Review

Mammalian complex I: A regulable and vulnerable pacemaker in mitochondrial respiratory function

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ABSTRACT

In this paper the regulatory features of complex I of mammalian and human mitochondria are reviewed. In a variety of mitotic cell-line cultures, activation in vivo of the cAMP cascade, or direct addition of cAMP, promotes the NADH–ubiquinone oxidoreductase activity of complex I and lower the cellular level of ROS. These effects of cAMP are found to be associated with PKA-mediated serine phosphorylation in the conserved C-terminus of the subunit of complex I encoded by the nuclear gene NDUFS4. PKA mediated phosphorylation of this Ser in the C-terminus of the protein promotes its mitochondrial import and maturation. Mass-spectrometry analysis of the phosphorylation pattern of complex I subunits is also reviewed.

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1. Introduction

Complex I of the respiratory chain catalyzes the oxidation of NADH by ubiquinone and conserves the free energy thus made available as a transmembrane proton gradient [1–4]. In mammalian mitochondria, complex I is made up of 45 subunits [5], of which seven are encoded by the mitochondrial genome, the others by nuclear genes [1–4]. Fourteen subunits of the mammalian enzyme, conserved from prokaryotes to humans, are likely to contribute the essential elements of the redox and proton translocating activity [1–4,11,12]. The NADH–ubiquinone oxidoreductase activity of the membrane associated complex I of mammals and certain prokaryotes is coupled with proton pumping from the inner (N) side to the outer (P) side of the membrane, with a mechanistic upper limit H+/e− stoicheiometry of 2, as compared to 2 and 1 in complex III and IV of the respiratory chain respectively [4,13–15].

A possible decline of the H+/e− stoicheiometry at the first coupling site of oxidative phosphorylation is an intriguing aspect that should be revisited in whole mitochondria and in the isolated complex I.

from FMN, the first redox acceptor from NADH, to protein stabilized semiquinones in the membrane hydrophobic moiety, from which electrons are finally delivered to the ubiquinone of the membrane pool [1–4,11,12]. The NADH–ubiquinone oxidoreductase activity of the membrane associated complex I of mammals and certain prokaryotes is coupled with proton pumping from the inner (N) side to the outer (P) side of the membrane, with a mechanistic upper limit H+/e− stoicheiometry of 2, as compared to 2 and 1 in complex III and IV of the respiratory chain respectively [4,13–15]. It has, however, been observed that, when in isolated mitochondria electron transfer in the respiratory chain is activated by the addition of a substrate of NADH-linked dehydrogenase, the H+/e− stoicheiometry for proton pumping at the first coupling site of the chain apparently decreases well below 2 as the rate of electron flow is raised [16].

In mitochondria isolated from different rat-tissues a decrease of the P/O ratio for oxidative phosphorylation at the first coupling site has also been observed at high rates of electron flow [17]. Occurrence of slip in the proton pump of both complex III and complex IV has been previously observed [15,18]. A possible decline of the H+/e− stoicheiometry at the first coupling site of oxidative phosphorylation is an intriguing aspect that should be revisited in whole mitochondria and in the isolated complex I.
There are observations pointing to a role in proton pumping of protein bound ubisemiquinone species \([12,19,20]\) and redox-linked protolytic conformational changes of subunits, like the PSST subunit in the bovine enzyme (NDUF57 gene, NuoB of the \(E. coli\) enzyme) \([21]\). The N2 iron–sulfur center bound to this subunit, serving as the final electron transfer center in the sequence of redox carriers from FMN to protein-stabilized ubisemiquinone, exhibits a pH dependent redox potential, due to pH changes of nearby amino acid residues \([22,23]\).

In 1994 Papa et al. \([24]\) proposed that a protein stabilized \(UQ^-/UQH_2\) couple, transferring \(2H^+\) per \(e^-\), could represent the critical element of the pump in complex I, in combination with cooperative \(H^+/e^-\) coupling in redox proteins. On this basis Papa et al. \([15]\) developed a Q-gated proton pump model (Fig. 1), in which cooperative redox linked \(H^+\) transfer by the N2-PSST subunit mediated proton consumption from the N space in the reduction of a protein stabilized \(UQ^-\) to \(UQH_2\). Protons were then released towards the P space, upon electron transfer from this \(UQH_2\), directly to the ubiquinone of the pool (version A) or to a protein-stabilized \(UQ/UQ^-\) couple and from this to the ubiquinone of the pool (version B). The work of Ohnishi and Salerno \([19,20]\), showing the existence in complex I of two protein-bound ubisemiquinone anions, one of which (\(UQ^-_{ni}\)) accumulates in the presence of transmembrane \(\Delta \mu H^+\) and is directly spin coupled with the N2 center at the inner membrane surface, seems to provide direct support for version (B) of the Q-gated proton pump mechanism shown in Fig. 1 \([15]\). The observed rate-dependent decline in the \(H^+/e^-\) stoichiometry for proton pumping at the first coupling site could be associated with modulation of the protonmotive electron transfer sequence in the model shown in Fig. 1.

The present paper reviews features of the regulation by cAMP of complex I and production of oxygen free radicals in mammalian and human cell cultures, the role of the NDUFS4 subunit and the phosphorylation pattern of constituent subunits of the complex.

### 2. Regulation by the cAMP cascade of complex I and oxygen free radical balance

In mammalian cells the redox activity of complex I changes substantially in the various phases of the cell cycle. Arrest of fibroblast division in the G0 phase, caused by serum deprivation, is accompanied by decrease in the NADH ubiquinone oxidoreductase activity of complex I as compared to cells in the exponential growth phase \([25–28]\). Fibroblast reintroduction in the replicative cycle by adding back serum to serum-starved cells results, after 24 h, in significant enhancement of the NADH ubiquinone oxidoreductase activity and content of complex I \([15]\). If instead of serum addition, the cAMP cascade is activated or dibutyryl-cAMP is directly added to serum-starved cells, in 30 min an interval in which serum has no effect, the NADH–ubiquinone oxidoreductase activity is activated more than two fold \([25–28]\). This activating effect of cAMP, which is abrogated by inhibitors of cAMP-dependent protein kinase A (PKA) \([27]\), is generally observed in a variety of mitotic cells (Fig. 2A) \([28]\). No change in the activity of complex IV is, on the other hand, caused by cAMP in this time interval \([25–28]\).

Confocal microscopy analysis has shown that the decline in the NADH–ubiquinone oxidoreductase activity in serum starved cells is accompanied by increase in the cellular level of \(H_2O_2\) and in the intramitochondrial level of \(O^-_2\) (Fig. 2B) \([28]\). The addition of dibutyryl-cAMP results, in the same time interval in which NADH–ubiquinone oxidoreductase is activated, in disappearance of \(H_2O_2\) and \(O^-_2\) from the cells. This effect of cAMP, which is also abrogated by inhibitors of PKA, was observed in all the cell lines in which the NADH–ubiquinone oxidoreductase was activated by the cyclic nucleotide (Fig. 2-B) \([28]\). Under these conditions cAMP did not exert any effect on components of ROS scavenger systems, like glucose-6-phosphate, glucose-6-phosphate dehydrogenase, total glutathione, reduced glutathione, glutathione reductase, Cu/ZnSOD, MnSOD, glutathione peroxidase, glutaredoxin, catalase, \([28]\). If one takes into account that complex I is the major site of \(O^-_2\) production in the respiratory chain \([4,29,30]\) it can be argued, from the above, that the decrease in the ROS level effected by cAMP is associated with the activation of the forward NADH–ubiquinone oxidoreductase activity.

In murine and human cell cultures, activation of the cAMP cascade or direct addition of cAMP has been found to promote, under conditions in which they result in activation of complex I and counteract ROS accumulation \([28]\), serine phosphorylation in the subunit of the complex encoded by the nuclear NDUFS4 gene \([25–27]\).

The human NDUFS4 gene, and the corresponding genes in mammals and birds encode for highly homologous proteins \([4,29,30]\). These proteins, but also those of \(Drosophila\) and \(Anopheles\), which present a low overall homology with the human species, have a highly conserved C-terminus in which a canonical RVSTK phosphorylation site in the last residues, with the highest phosphorylation score for phosphorylation by PKA, is present. The human NDUFS4 gene has, also, been found to be a hotspot for mutations. So far, 5 different pathological homozygous mutations have, in fact, been reported for this gene \([4,26,31–33]\) (Fig. 5).

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Fig. 1. Q-gated proton pump model in Complex I. A protein stabilized \(UQ^-\) takes up \(2H^+\) from the N space upon its reduction to \(UQH_2\) by the N2 center. Upon oxidation of \(UQH_2\) to \(UQ^-\), directly by UQ of the pool (Version A) or via a protein stabilized UQ/UQ^- couple (acting as an electron transfer center), \(2H^+\) are released in the P space. Reproduced with permission from Papa et al. \([15]\).
Results presented in Fig. 6 show that stimulation of the NADH–ubiquinone oxidoreductase activity of complex I, produced in mouse myoblast cultures by enhancement of intracellular cAMP with cholera toxin, is accompanied by serine phosphorylation in the 18 kDa protein band of the complex (Panel A). Direct treatment of the mitoplasts isolated from myoblasts with dibutyryl-cAMP promoted, in fact, serine phosphorylation in the RVSTK C-terminus site of the NDUFS4 protein (Panel B), recognized by a specific antibody raised against a synthetic peptide of the phosphorylated C-terminus of the protein [27]. This phosphorylation and the accompanying stimulation of NADH–ubiquinone oxidoreductase activity, produced by dibutyryl-cAMP, were both abolished by the PKA inhibitor, H89 (Panel B).

Involvement of phosphorylation of the NDUFS4 subunit, in the modulation of the redox activity of complex I, is supported by the finding that in a neurological patient a pathological 466–470 AAGTC base duplication in the NDUFS4 gene, which results in a frame shift with 14 amino acids elongation of the protein and abrogation of the serine phosphorylation site, caused disappearance of the subunit encoded by the gene and abolishment of the cAMP-dependent serine phosphorylation activity, produced by dibutyryl-cAMP, were both abolished by the PKA inhibitor, H89 (Panel B).

Fig. 2. Effect of cAMP on the activities of NADH-UQ oxidoreductase of complex I, cytochrome c oxidase of complex IV and ROS levels in different human and murine cell cultures. Cells grown in the exponential phase in the presence of 10% fetal bovine serum (FBS) in DMEM (a) were brought into G0 phase by 48 h serum deprivation (0.5% FBS) (b). Serum starved cells were then treated for 60 min with 100 µM dibutyryl-cAMP+ 100 µM IBMX (c). Panel A, complex I and complex IV activities represent the means±S.E.M. of three or more determinations in mitoplasts isolated from the cells. Panel B, confocal microscopy quantitative analysis was used to determine the cellular content of H2O2 by the green fluorescence of 2′-7′-dichlorofluorescein-diacetate (DCF-DA) and mitochondrial O2− by the red fluorescence of the MitoSox dye. Reproduced with permission from [28].

Fig. 3. Effect of serum starvation and cAMP on ROS scavenger systems in different human and murine cell cultures. Changes in the pattern of components of ROS scavenger systems in cell cultures. 1, changes induced in cell cultures by serum starvation for 48 h vs cells in the exponential growth phase; 2, changes after 60 min. treatment with 100 µM dibutyryl-cAMP+ 100 µM IBMX of serum starved cells, vs starved cells. Table compiled from data in [28].

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findings provided a clue to identify the mechanism by which phosphorylation of the NDUFS4 subunit could modulate, at least in cell cultures in vivo, the redox activity of complex I. It has to be stressed that cAMP fails to activate complex I in isolated mammalian mitochondria or in the purified bovine complex (supplemented with PKA) [34]. Could phosphorylation of the NDUFS4 subunit modulate the activity of complex I by promoting import/assembly of complex I? Whilst we carried out experiments on the mitochondrial import of the NDUFS4 protein a paper of Lazarou et al. [33] was published showing that in vitro synthesized NDUFS4 protein is, in less than 60 min, incorporated in mature complex I in isolated human fibroblast mitochondria, apparently in exchange with preexisting, possibly damaged, subunit in the complex. These authors also showed that in vitro synthesized NDUFS4 subunit restored a last step in complex I assembly, impaired in fibroblasts from a patient with NDUFS4 mutation [33]. In Fig. 7 experimental observations on the import into isolated mitochondria of [35S]Met-labelled NDUFS4 and ESSS human subunits of complex I, synthesized in vitro in the reticulocyte system, are presented. The human NDUFS4 has two conserved serine phosphorylation consensus sites one in the NH2-terminal leader sequence, the other in the carboxy-terminal tail (Fig. 4). The human ESSS subunit, encoded by the nuclear gene NDUFB11, has only one serine phosphorylation consensus site, at the junction between the leader and the mature sequence. The bovine ESSS also has a consensus serine phosphorylation site in the mature sequence [35], which is, however, absent in the human subunit (EMBL Data Bank). The precursor forms of the [35S]Met labelled NDUFS4 and ESSS proteins did bind to the mitochondrial surface, where both were digested by added trypsin. The ESSS protein was almost completely imported into mitochondria and converted to the mature, trypsin resistant, form by the canonical Δψ and ATP dependent translocation process [36]. Under the same conditions the import into mitochondria and maturation of the NDUFS4 protein was poor (compare panels A and B of Fig. 7). Addition to the import system of PKA and ATP, which had no effect on the binding of [35S]Met-labelled NUDFS4 and ESSS precursor proteins to the surface of mitochondria, promoted Δψ dependent accumulation into mitochondria of the mature form of the NDUFS4 protein. The addition of AP suppressed, on the contrary, the appearance in mitochondria of the mature NDUFS4 protein (panel C, Fig. 7). At difference with these effects on the NDUFS4 protein, PKA and AP had no influence on the accumulation in mitochondria of the mature form of the ESSS protein (panel D, Fig. 7).

Fig. 4. Sequence alignment of the 18 kDa (NDUFS4) subunit of mitochondrial complex I in higher eukaryotes (Human gi: 3287881, Bovine gi: 400578, Mouse gi: 6754814, Gallus gi: 1910803, Anophele gi: 3199457, Drosophila gi: 17944526). Sequences were obtained from Swiss-Prot Database and aligned with EMBL engine. Synthetic peptides used to produce antibodies against the amino- and carboxy-terminal regions of the protein are shaded in gray. The highly conserved C-terminal segment with the putative canonical phosphorylation consensus sites (RVS) for PKA is boxed. Known mitochondrial import sequences are underlined. Alternative cleavage sites of presequences are indicated by arrows.

Fig. 5. Schematic representation of 5 different NDUFS4 gene mutations found in neurological patients with Complex I deficiency. The IVS1nt-1,G→A splice acceptor site mutation in intron 1 produced a transcript skipping exon 2; the AAGTC 466–470 Dupl caused a frame shift with 14 amino acids elongation of the protein and abrogated the phosphorylation site in the C-terminus. The other mutations introduced premature termination codons. From refs. [4,31,32].
Site specific S173A code substitution in the cDNA utilized for in vitro synthesis of the NDUFS4 protein was without effect on the binding of the [35S]Met labelled NDUFS4 precursor protein at the mitochondrial outer surface, but abolished completely the appearance of the mature NDUFS4 protein in the inner mitochondrial compartment (panel E, Fig. 7).

In HeLa cells transfected with a construct of wild NDUFS4 cDNA, the precursor form of the overexpressed protein was detectable in the cytosolic fraction, whilst in the mitochondrial fraction only the mature NDUFS4 protein was present (Fig. 8). Treatment of transfected HeLa cells with H89, prevented the accumulation in mitochondria of the mature NDUFS4 protein. When HeLa cells were transfected with a construct of the NDUFS4 cDNA with S173A substitution, the precursor form of the NDUFS4 protein accumulated in the cytosol, but no mature NDUFS4 protein, in addition to the endogenous content, appeared in mitochondria (Fig. 8).

Semi-quantitative analysis of the precursor and mature [35S]Met-labelled NDUFS4 protein in the cytosolic and mitochondrial fractions in the course of the import of the protein showed that, in the presence of PKA, no trace of the mature NDUFS4 was detectable in the supernatant during its progressive accumulation in mitochondria (Fig. 9-A). Parallel experiments revealed that, in the presence of AP, which depressed accumulation of [35S]Met-labelled mature NDUFS4 protein in mitochondria, a significant amount of the mature form rapidly appeared in the supernatant, from where it then progressively disappeared (Fig. 9-A). Addition of the cytosolic Hsp70 antibody to the in vitro mitochondrial import mixture had no effect on the binding of [35S]Met-labelled precursor of the NDUFS4 protein to the mitochondrial surface but depressed the PKA dependent accumulation in mitochondria of the mature form of the protein which now appeared in the supernatant (Fig. 9-B). Under the same experimental condition a β-actin antibody had no effect on the accumulation of the mature NDUFS4 protein in mitochondria, neither it caused its appearance in the supernatant (Fig. 9-B).

3. Phosphorylation of complex I subunits

Papa et al. first found that in isolated bovine heart mitochondria cAMP promotes, among others, phosphorylation of 18 kDa protein(s) [37], which were shown to be associated with the inner mitochondrial membrane [38] and the isolated complex I [39]. Edman sequencing of the 18 kDa protein band of the purified complex I showed the presence of two subunits of complex I, namely the GDHG and AQDQ proteins (NDUFS4 gene), the latter of which corresponded to the band with phosphorylated-serine [39]. Subsequent work of Chen et al. confirmed PKA-dependent phosphorylation of 18 kDa protein(s) in bovine heart mitochondria [35]. Extensive analytical work of these authors, using 32P-radiolabelling, reverse phase HPLC, mass spectrometry and Edman sequencing showed, in the 18 kDa bands of bovine complex I a PKA-dependent serine phosphorylation of the ESSS subunit, but did not reveal phosphorylation of the AQDQ. It should be recalled that the ESSS protein is difficult to be sequenced and was identified as a constituent complex I subunit by Carroll et al. [40] after our work of 1996 [39]. These observations prompted us to revisit the phosphorylation of 18 kDa protein(s) of purified bovine heart complex I by 2D isoelectrofocusing/SDS-PAGE electrophoresis, radiolabelling, immunoblotting and mass spectrometry. The 2D electrophoretic separation showed the presence in complex I of a 32P-labelled protein band, recognized by the antibody specific for the serine-phosphorylated carboxy-terminus of AQDQ, with an alkaline isoelectric point and M.W. proper of this subunit (unpublished observation of the authors). It should be noted that the ESSS protein which has an acidic isoelectric point, since of its hydrophobic nature, does not enter the 3–10 pH gradient strip used for the analysis [40].

Results of systematic mass spectrometry analysis of the phosphorylation pattern of subunits of complex I isolated from bovine heart mitochondria are summarized in Table 1 [41]. Phosphopeptide directed MS3 mass spectrometry confirmed the presence of serine phosphorylated 42 kDa [42]. ESSS [35] and B14.5a [43] subunits. In particular whilst phosphorylated ESSS was found only in the complex from mitochondria incubated with ATP and PKA, the phosphorylated forms of 42 kDa and B14.5a were found in the samples from both control and PKA treated mitochondria. The same analytical procedure revealed two new threonine-phosphorylated subunits of complex I, namely B14.5b and B16.6. Mass spectrometry detected both the phosphorylated and non phosphorylated form of these two proteins as well as of the 42 kDa and B14.5a subunits. Thus these four subunits undergo a dynamic condition of phosphorylation, as expected for a
physiological regulatory process. Both B14.5a and B14.5b subunits are N-acetylated [44]. Phosphorylation of these subunits, which are located at the matrix side and in the membrane arm of the complex, might have an impact on its conformation and activity. Subunit B16.6 has been identified as the product of the GRIM-19 gene [9]. This subunit seems to be essential for the assembly or stability of complex I and cell death [45]. The finding that the deletion of the C-terminus of GRIM19, where the threonine phosphorylation site is located, suppresses its death promoting effect seems to be relevant in this context [45,46].

In agreement with the observations of Chen et al [35], our attempts to verify by mass spectrometry phosphorylation of the AODQ subunit in PKA treated bovine heart complex I have so far been unsuccessful. The specific location of the serine phosphorylation site is located, suppresses its death promoting effect seems to be relevant in this context [45,46].

![Fig. 7. Mitochondrial import of NDUFS4 and ESSS subunits of complex I and effect of PKA and AP on their in vitro mitochondrial import. Full-length human NDUFS4 wild type (panels A, C and E), mutated Ser173Ala NDUFS4 (panel E) and ESSS (panels B and D) cDNAs were cloned in pCDNA3.1(+)/ vector containing the T7 promoter and used for in vitro transcription and translation in Rabbit Reticulocyte Lysate System (RRL). Proteins obtained by the RRL translation system, were added to the import mixture containing isolated rat liver mitochondria and an ATP energy supplying system. After 60 min. incubation at 30 °C mitochondria were spun down and analyzed by autoradiography. Panels A and B, lanes 1, aliquots of the RRL translation mixture were directly analyzed by autoradiography. Lanes 2–6, RRL translation mixture were added to the import mixture, containing rat liver mitochondria. Mitochondria were spun down from the import mixture before (lanes 2) or after trypsin treatment, in the absence (lanes 3) or in the presence of Triton X-100 (lanes 4). Lanes 5: valinomycin was added to the import mixture. Lanes 6: the energy supply mixture was omitted. Aliquots of the samples were subjected to western blotting with antibody against the 39-kDa subunit of complex I. Panel C, lane 1 autoradiogram of the RRL translation mixture; lane 2, control import mixture; lane 3 and 7, import mixture supplemented with PKA plus NaF; lane 4, after 60 min of import incubation, in the presence of PKA, trypsin was added; lane 5, as lane 4 with the addition of Triton X-100 together with trypsin; lane 6, import incubation in the presence of; lane 8, import incubation in the presence of PKA and valinomycin. Aliquots of the samples were subjected to western blotting with antibody against the 39-kDa subunit of complex I. Panel D, lane 1 autoradiogram of the RRL translation mixture; lane 2, control import mixture; lane 3, import mixture supplemented with PKA; import incubation in the presence of PKA followed by trypsin treatment; lane 4, after 60 min of import incubation, in the presence of PKA and valinomycin. Aliquots of the samples were subjected to western blotting with antibody against the 39-kDa subunit of complex I. Panel E, the mitochondrial import/maturatation of the radiolabelled native and mutated NDUFS4 protein was analyzed as described above. Lanes 1, direct autoradiography of the RRL translation mixtures; lanes 2 and 3, no addition (autodigraphography before, lanes 2, and after trypsin treatment of mitochondria, lanes 3); lanes 4 and 5, import incubation in the presence of PKA plus NaF (autodigraphy before, lanes 4, and after trypsin treatment of mitochondria, lanes 5); lanes 6 and 7, import incubation in the presence of 10 U AP (autodigraphy before, lanes 6, and after trypsin treatment of mitochondria, lanes 7). Reproduced with permission from [36]. Panels A and B from Fig. 3 in [36] and panels C, D and E from Fig. 4 in [36]. See [36] for experimental details.

Fig. 7. Mitochondrial import of NDUFS4 and ESSS subunits of complex I and effect of PKA and AP on their in vitro mitochondrial import. Full-length human NDUFS4 wild type (panels A, C and E), mutated Ser173Ala NDUFS4 (panel E) and ESSS (panels B and D) cDNAs were cloned in pCDNA3.1(+)/ vector containing the T7 promoter and used for in vitro transcription and translation in Rabbit Reticulocyte Lysate System (RRL). Proteins obtained by the RRL translation system, were added to the import mixture containing isolated rat liver mitochondria and an ATP energy supplying system. After 60 min. incubation at 30 °C mitochondria were spun down and analyzed by autoradiography. Panels A and B, lanes 1, aliquots of the RRL translation mixture were directly analyzed by autoradiography. Lanes 2–6, RRL translation mixture were added to the import mixture, containing rat liver mitochondria. Mitochondria were spun down from the import mixture before (lanes 2) or after trypsin treatment, in the absence (lanes 3) or in the presence of Triton X-100 (lanes 4). Lanes 5: valinomycin was added to the import mixture. Lanes 6: the energy supply mixture was omitted. Aliquots of the samples were subjected to western blotting with antibody against the 39-kDa subunit of complex I. Panel C, lane 1 autoradiogram of the RRL translation mixture; lane 2, control import mixture; lane 3 and 7, import mixture supplemented with PKA plus NaF; lane 4, after 60 min of import incubation, in the presence of PKA, trypsin was added; lane 5, as lane 4 with the addition of Triton X-100 together with trypsin; lane 6, import incubation in the presence of; lane 8, import incubation in the presence of PKA and valinomycin. Aliquots of the samples were subjected to western blotting with antibody against the 39-kDa subunit of complex I. Panel D, lane 1 autoradiogram of the RRL translation mixture; lane 2, control import mixture; lane 3, import mixture supplemented with PKA; import incubation in the presence of PKA followed by trypsin treatment; lane 4, after 60 min of import incubation, in the presence of PKA and valinomycin. Aliquots of the samples were subjected to western blotting with antibody against the 39-kDa subunit of complex I. Panel E, the mitochondrial import/maturatation of the radiolabelled native and mutated NDUFS4 protein was analyzed as described above. Lanes 1, direct autoradiography of the RRL translation mixtures; lanes 2 and 3, no addition (autodigraphography before, lanes 2, and after trypsin treatment of mitochondria, lanes 3); lanes 4 and 5, import incubation in the presence of PKA plus NaF (autodigraphy before, lanes 4, and after trypsin treatment of mitochondria, lanes 5); lanes 6 and 7, import incubation in the presence of 10 U AP (autodigraphy before, lanes 6, and after trypsin treatment of mitochondria, lanes 7). Reproduced with permission from [36]. Panels A and B from Fig. 3 in [36] and panels C, D and E from Fig. 4 in [36]. See [36] for experimental details.
with specific antibodies should help to clarify the phosphorylation pattern of this and other subunits of complex I [35, 41, 47, 48].

4. Conclusions

The mitochondrial import of the NDUFS4 subunit, which is essential for the last step of the assembly of a functional complex [6, 7, 33], is promoted by its PKA-dependent phosphorylation at serine 173 in the C-terminus [36]. It has been reported that the mitochondrial import of other proteins like cytochrome P4502B1 [49], CYP2E1 [50] and glutathione S-transferase [51] is also promoted by PKA-mediated phosphorylation. Mitochondrial import of nuclear encoded subunits of oxidative phosphorylation complexes is mediated by the concerted action of cytosolic Hsp chaperons, the TOM/TIM multiprotein complexes, inner-mitochondrial compartment maturase(s) and ATP-dependent mitochondrial chaperons [52]. The precursor proteins are brought in an extended translocation-competent configuration and pushed into the TOM/TIM membrane system by cytosolic Hsp chaperons [53]. Active translocation of the precursors in the TOM/TIM system, driven by the membrane potential, is further assisted by the ATP-dependent pulling activity of mitochondrial Hsp proteins [54]. Translocation of proteins by the TOM-TIM system is a reversible process. Intramitochondrial processed mature proteins can undergo retrograde diffusion into the cytosol [53, 55]. The findings reviewed in section 2 (Fig. 9) indicate that phosphorylation of the NDUFS4 protein, rather than stimulating the active penetration of the protein into mitochondria, where it is proteolytically converted to the mature form, it inhibits passive retrograde diffusion of the mature NDUFS4 protein into the cytosol where it is proteolytically degraded. The inhibition by PKA of retrograde release from mitochondria of the mature NDUFS4 protein appears to be associated with binding of the C-terminus phosphorylated protein to the cytosolic Hsp70. This can contribute to hold transiently the C-terminus phosphorylated protein to cytosolic Hsp70. After incubation in the presence of AP plus cHsp70 antibody; lane 4, import incubation in the presence of PKA plus cHsp70 antibody; lane 5, import incubation in the presence of PKA plus β-actin antibody; lane 6, import incubation in the presence of PKA plus β-actin antibody. Reproduced with permission from [36], see this reference for details.

Table 1

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The phosphopeptides were detected after their enrichment by TiO2 column chromatography of the trypsin in-gel digest. Nonphosphorylated peptides were detected in the trypsin in-gel digest before TiO2 enrichment. Phosphorylation analysis was performed by MS2 mass spectrometry. Data reproduced with permission from [41].
import of the NDUFS4 subunit is directly involved in the promoting effect exerted by cAMP, in cell cultures in vivo, on the activity of complex I and prevention of cellular accumulation of reactive oxygen species.

The NADH–ubiquinone oxidoreductase activity is impaired and oxygen superoxide production enhanced in complex I by thiol oxidation and mixed disulfide glutathionylation of some of its subunits, like the 75 kDa (NDUF51 gene), 51 kDa (NDUFV1 gene) and TYKY (NDUF8 gene) [56,57]. A pathological homozygous hereditary Q522 K replacement in the 75 kDa subunit (NDUF51 gene) has been found to result in decreased content of the protein, impairment of the NADH ubiquinone oxidoreductase activity, mitochondrial membrane potential and cellular ROS accumulation [58] (Fig. 11). These last effects were counteracted by cAMP.

The phosphorylation state of the NDUFS4 subunit, as well as of other subunits of complex I [41–43,47,48,58–60], by PKA, or different protein kinases which impinge on mitochondrial functions [61,62], may be interrelated with the redox state of the subunits, their susceptibility to oxidative damage and/or proteolytic turnover [63]. Disregulation of these processes appears to be involved in complex I dysfunction associated with neurological disorders [62,64].

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Fig. 10. Scheme describing the impact of serine phosphorylation in the C-terminus on the mitochondrial import of the NDUFS4 subunit of complex I. Phosphorylation of NDUFS4 C-terminus mediates binding of the NDUFS4 protein to the cytosolic Hsp70. Binding of the NDUFS4/Hsp70 complex to TOM contributes to maintain the NDUFS4 precursor protein in unfolded translocation competent configuration and prevents retrograde release from mitochondria of the mature protein from which the positively charged leader sequence has been removed.
membrane potential. B, DCF-DA detection of H₂O₂ (green fluorescence). C, Vmax of NADH-UQ oxidoreductase activity. Reproduced with permission from [58], see this reference for details.

Fig. 11. Rescue effect of dibutyryl-cAMP on the depression of NADH-ubiquinone oxidoreductase, mitochondrial membrane potential and accumulation of H₂O₂ in fibroblasts from a patient with NDUF51 mutation. The patient harboured a homozygous C1564A mutation in NDUF51 gene, 75 kDa Fe-S protein, (Q522K replacement). (a), control human fibroblasts. (b), NDUF51 mutant fibroblasts exposed to 100 μM dibutyryl-cAMP for 60 min. A, MitoCapture for detection of low (green fluorescence) and high (red fluorescence) mitochondrial membrane potential. B, DCF-DA detection of H₂O₂ (green fluorescence). C, Vmax of NADH-UQ oxidoreductase activity. Reproduced with permission from [58], see this reference for details.

References


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