTORAL SCHOOL

The connection between the mitochondrial DNA and the oxidative protein folding apparatus

Ph.D. theses

Author: Tibor Balogh
Supervisor: András Szarka, Ph.D.

Budapest University of Technology and Economics
Department of Applied Biotechnology and Food Science
Laboratory of Molecular Biology and Biochemistry

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1 Introduction

The biological activity of a protein is determined by its three-dimensional (3D) native structure, which in turn is defined by its amino acid sequence. Disulphide bridges formed between cysteine residues can stabilize the native structure of proteins more effectively. The prokaryotic periplasm and the endoplasmic reticulum of eukaryotes were considered as the only compartments for enzyme mediated formation of stable disulphide bonds. Recently, the mitochondrial intermembrane space has emerged as the third protein-oxidizing compartment.

In special cases, mitochondrial disorders can cause the failure of protein folding. It is well known that the mutations of mitochondrial DNA can cause serious, often fatal neurodegenerative symptoms. The mitochondrial electron transfer chain functions also as the final electron acceptor of the mitochondrial oxidative folding machinery. Thus the injured process of protein folding caused by mtDNA damage can lead to the accumulation of misfolded proteins, which may further aggravates the situation.

Dehydroascorbic acid (DHA), the most oxidized form of vitamin C is able to oxidize the protein disulphide isomerase (PDI) enzyme, which is considered, as the central member of the disulphide relay system in the endoplasmic reticulum. Thus DHA may contribute to the maintenance of oxidative protein folding.

In our experiments we investigated primarily the effects of mtDNA damage on oxidative folding process. MtDNA depleted, ρ^0 cell lines were used as models organisms. Furthermore, the possible electron acceptor role of dehydroascorbic acid in the mitochondrial oxidative folding process was investigated. The analytic performance of different spectrophotometric and fluorometric methods for vitamin C determination was studied to get an objective picture on their applicability.

2 Background

2.1 The importance of oxidative protein folding

Although several types of oxidative protein modifications have an impact on folding, traditionally, the use of the term is restricted to disulphide bond formation. Disulphide bonds between cysteine residues can significantly contribute to the formation of thermodynamically stable structure of proteins.[1] Such bonds can stabilize the native structure significantly, both by lowering the entropy of the unfolded state and by forming stabilizing interactions in the native state.[2] This effect has a high importance in the case of secreted proteins, because it helps to maintain their native structure in the mainly oxidizing extracellular milieu.

Recently, it was discovered, that beyond prokaryotic periplasm and endoplasmic reticulum of eukaryotes, the mitochondrial intermembrane space is the third protein-oxidizing compartment.

The most important chemical reaction of disulphide bond formation is the thiol-disulphide exchange:

$$R_1S^- + R_2SSR_3 \rightarrow R_2S^- + R_1SSR_3$$

[1] Betz SF (1993). Protein Sci 2:1551–8.
[2] Narayan M, Welker E, Wedemeyer WJ, Scheraga H a (2000). Acc Chem Res 33:805–12.

The core of this reaction is that a thiolate anion R_1S^- displaces one sulphur of the disulphide bond of R_2SSR_3 molecule. In the transition state, the negative charge of the thiolate appears to be delocalized among the three sulphur atoms. Protein disulphide bonds are formed and reduced by two such thiol-disulphide exchange reactions with a redox reagent, the first of which involves the formation of a mixed disulphide bond between the protein and the redox reagent. Then an additional thiol group of the same protein reacts with the mixed disulphide, which leads to the dissolution of the intermolecular cross-link, and formation of an intramolecular disulphide bond.[2]

In the process of oxidative folding, proteins are usually oxidized by an oxidoreductase enzyme, which in fact "carries" the disulphide bonds generated by sulfhydryl oxidases to the target proteins. The oxidizing capacity for these processes is generally provided by the molecular oxygen.[3]

2.2 The role of mitochondrial DNA in cells

Mitochondria are the only location of extra-chromosomal DNA within the cell (except in plant chloroplasts). The mitochondrial genome consists of a multicopy, circular dsDNA molecule (16.6 kb in humans), which encodes 13 essential polypeptides of the OXPHOS system and the necessary RNA machinery (2 rRNAs and 22 tRNAs) for their translation within the organelle. The remaining protein subunits that make up the respiratory-chain complexes, together with those required for mtDNA maintenance, are nuclear-encoded, synthesized on cytoplasmic ribosomes, and are specifically targeted and sorted to their correct location within the organelle.[4] Historically, mitochondria have been considered as discrete, noncommunicative entities, but recent evidence indicates that quite the opposite is true with frequent fission and fusion events allowing exchange of genetic material between mitochondria.[5]

Mitochondrial DNA mutations contribute to a variety of biochemical abnormalities in living cells, which can affect the operation of whole organs or organ-systems. In order to investigate the biochemical abnormalities caused by mtDNA defects, we can produce cell lines that have been completely depleted of their endogenous mtDNA. These cell lines are generally called ρ^0 cell lines.[6]

The classic method to generate ρ^0 cell lines is the long-term treatment of cells with ethidium bromide. Ethidium bromide is a phenanthridine dye, which preferentially intercalates between the strands of double stranded DNA molecules. The chemical is a potent inhibitor of mtDNA synthesis, but has no effect on the replication of nuclear DNA.[7]

2.3 The mitochondrial oxidative folding

Except the 13 mtDNA encoded electron transfer chain proteins, majority of mitochondrial proteins use sophisticated import processes to reach their intended location in one of the submitochondrial compartments.[8]

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[3] Riemer J, Bulleid N, Herrmann JM (2009). Science 324:1284–7.
[4] Taylor RW, Turnbull DM (2005). Nat Rev Genet 6:389–402.
[5] McFarland R, Turnbull DM (2009). J Intern Med 265:210–28.
[6] Armand R, Channon JY, Kintner J, et al. (2004). Toxicol Appl Pharmacol 196:68–79.
[7] Horwitz HB, Holt CE (1971). J Cell Biol 49:546–53.
[8] Sideris DP, Tokatlidis K (2010). Antioxid Redox Signal 13:1189–204.

Most of the substrate proteins of the mitochondrial disulphide relay system have a low molecular weight and contain a characteristic helix-turn-helix structure, in which the two helices are connected through a disulphide bridge. The main character of the mitochondrial disulphide formation machinery is the oxidoreductase MIA40 (Mitochondrial Import and Assembly), which is anchored to the inner mitochondrial membrane in fungi, whereas its homologs of plants and animals are soluble proteins in the IMS. A conserved redox-active disulphide bond situated in the active site of the enzyme is able to oxidize the cysteine residues of polypeptide chains imported into the IMS.[3] MIA40 becomes reduced while it mediates the formation of disulphide bonds into the target proteins. It should be oxidized before the next catalytic cycle. This reoxidation is performed by the ALR (Augmenter of Liver Regeneration) protein, which belongs to the group of sulfhydryl oxidase flavoproteins. The reduced ALR can pass the electrons to the cytochrome c via its cytochrome c reductase activity. Cytochrome c delivers the electrons to respiratory COMPLEX IV (cytochrome c oxidase), which uses them altogether with the electrons delivered from terminal oxidation to reduce respiratory oxygen to water.[9]

2.4 Mitochondrial dehydroascorbic acid transport

Although the first report on mitochondrial vitamin C transport was published more than 30 years ago, many details of the transport became evident in the last few years. A stereo-selective mitochondrial D-glucose uptake mechanism, which competes with the transport of dehydroascorbic acid (DHA), was found in the mitochondria from mammalian cells. In accordance with former computational analysis of human GLUT hexose transporter isoforms, the presence of GLUT1 in mitochondrial membrane was verified by experimental observations, too.[10] More recently, GLUT10 was also reported to be localized to the mitochondria of mouse aortic smooth muscle cells and insulin-stimulated adipocytes.[11]

Up to now DHA was considered to be the transported form of vitamin C through the mitochondrial inner membrane. However, in recent studies on U937 then HEK-293 cells mitochondrial expression of sodium dependent ascorbic acid transporter, SVCT2 was also reported.[12,13]

2.5 The role of dehydroascorbic acid in the oxidative protein folding

Together with the isolation of protein disulphide isomerase (PDI), the oxidoreductase of endoplasmic reticulum, a heat-stable factor was described in the formation of disulphide bonds (oxidative protein folding). This heat-stable factor could be replaced by the oxidized form of vitamin C, DHA.[14] Based on this fact, DHA can be considered as a potential electron acceptor of oxidative protein folding.

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[9] Allen S, Balabanidou V, Sideris DP, et al. (2005). J Mol Biol 353:937–44.
[10] KC S, Cárcamo JM, Golde DW (2005). FASEB J 19:1657–67.
[11] Lee Y-C, Huang H-Y, Chang C-J, et al. (2010). Hum Mol Genet 19:3721–33.
[12] Azzolini C, Fiorani M, Cerioni L, et al. (2013). IUBMB Life 65:149–53.
[13] Muñoz-Montesino C, Roa FJ, Peña E, et al. (2014). Free Radic Biol Med 70:241–54.
[14] Venetianer P, Straub FB (1964). Biochim Biophys Acta 89:189–90.

3 Experimental

3.1 Cell culture

HepG2 human hepatocyte carcinoma, MCF7 human breast adenocarcinoma, SH-SY5Y human neuroblastoma cell lines were obtained from Sigma, and grown at 37 °C, under 5% CO_2 atmosphere following the instructions of the distributor.

3.2 Preparation and maintenance of ρ^0 cell lines

The generation of ρ^0 cells has been achieved by the protocol of King and Attardi.[15] In the case of SH-SY5Y cells, the parameters of the treatment were modified following the recommendations of Trimmer et al.[16]

The depletion of mtDNA was verified by real-time PCR. Total DNA for the subsequent PCR amplification was isolated from cells by using Wizard SV Genomic DNA Purification System (Promega) following the manufacturer's protocol.

3.3 Inhibition of mitochondrial respiratory chain complexes

In order to block the different mitochondrial respiratory complexes, HepG2 cells were incubated for 24 h in complete media, supplemented with the inhibitors of different respiratory complexes. The following inhibitors were applied: rotenone for COMPLEX I, at final concentration of 0.025 μM, thenoyltrifluoroacetone (TTFA) for COMPLEX II, at final concentration of 200 μM, antimycin A for COMPLEX III, at final concentration of 0.0025 μM, sodium-azide for COMPLEX IV at final concentration of 250 μM. Furthermore, the well-known uncoupling agent, 2,4-dinitrophenol (DNP) was used at a final concentration of 100 μM to inhibit the ATP production of the mitochondrial ATP-synthase (COMPLEX V). O_2-consumption of the cells was measured with oxygraph to verify the effectiveness of inhibitor-treatments.

3.4 Determination of expression of mitochondrial folding enzymes with Western blot analysis

The cells were harvested and total protein content was isolated using lysis buffer (150 mM NaCl; 1 % NP40; 0.1 % SDS; 50 mM Tris-HCl, pH 8).

50 μg amounts of total protein samples were loaded onto 15 % polyacrylamide gel under reducing conditions and transferred to a 0.45 μm nitrocellulose membrane.

After blotting the membranes were blocked for 2 h. Then the membranes were incubated overnight at 4 °C with primary antibodies. Subsequently the membranes were incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Finally ECL reagent was used to detect the signals on the light-sensitive film.

3.5 Determination of ALR mRNA levels with RTqPCR analysis

Total RNA was purified using innuPREP RNA Mini Kit (Analytikjena). 2 μg of total RNA was reverse transcribed using RevertAid First Strand cDNA Synthesis Kit

[15] King MP, Attardi G (1996). Methods Enzymol 264:304–13.
[16] Trimmer PA, Keeney PM, Borland MK, et al. (2004). Neurobiol Dis 15:29–39.

(Thermo Scientific) following the manufacturer's protocol. Transcript levels of ALR and 18S rRNA as internal control were quantified by real-time PCR analysis.

3.6 Investigation of the mitochondrial localization of vitamin C transporters using *in silico* prediction tools

The sequences of all GLUT and SVCT transport proteins and four different carrier proteins with known mitochondrial location (Mitochondrial pyruvate carrier 2,MPC2; Mitochondrial phosphate carrier protein, MPCP; Dicarboxylate carrier ,DIC; Carnitine O-palmitoyl transferase 2, CPT2) were downloaded from the Uniprot database (http://www.uniprot.org/).

The first 60 amino acid residues of proteins were used for prediction analysis of transporters.

The subcellular localization of proteins (GLUT1-14 and SVCT1,2) was predicted by an *in silico* analysis using eight different prediction software: Target P, Mitoprot II, Predotar, Psort II, MultiLoc/TargetLoc, ngLOC, YLoc, CELLO v2.5.

3.7 Determination of dehydroascorbic acid consumption of mtDNS deficient HepG2 cells

DHA stock solution was prepared by oxidizing 100 mM L-ascorbic acid solution with bromine.

Both ρ^0 and parental HepG2 cells were treated for 4 h with DHA at a final concentration of 1 mM. The growth media on plates were replaced in every half hour to a fresh supply. Samples were taken in every hour. The DHA content of samples was determined by HPLC analysis.

3.8 Preparation of plant samples for investigation of vitamin C determination methods

Vitamin C content of four different fruit and vegetable species were determined to check the applicability of ascorbic acid determination methods. The following samples were measured: lemon (*Citrus medica*), orange (*Citrus sinensis*), pepper (*Capsicum annuum*), tomato (*Solanum lycopersicum*).

All the sample extracts were prepared by slicing the fruit or vegetable into small pieces and immediately freezing in liquid nitrogen. Thereafter, the samples were smashed using a porcelain mortar and pestle, then known amounts of the frozen tissue samples were homogenised in 5% acetic acid solution. The volumes of the homogenates were recorded and the samples were centrifuged at 16 500 *g* for 5 min at 4 °C.

3.9 2,2'-bipyridyl method for vitamin C determination

75 µl 85% orthophosphoric acid, 750 µl 1% 2,2'-bipyridyl, and 300 µl 1% $FeCl_3$ solutions were added to 300 µl of samples in a microcentrifuge tube. The samples were incubated for 60 min at room temperature, then they were centrifuged at 16 500 *g* for 5 min. 350 µl of the supernatant was transferred into a well of a microtiter plate. Each sample were analysed in three replicates. The absorbance values were read at 525 nm.

3.10 Enzymatic methods for vitamin C determination (OPDA, OPDA-fluorometric)

300 µl of o-phenylenediamine (OPDA) solution (4.6 mM in 0.1 M phosphate buffer) and 15 µl ascorbic acid oxidase enzyme solution (10 U ml^{-1} in 0.1 M phosphate buffer) were added to 37.5 µl sample, control, or standard solution in a microtiter plate well. The absorbance has been measured at 340 nm after shaking and 10 min of incubation at room temperature. Alternatively, fluorescence was measured using 340 nm for excitation and 420 nm for emission.

3.11 HPLC (reference) method for vitamin C determination

The isocratic reverse phase HPLC analysis was carried out with a Waters Series 2690 Separations Module and Teknokroma NUCLEOSIL 100 C18 column, as described by Szarka et al.[17] The isocratic mobile phase was 0.1 M NaH2PO4 and 0.2 mM Na2EDTA, adjusted to pH 3.1 with orthophosphoric acid, and we applied a flow rate of 1 ml/min. The absorbance of ascorbic acid was measured at 254 nm with SPD-10A UV-VIS.

4 Results

4.1 The effect of mitochondrial DNA depletion on the expression of ALR protein

In the first set of experiments mtDNA depleted, ρ^0 cell line has been prepared from HepG2 human hepatocyte carcinoma parental cell line. The selective loss of mtDNA and the lack of functional respiratory chain have been followed by real-time PCR and by the measurement of cellular oxygen consumption.

Since ALR is also involved in liver regeneration, two other, non-hepatic (MCF7 human breast adenocarcinoma and SH-SY5Y human neuroblastoma) cell lines were chosen to prepare ρ^0 cell lines.

We found that the depletion of mtDNA did not cause any difference in the mRNA level of the ALR in the investigated cell lines, however, it caused a markedly difference in the expression of ALR at the protein level. The effect of mtDNA depletion on the protein level of ALR has been proved to be not liver specific, since the phenomenon could also be observed in the case of the other two, non-hepatic (MCF7 and SH-SY5Y) cell lines. We also found that the elevation of the protein level of ALR did not accompanied by the elevation of MIA40, the other member of the mitochondrial disulphide relay system.

Thereafter, we make an attempt to find the regulatory factor of this significant change in the expression of ALR.

The first possible choice can be the level of ATP. It is well known that the depletion of ALR leads to the reduction of ATP level.[18] Thus the reduced ATP level due to the lack of functional mitochondrial electron transfer chain in ρ^0 cell lines may provoke the elevation of the protein level of ALR. To check this option, HepG2 cells were treated with different respiratory complex inhibitors and an uncoupling agent to

[17] Szarka A, Stadler K, Jenei V, et al. (2002). J Bioenerg Biomembr 34:317–23.
[18] Gandhi CR, Chaillet JR, Nalesnik M a., et al. (2015). Gastroenterology 148:379–391.

gain a decreased ATP level. However, the possibility of ATP as a regulatory factor for ALR expression should have ruled out because the reduced ATP level due to the treatment of the parental cell line with different respiratory inhibitors and uncoupling agent could not provoke any elevation in the protein level of ALR.

The second candidate for the regulation of ALR might have been the elevated level of reactive oxygen species, since the dysfunction of the electron transport chain in ρ^0 cells was associated with an increase in ROS generation.[19] However the elevation of ROS by the blockage of COMPLEX III has not accompanied by the elevation of ALR. As the reduction of ROS level due to the treatment of the parental cells by the uncoupling agent 2,4-dinitrophenol has not caused any alteration in the level of ALR.

The observed caspase-3 activation due to the silence or knock out of ALR[20] raise the third possibility that ALR has an anti-apoptotic role in the case of ρ^0 cells. This possibility cannot be ruled out since ρ^0 cells showed resistance against apoptosis.[21]

The fact that the protein level of the other member of the mitochondrial disulphide relay system, MIA40 has not changed together with the level of ALR suggest that the elevation of ALR may be independent from the mitochondrial oxidative folding; but there is a possibility that the increasing ALR expression solves the problem with the increasing capacity in carrying electrons from MIA40, thus elevation of MIA40 remains unnecessary.

It seems that the level of mtDNA and/or its products may have a regulatory role on the protein level of ALR. Since it is well-known that ALR contributes to mitochondrial biogenesis and maintenance[18], the liver independent up-regulation of ALR can be a part of the adaptive response in ρ^0 cells that preserves the structural integrity of the mitochondrial inner membrane as well as the transmembrane electrochemical gradient despite the absence of protein components encoded by the mtDNA.

4.2 The mitochondrial localization of vitamin C transporters, based on *in silico* investigation

Four carrier proteins with known mitochondrial localization were chosen as so called mitochondrial standards (etalons). The mitochondrial localization scores for these mitochondrial etalon proteins similarly to the members of GLUT and SVCT families were calculated by eight different prediction tools.

Similar to the mitochondrial etalon proteins all prediction tools (except ngLOC) gave a good chance for the mitochondrial localization of GLUT1. Our prediction results support the mitochondrial localization of GLUT1 the earlier findings of KC et al. based on their GLUT1-EGFP and immunoblot observations.[10]

Although GLUT2, 3, 4 and 8 are considered as efficient dehydroascorbic acid transporters their mitochondrial localization is not likely. On one hand all these transport proteins got low mitochondrial localization scores, on the other hand no experimental data imply to their mitochondrial localization.

Five prediction tools out of the eight give higher score for the mitochondrial localization of GLUT9. GLUT9 (or SLC2A9) is a member of the GLUT family but is now primarily described as a novel high-capacity urate transporter, which can

[19] Miranda S, Foncea R, Guerrero J, Leighton F (1999). Biochem Biophys Res Commun 258:44–49.
[20] Polimeno L, Pesetti B, De Santis F, et al. (2012). Cell Death Dis 3:e289.
[21] Lee MS, Kim JY, Park SY (2004). Ann N Y Acad Sci 1011:146–153.

exchange both glucose and fructose for urate.[22] Hence we can hypothesize the following model, SLC2A9a localized in the mitochondrial membrane transports urate in a voltage-dependent fashion from the mitochondrial matrix to the cytosol, while it mediates the uptake of glucose or its relative molecule DHA.

The really low scores got for the mitochondrial localization of GLUT10 caused a real surprise, because these results oppose several earlier experimental findings.[11] In a very recent study on HEK-293 cells the absence of expression of GLUT10 was found, therefore the authors discarded the participation of GLUT10 as a mitochondrial DHA transporter in these cells.[13] Our extremely low mitochondrial scores also query the role of GLUT10 as a mitochondrial (vitamin C) transporter.

All prediction tools (except MultiLoc and ngLoc) gave high score for the mitochondrial localization of GLUT11. GLUT11 immunoreactive proteins were localized at the cell surface and in an intracellular compartment.[23] It cannot be ruled out that the observed intracellular compartment is the mitochondrion.

GLUT12 has not got high mitochondrial localization scores. In accordance with our results it appears to localize to the Golgi apparatus and to the plasma membrane.[24]

The likelihood of mitochondrial localization for all the isoforms of GLUT13 (HMIT) is high since most prediction tools gave high scores. GLUT13 or HMIT is a H^+ - myoinositol cotransporter.[25] Although the driving force (H^+ gradient) is given in the case of mitochondria the role of HMIT in mitochondrial vitamin C transport is not likely since no sugar transport activity has been found for HMIT.[26]

GLUT14 appears to be a consequence of a gene duplication of GLUT3.[27] Hence it is not surprising that its mitochondrial localization similar to GLUT3 is not likely.

In accordance with former experimental results, SVCT2 but not SVCT1 got good scores for its mitochondrial localization.[13] The ionic milieu of mitochondria enables SVCT2 to function as a (low-affinity) ascorbic acid transporter in the mitochondrial membrane.

4.3 The effect of mtDNA depletion on the dehydroascorbic acid uptake of cells

The DHA-consumption of HepG2 cells with respiratory chain deficiency was investigated. As in our previous experiments, mtDNA depleted ρ^0 cells were used as a model.

According to our results, the DHA-consumption of ρ^0 cells has been significantly increased in the second hour of the four-hour treatment, and then it has set on a moderate level. The control cells have reached this maximum value faster, than the ρ^0 cells.

Clear difference could be observed in DHA consumption of control and ρ^0 cells. We plan to perform AMS-labeling of the IMS-localized substrate proteins in both control and ρ^0 cells.

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[22] Caulfield MJ, Munroe PB, O'Neill D, et al. (2008). PLoS Med 5:e197.
[23] Scheepers A, Schmidt S, Manolescu A, et al. (2005). Mol Membr Biol 22:339–51.
[24] Flessner LB, Moley KH (2009). Traffic 10:324–33.
[25] Uldry M, Ibberson M, Horisberger JD, et al. (2001). EMBO J 20:4467–77.
[26] Di Daniel E, Mok MH, Mead E, et al. (2009). BMC Cell Biol 10:54.
[27] Augustin R (2010). IUBMB Life 62:315–33.

4.4 The applicability of vitamin C determination

Three ascorbic acid determination methods have been compared with each other and with the so-called etalon HPLC method to get an objective picture about their applicability.

The 2,2'-bipyridyl method and another method using ascorbic acid oxidase enzyme and OPDA were also tested with absorbance and fluorescence detection mode (OPDA and OPDA-fluorometric).

Based on our measurements and calculations the conclusions are the following:

The OPDA-fluorometric method can be suggested for the determination of samples containing the ascorbate at low concentrations because of its low LoD and LoQ values. However, this method can be characterized by the narrowest linear range. Furthermore, the ascorbate contents of different fruit and vegetable samples have been slightly underestimated by this method, probably due to the derivatization of ascorbate.

Unfortunately, the analytical properties of the OPDA method with spectrophotometric detection have been lagging far behind the others. Hence, we cannot recommend the application of this method.

The 2,2'-bipyridyl method could give a balanced performance for all tests. Furthermore, the results gained by the 2,2'-bipyridyl method are the closest to the results of the reference HPLC method in the case of fruit and vegetable samples. The specificity issue of the method can be diminished by the application of orthophosphoric acid.

Similarly to the OPDA-fluorometric method, the throughput of the 2,2'-bipyridyl method could be significantly increased by detecting the absorbance or the fluorescence with a plate reader.

Considering these facts, the 2,2'-bipyridyl method seems to be the most suitable method for vitamin C analysis in small or middle sized laboratories, with a sample number of up to several hundreds.

However, in basic research laboratories the samples usually show a really wide variety of analyte concentration and composition. Under circumstances like these, only the HPLC method can offer an appropriate selectivity, sensitivity and reliability.

5 Theses

1) The expression of ALR (Augmenter of Liver Regeneration) was increased significantly at protein level, but remained unchanged at mRNA level due to the depletion of mitochondrial DNA content of different human cell lines. The effect is not tissue-specific, since it was independent of the origin of the investigated cell lines (HepG2 human hepatocyte carcinoma, MCF7 human breast adenocarcinoma, SH-SY5Y human neuroblastoma cell lines) [1].
2) The regulatory role of ATP and ROS levels could be ruled out because the treatment of the parental cell line with different respiratory inhibitors and uncoupling agent could not provoke any changes in the protein level of ALR [1].
3) The role of GLUT1 as a mitochondrial dehydroascorbate transporter could be reinforced by *in silico* predictions however the mitochondrial presence of GLUT10 is not likely since this transport protein got far the lowest mitochondrial localization scores [2].
4) The possible roles of GLUT9 and 11 in mitochondrial vitamin C transport can be proposed leastwise on the base of their computational localization analysis [2].
5) Using prediction tools, we confirmed the mitochondrial localization of SVCT2, which has already been assumed based on former experimental results [2].
6) The accuracy and precision of vitamin C determination by ascorbate oxidase and o-phenylenediamine with fluorometric detection exceed the corresponding parameters of reverse phase HPLC method. However, the application of this method is limited by its narrow linear range, and the distortion caused by the disruption of ascorbic acid - dehydroascorbic acid balance [3].
7) The 2,2'-bipyridyl method for vitamin C determination could give a well-balanced performance (linear range: 5 - 1000 µmol/l, average accuracy: 8.16 %, average precision: 9.67 µmol/l, limit of detection: 1.34 µmol/l, limit of quantification: 4.46 µmol/l). On the base of its analytical features the 2,2'-bipyridyl method seems to be a suitable method for vitamin C analysis in small or middle sized food analytic laboratories [3].

6 Possible application of the results

The proper folding of proteins produced by recombinant DNA technology is as important as their translation. One of the major challenges of the production of monoclonal antibodies is their post-translation modification (folding). The proper folding of proteins is the prerequisite of their proper function. Hence the investigation of the detailed mechanism of mammalian (mitochondrial) folding apparatus in different conditions has both industrial (biotechnology) and medical benefits. The described overexpression of ALR protein in mtDNA depleted cell lines may contribute to this beneficial process. Similarly the in silico clarification of mitochondrial vitamin C transport has important medical relevance, especially in arterial tortuosity syndrome. On the other hand the comparison of different methods for vitamin C determination can be used directly in small and middle sized food analytical laboratories.

7 Publications

Publications related to the dissertation:

1) **Tibor Balogh**, Tamás Lőrincz, Ibolya Stiller, József Mandl, Gábor Bánhegyi, András Szarka: The Level of ALR is Regulated by the Quantity of Mitochondrial DNA; Pathology and Oncology Research, DOI: 10.1007/s12253-015-0020-y (2015), IF: 1,855

2) András Szarka, **Tibor Balogh:** In silico aided thoughts on mitochondrial vitamin C transport; Journal of Theoretical Biology 365, 181-189, DOI: 10.1016/j.jtbi.2014.10.015 (2015), IF: 2,116

3) **Tibor Balogh**, András Szarka: A comparative study: Methods for the determination of ascorbic acid in small and middle sized food analytic laboratories; Acta Alimentaria, DOI: 10.1556/AAlim.2015.0017 (2015), IF: 0,427

4) **Tibor Balogh**, András Szarka: Egy multifunkcionális fehérje: az ALR; Orvosi Hetilap 156 (13), 503-509, DOI: 10.1556/OH.2015.30119 (2015)

Oral presentations:

Tibor Balogh, Tamás Lőrincz: Mitokondriális genom és fehérje folding; Számoljon velünk! Kutatás-fejlesztés és innováció a Műegyetemen 2013; Biotechnológia, egészség- és környezetvédelem; Budapesti Műszaki és Gazdaságtudományi Egyetem; 21 June 2013

Tibor Balogh, András Szarka: Gyors analitikai módszerek élelmiszerminták C-vitamin tartalmának meghatározására; Táplálkozástudományi Kutatások II. Workshop; Innováció – Táplálkozás – Egészség – Marketing; Kaposvári Egyetem; 10 December 2012

Poster presentations:

Tibor Balogh, Tamás Lőrincz, József Mandl, András Szarka: ALR: kapocs a mitokondriális DNS és oxidatív folding között; MedInProt konferencia 3.; Eötvös Lóránd Tudományegyetem; 14 November 2015

Other publication:

Tibor Balogh, András Szarka: Napfény és C-vitamin; Élelmiszer - Tudomány Technológia 67 (3), 27-28 (2013)

Other poster presentation:

Péter Hajdinák, Ádám Czobor, Veronika Deák, **Tibor Balogh**, András Szarka: The effect of HrpWpto and HrpZpto treatment on ascorbate metabolism; 9th International Conference for Plant Mitochondrial Biology; Wrocław; 17-22 May 2015