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Fatty acid cycling mechanism and mitochondrial uncoupling proteins

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Abstract

We hypothesize that fatty acid-induced uncoupling serves in bioenergetic systems to set the optimum efficiency and tune the degree of coupling of oxidative phosphorylation. Uncoupling results from fatty acid cycling, enabled by several phylogenetically specialized proteins and, to a lesser extent, by other mitochondrial carriers. It is suggested that the regulated uncoupling in mammalian mitochondria is provided by uncoupling proteins UCP-1, UCP-2 and UCP-3, whereas in plant mitochondria by PUMP and *St*UCP, all belonging to the gene family of mitochondrial carriers. UCP-1, and hypothetically UCP-3, serve mostly to provide nonshivering thermogenesis in brown adipose tissue and skeletal muscle, respectively. Fatty acid cycling was documented for UCP-1, PUMP and ADP/ATP carrier, and is predicted also for UCP-2 and UCP-3. UCP-1 mediates a purine nucleotide-sensitive uniport of monovalent unipolar anions, including anionic fatty acids. The return of protonated fatty acid leads to H^+ uniport and uncoupling. UCP-2 is probably involved in the regulation of body weight and energy balance, in fever, and defense against generation of reactive oxygen species. PUMP has been discovered in potato tubers and immunologically detected in fruits and corn, whereas *St*UCP has been cloned and sequenced from a potato gene library. PUMP is supposed to act in the termination of synthetic processes in mature fruits and during the climacteric respiratory rise. © 1998 Elsevier Science B.V.

Keywords: Fatty acid cycling mechanism; Uncoupling; Mitochondrial uncoupling protein; Plant uncoupling mitochondrial protein

1. Introduction

According to Stucki [1], mitochondrial oxidative phosphorylation with an external load requires a degree of coupling less than unity to reach the most efficient state. This state exists when so-called conductance matching conditions are fulfilled [1] that relate conductance of phosphorylation to conductance of all ATP-utilizing processes [1]. We hypothesize that this optimum, i.e. a lower degree of coupling, may be set by fatty acid-induced partial uncoupling [2,3], and that this necessity has been reflected during phylogenesis by the development of several uncou-

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pling proteins and a fatty acid cycling mechanism. Fatty acids (FAs) have long been known as weak uncouplers of oxidative phosphorylation [4], but the mechanism of their effects has remained obscure until Skulachev introduced the hypothesis of fatty acid cycling [5], assuming the ability of some inner membrane proteins to translocate fatty acid anions. If this occurs, fatty acid can return in a protonated form, and thereby translocate H^+. Experiments supporting this mechanism were reported for the ADP/ATP carrier [2,6,7] and two uncoupling proteins [3,8–11]. Nevertheless, the possibility of the phylogenetically developed mechanism that tunes the efficiency of oxidative phosphorylation arrived with the recent discovery of novel and rather ubiquitous uncoupling proteins in rat and human mitochondria and in plants. Their function may also explain the phenomenon previously referred to as a leak [12]. It is important to note that some heat production is always a byproduct of partial uncoupling, at least in the case of UCP-1, where it was evolved as the main function. The same function is hypothesized for UCP-3.

Among new mitochondrial uncoupling proteins, mammalian (human) UCP-2 [13,14] and UCP-3 [15,16]) were revealed in 1997 by genetic approaches on the basis of their extensive homologies with UCP-1 [17,18], a 33-kDa protein specific for brown adipose tissue (BAT), described first in 1976 [19]. UCP-2 was found in the mouse and human genome by screening the expressed sequence tag (EST) libraries, and in humans it is 59% identical with hUCP-1. Its mRNA has been identified in heart, BAT, white fat, skeletal muscle, kidney, lung, placenta, and tissues of the immune system, including leukocytes and macrophages [13–15]. UCP-2 has been proposed to regulate body weight and link thermoregulation with the inflammatory stimulus [13,14] and defend against generation of reactive oxygen species [20]. UCP-2 mRNA is not regulated by norepinephrine, but rather by leptin, the adipocyte hormone that might upregulate UCP-2 in tissues expressing the leptin receptor OB-R [21]. UCP-3, 57% identical with UCP-1 in humans, was found by reverse transcription PCR [15] and in the EST library [16], and was recognized to be specific for skeletal muscle and BAT, thereby becoming a potential candidate for epinephrine-induced muscle nonshivering thermogenesis, prevention of oxygen damage in myocytes, or, in view of its expression in BAT and upregulation by leptin [22], in prevention of obesity.

Plant uncoupling mitochondrial protein (PUMP) has been discovered in potato tubers by Vercesi et al. [23] in 1995. Its fatty acid-mediated H^+ transporting function has been confirmed in studies of potato and tomato mitochondria [3,24] and in the reconstituted system [9], whereas its regulation by purine nucleotides was recognized from the corresponding weak inhibition [3,9] and photoaffinity labelling by azido ATP [25]. Immunologically, PUMP has been identified in various tropical and mild climate fruits [26], mostly climacterics (i.e. those which exhibit a respiratory rise during ripening) and in corn. Potato gene library screening led to the discovery of S*t*UCP, a 44% homologous protein to hUCP-1 [27]. Its mRNA has been detected in leaves, roots, stem, and fruits of potato, but not in tubers [27]. It is not yet known whether PUMP is identical with S*t*UCP.

2. Materials and methods

Illustrative experiments (Figs. 2–4) were conducted as previously published. ^{3}H-Azidofatty acid labelling of rat heart submitochondrial particles (SMPs) was conducted, as described, for heart mitochondria [2], by 20 min incubation with a label, 10 min UV illumination, and extensive washing with BSA by ultracentrifugation. SMP proteins were separated on SDS–PAGE gels, stained with Coomassie blue, treated by autoradiography enhancer Entensify (NEN), dried and exposed to Kodak X-ray films [2]. AAC was identified by comparison of the obtained labelled bands with the labelled 30-kDa bands of the hydroxylapatite eluate of Triton X-100-extracted mitochondria [2] and with Western blots using anti-AAC antibodies [2].

Mitochondria-enriched fractions were prepared by the differential centrifugation from fruit homogenates, the pH of which was carefully adjusted by KOH during homogenization [24]. Solubilized samples were run on SDS–PAGE minigels (Bio-Rad), electrotransferred to Hybond-N nylon membranes (Amersham), blocked in 20 mM Tris, pH 7.4, containing 137 mM NaCl, 0.1% (v/v) Tween 20 and 5% (w/v) no-fat dry milk, and incubated with anti-potato-PUMP polyclonal antibodies [24] and with

anti-rabbit IgG alkaline phosphatase-conjugated antibodies (Sigma). The membranes were incubated for 20 min in the dark in 100 mM Tris–Cl, pH 9.5, 100 mM NaCl and a 1:1000 solution of CSPD (Tropix, USA). The bands were detected by chemiluminescence and photographed with a Kodak Digital Science EDAS system with a DC 40 camera.

The DNY1 yeast strain was a kind gift of D. Nelson (Coll. Medicine, Memphis, TN, USA) and was grown by galactose induction in 5-l fermentors. Yeast mitochondria were isolated using Zymolyase (Seikagaku, Japan), as described in Ref. [28]. Oxygen consumption was measured by a Clark polarographic probe in 650 mM mannitol, 20 mM Tris–MOPS, 0.5 mM Tris–EGTA, 2 mM $MgCl_2$, 10 mM K_2HPO_4, pH 6.8, containing 5 μM rotenone. Membrane potential was monitored fluorometrically with 5 μM safranine [3] in 100 mM KCl, 20 mM Tris–Cl, 5 mM K_2HPO_4, pH 6.8, and 5 μM rotenone.

3. Fatty acids as mediators of uncoupling

Having a large partition coefficient for biological membranes, fatty acids underlie the basic thermodynamics equilibria that encompass the whole system, i.e. the aqueous plus membrane phase [10]. A simple addition of a fatty acid to a *cis* side of the membrane represents a nonequilibrium state that approaches toward equilibrium as follows (Fig. 1, 'flip-flop acidification'). It can be viewed as several subsequent steps [10]. In step 1, equilibrium is attained first in the external aqueous space as an acid–base equilibrium. Step 2 represents partitioning into the membrane, and step 3 the acid–base equilibrium in the external membrane leaflet. This intermediate state is still a nonequilibrium state with regards to the membrane. Since only the neutral fatty acids can rapidly flip-flop across the membrane, the final equilibrium might be reached via two sub-

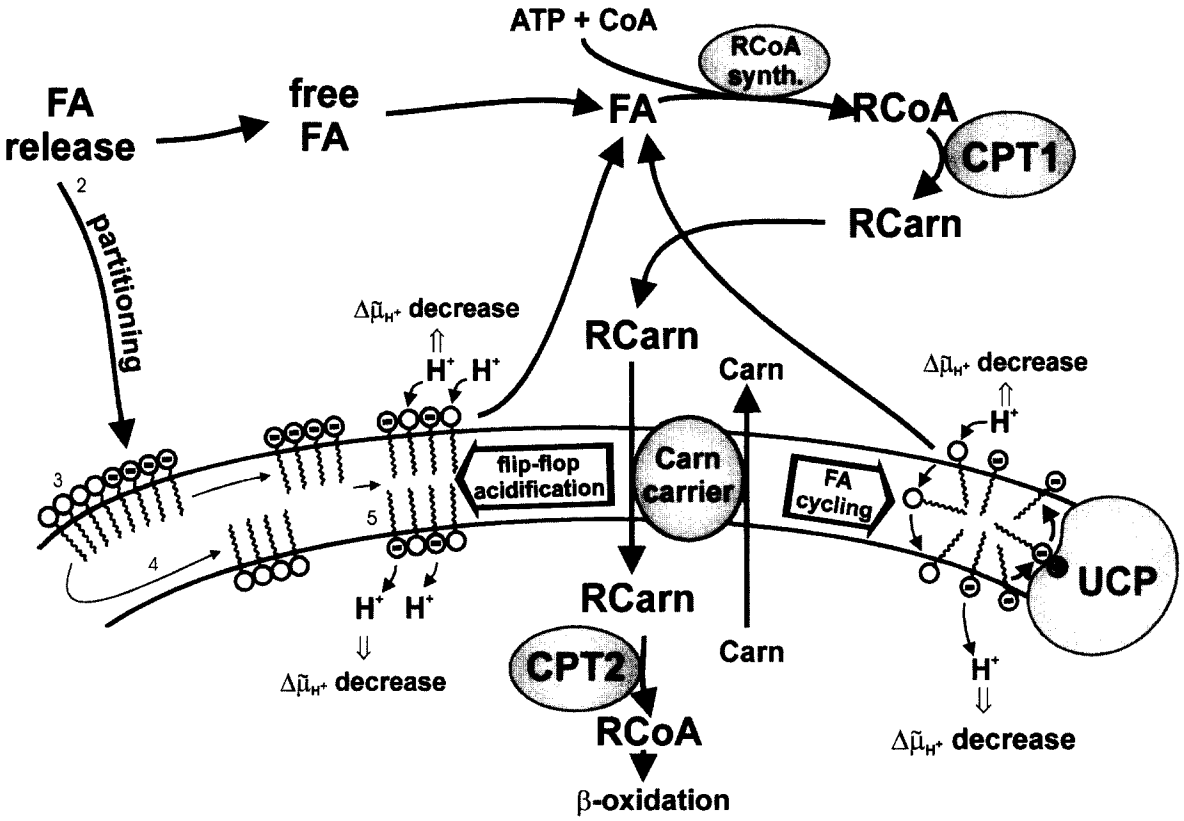

Fig. 1. Three major roles of fatty acids in bioenergetics: (I) transient FA-induced matrix acidification ('flip-flop acidification'); (II) fatty acid cycling enabled by uncoupling proteins and other carriers ('FA cycling'); and (III) carnitine cycle and β-oxidation. RCoA synth., Acyl-CoA synthase; CPTI and CPTII, outer and inner carnitine palmitoyl transferase; Carn. carrier, carnitine carrier or carnitine/acylcarnitine translocase.

sequent steps: step 4, a flip-flop transfer, equilibrating a number of fatty acid molecules in the two opposite membrane leaflets; and step 5, attaining the acid–base equilibrium in the inner leaflet. The latter leads to the acidification of liposome interior upon the addition of FA, as observed experimentally [10,11,29]. It must be assumed that such flip-flop acidification proceeds transiently in mitochondria, when FAs are released from the triglyceride storages and other sources (Fig. 1). This flip-flop acidification causes a transient decrease in the protonmotive force that is observed experimentally upon the FA addition to all types of energized mitochondria (e.g. Ref. [3]).

Accepting Skulachev's hypothesis [5] that several integral inner membrane proteins, namely the anion carriers, are able to mediate uniport of anionic FAs, we deal with the second, less-transient, mechanism of uncoupling (Fig. 1, 'FA cycling'). FA anions are repulsed via these proteins to the external leaflet of the inner membrane, where they become protonated and subsequently return in their neutral form to the inner leaflet, thereby translocating H^+. As a result, mitochondria are being uncoupled as long as the cycle lasts. The great advantage of this mechanism is that it can be regulated in different ways—in each protein differently—and lasts until all nonesterified FAs are depleted from the membrane and binding sites on proteins, as well as from both cytosolic and matrix aqueous compartments. A major depleting mechanism is illustrated in Fig. 1, the so-called carnitine cycle, that passes FAs to β-oxidation into the matrix. The carnitine cycle is inhibited by malonyl-CoA and has important organ-dependent regulatory links towards de novo lipogenesis and FA elongation [30].

4. Proteins mediating fatty acid cycling

In general, one could imagine that any integral membrane protein, possessing, for example, positively charged residues in the hydrophobic core of the membrane, could attract the anionic carboxylic headgroup of FA and permit its passage to the other side of the membrane. Indeed, the majority of members of the mitochondrial gene family contain such charges [31]. Nevertheless, as it can be hypothesized in relation to the discoveries of novel UCPs, the phylogenesis probably designed several UCPs to be specialized for this task. Among them, UCP-1 [8,11] and PUMP [9], as experiments supported, participate in the FA cycling. The cycling has probably evolved as a side function in other carrier proteins, such as in the ADP/ATP carrier for which also the results supporting FA cycling have been reported [3,6,7]. We suggest that UCP-2 and UCP-3 also provide FA cycling leading to uncoupling, and that the major control over FA cycling is provided by proper regulatory mechanisms. These mechanisms had to be developed during phylogenesis, and certainly must exist for UCP-2 (unless becoming fatal, e.g. in the beating heart) and for UCP-3 (if open constantly, muscle contraction would be harmed). Their nature is presently unknown. On a longer time-scale regulation, their transcription is stimulated by leptin and other factors [21,22].

All UCPs belong to the gene family of mitochondrial anion carrier proteins (MACP), that includes the ADP/ATP carrier, phosphate, oxoglutarate and other carriers [17,32]. Twenty-two new carriers (altogether 29 members) of MACP have been identified in the yeast genome [31]. All members of the MACP family have a triad structure with three internal repeats that are homologous with each other and form six membrane-spanning quasi-α-helices [17,31,32]. In UCPs, the C- and N-ends are located on the cytosolic side of the membrane, resulting in four cytosolic and three matrix segments. A proline begins each matrix segment, followed by a characteristic MACP signature: P-ϕ-(DE)-(ϕT)-(ϕT)-(RK)-(Tϕ)-(RK)-ϕ (where ϕ is a hydrophobic residue) [31].

5. Fatty acid-binding sites on the mitochondrial inner membrane proteins

The existence of specific FA-binding sites on the mitochondrial inner membrane proteins has been suggested by photoaffinity labelling studies of mitochondria [2,33,34] and isolated proteins [2,34]. The specificity is provided at least by the fact that the [$^3H_4$]azido-FA did label the most hydrophobic proteins, those which are not retained in the hydroxylapatite column (mostly, the carriers of MACP family). Therefore, in rat heart SMPs (Fig. 2) the labelled ADP/ATP carrier [2] and several other labelled bands

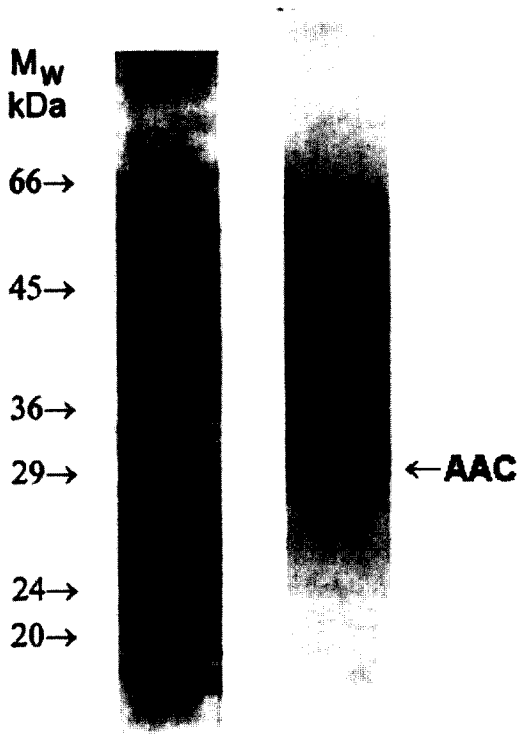

Fig. 2. Photoaffinity labelling of rat heart submitochondrial particles with 16-(4-azido-2-nitrophenylamino)-[$^3H_4$]hexadecanoic acid. Electronic photographs of dried SDS–PAGE (left panel, numbers indicate molecular mass of standards) and the corresponding autoradiogram (right panel) are shown. Rat heart SMPs were photolabelled using similar protocol as described for mitochondria [2,34].

have been identified. In BAT mitochondria, a major labelled band was the 32-kDa uncoupling protein UCP-1 [33,34], that can bind the photoaffinity label in a quite low stoichiometry (less then 0.5 per dimer) [34]. The existence of a FA binding site in UCP-1 has been independently demonstrated by EPR studies with 5-doxyl-stearic acid, that exhibits a specific EPR signal of the immobilized probe which was prevented by alkylsulfonates, and modulated by the addition of nucleotides [35]. The latter changes probably reflect the allosteric nucleotide-mediated gating. The participation of the FA-binding sites in the cycling mechanism has been demonstrated for the UCP-1 and ADP/ATP carriers, and is discussed below.

6. UCP-1, 'classic' mitochondrial uncoupling protein

BAT is an organ of nonshivering thermogenesis in newborns, cold-acclimated and hibernating mammals, and in overfed rodents [18]. A typical BAT cell is loaded with mitochondria containing the tissue-specific UCP-1. Since the majority of blood flow is passing through BAT, UCP-1 can provide efficient and regulated nonshivering thermogenesis to the whole body. UCP-1 expression is acutely regulated by norepinephrine via β_3-adrenergic receptor. This activation also stimulates UCP-1 function and cell differentiation in pre-adipocytes [18]. UCP-1 mRNA increases during diet-induced or cold-induced thermogenesis [18]. Human UCP-1 has 305 amino acids (M_r 32 786) and a polarity index (PI) of 41%, whereas rat UCP-1 has 306 amino acids (M_r 33 042) and a PI of 42% [36].

UCP-1 catalyzes the purine nucleotide (PN)-sensitive flip-flop transport of the anionic fatty acid head group from one side of the membrane to the other (Fig. 1, 'FA cycling'). FA, then, becomes protonated and rapidly flip-flops back with its proton. This UCP-1 action enables FAs to behave as cycling protonophores. UCP-1 also catalyzes electrophoretic, PN-sensitive transport of monovalent unipolar anions, including halides, nitrate, oxohalogenides, monovalent phosphate analogs, pyruvate, and other α-ketocarboxylates, short- medium- and long-chain alkylsulfonates, and unipolar arylsulfonates [37]. Anions with higher hydrophobicity (longer chain) are translocated faster with higher affinities (lower K_m values) [8,37]. It is predicted that the transport pathway possesses an internal weak binding site (energy well) near the mid-point of the membrane and to lie on an *outside* surface of the *trans*-membrane helix, at the protein–lipid interface [8]. Hydrophilic anions, such as $Hepes^-$, Tes^-, and bipolar anions, such as aminobenzenesulfonate, do not inhibit transport, even of closely related structures [37]. This implies that the 'surface' binding site for transported anions is shielded from the aqueous phase, and that this site is located below the lipid head groups at the level of acyl–glycerol linkages. This is precisely the equilibrium position of FA and the alkylsulfonate head-group in the membrane. Cl^- is transported with very low affinity (K_m about 140 mM) and is thought to access the internal energy well by brute force—i.e. by thermal bombardment of the membrane. This is in contrast to the high affinities of FA, and the long-chain alkylsulfonates (K_m of 10–15 μM) [8].

Support for the FA cycling mechanism (Fig. 1) was provided by the following: (i) the 'external'

translocation sites are shielded, as demonstrated by the failure of non-transported substrate analogs to inhibit transport [8,37]). (ii) Long-chain alkylsulfonates, which are very strong acids, fail to catalyze the H^+ flux mediated by UCP1. Nevertheless, they are transported at high rates and are competitive inhibitors of FA-induced H^+ transport [8]. This implies that their analogs, FA anions, are transported by UCP-1. (iii) FA inhibits Cl^- and alkylsulfonate transport [8]. (iv) Inactive FAs, such as 12-hydroxylauric acid, are unable to flip-flop across the lipid bilayer [10], presumably because they partition into the membrane in a U-shape. These FAs also fail to induce H^+ transport with UCP1, to inhibit Cl^- uniport via UCP1, and to be transported by UCP-1 as anions [11]. Therefore, the inability to flip-flop is associated with a lack of effects on the UCP-1 function.

Purine nucleotide (PN) binding from the cytosolic side, decreasing with increasing pH and by Mg^{2+} [17,18,38], represents a key physiological regulator. The regulatory PN binding site is purely allosteric, as confirmed by noncompetitive kinetics and by the lack of FA influence on the K_i for PN inhibition [39]. The site has a high affinity to PN di- and triphosphates and to AMP-PNP, ATP-γ-S, 8-Br-ATP, 8-azido-ATP; with a low affinity to PN monophosphates or to AMP-CH_2-PP [17]. Its location has been mapped by photoaffinity labeling and site-directed mutagenesis, and turns out to be very extensive [28,38]. The sugar-based moiety interacts with the matrix segment D233–E261 that connects the fifth and sixth *trans*-membrane helices. The nucleoside phosphates interact with three arginines that are located on helices 2, 4, and 6 [28]. Therefore, PN 'backs' into a cavity formed by all six helices, and the sugar-base reacts with the matrix segment. This interaction presumably confers specificity among nucleotides, and induces conformational changes required for gating [35].

7. UCP-2 and UCP-3

Both homologs of UCP-1 are expected to provide FA cycling. The existence of UCP-2 in the tissues, such as the heart, in which FAs are the preferred substrates, possesses a functional problem. It must be strictly regulated in the working heart, ideally to reach a partial uncoupling required for Stucki's optimum efficiency [1]. Uncoupling in lymphocytes may lead to anaerobic metabolism and cellular acidification, that causes the detachment of lymphocytes. UCP-3 is expected to underlie a switch-on/switch-off type of regulation, while providing thermogenesis at the resting state of skeletal muscle. The regulatory factor(s) are unknown.

8. PUMP, a plant uncoupling mitochondrial protein

PUMP has been characterized as a hydrophobic protein that is not retained on hydroxylapatite in a detergent micellar solution [3,9,23]. Antibodies raised against potato PUMP identified a 32-kDa PUMP band and its dimer in tomato, corn, and avocado mitochondria [24,26] and in mitochondrial fractions prepared from homogenates of many tropical and mild climate fruits, such as banana, apple, peach, melons, orange (Fig. 3), tomato [24] and other fruits [26]. Related to FAs, PUMP behaves exactly as UCP-1 [3,9], therefore it is ascribed to have the ability to translocate FA anions, and to mediate FA cycling. PUMP is weakly inhibited by purine nucleotides and translocates alkylsulfonates, but not small hydrophilic anions such as Cl^- and pyruvate [3,9].

In plants, uncoupling mediated by PUMP might serve to optimize oxidative phosphorylation, as well as to switch-off rapidly ATP production [3,9], thus contributing to the termination of synthetic processes during the final stages of seed formation and senescence. It has also been hypothesized that PUMP can be responsible for the climacteric respiratory burst [3,24,26], a transient stage, existing in many fruits during their ripening. In tomatoes, a state of mitochondrial uncoupling has been correlated with the amount of FA present [24]. Thus, mitochondria from green immature tomatoes were very well coupled; those from an intermediate, but pre-climacteric state, were partially uncoupled; and mitochondria from red (ripe) fruits, which passed the climacterics, were completely uncoupled. PUMP has been isolated in a reconstitutively active form, and immunologically detected in all of these stages [24].

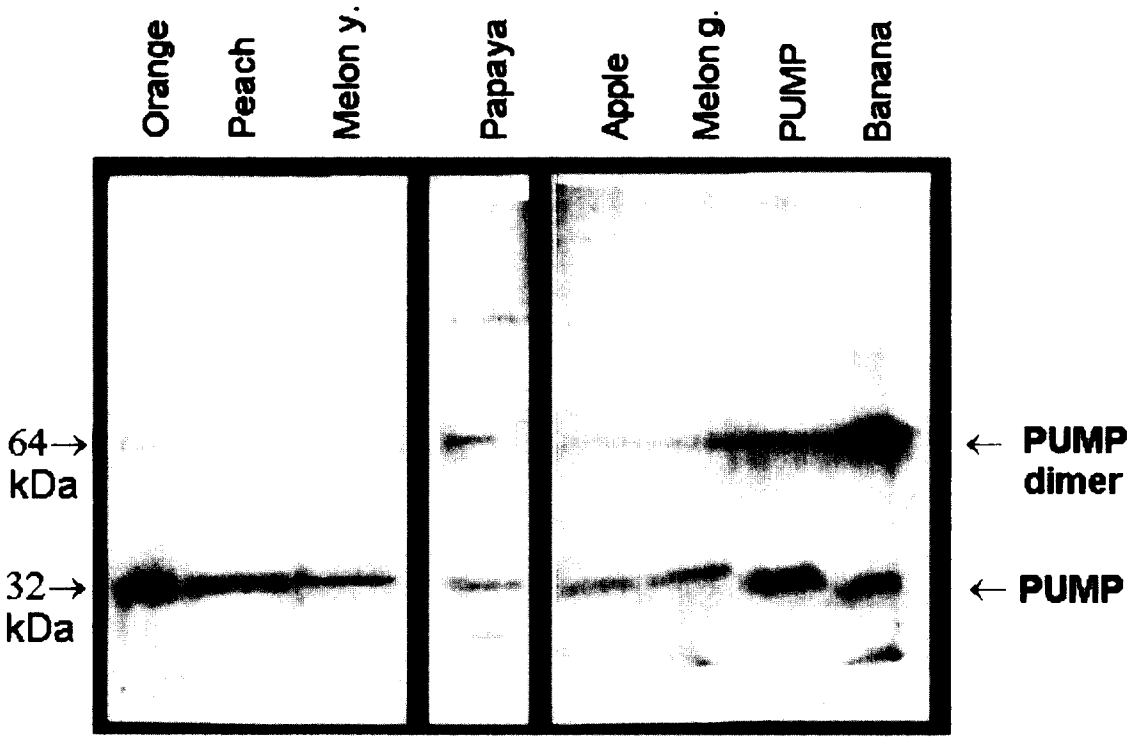

Fig. 3. Western blots of mitochondrial fractions of various fruits. (Melon y., yellow skin; Melon g., green skin). Primary antibodies were anti-potato-PUMP and secondary antibodies were alkaline phosphatase-conjugated anti-IgG. Development was done by chemiluminescence (CSPD, Tropix, USA, cf. Ref. [24]), using Amersham autoradiography films, which were photographed with a Kodak Digital Science EDAS system with a DC 40 camera.

9. ADP/ATP carrier

The mitochondrial ADP/ATP carrier (AAC) has been originally suggested by Skulachev to allow for FA cycling due to the observation that carboxyatractyloside (CAT) partially prevents FA-induced uncoupling [5]. Later, it was demonstrated that FA-induced H^+ transport in proteoliposomes with the reconstituted AAC is partially inhibited by CAT and bonkrekic acid [6]. On the contrary, FA-induced uncoupling in yeast mitochondria of the mutant strain DNY1 was not sensitive to CAT (Fig. 4). DNY1 has the deleted AAC2 gene, coding the functional AAC. Similar results were independently reported for a JL-1-3 strain, having deleted all AAC genes (AAC1, AAC2 and AAC3) [7], or the *op1* mutant, in which R96H substitution is present in the product of AAC2 gene [7], Note, the products of AAC1 and AAC3 isogenes do not participate at the ADP/ATP transport [7]. The inability of AAC, with the R96H substitution, to transport FA anions suggests that the Arg^{96} could be the residue attracting the anionic headgroup. Moreover, the azido-FA-induced uncoupling was turned back to a coupled state upon UV irradiation [2]. Thus, the covalently attached azido-FA was unable to induce coupling. All these results support the existence of fatty acid cycling mediated by AAC.

It should be asked, however, whether cycling mediated by proteins nonspecialized for this purpose takes place in vivo. Actually, FAs and their CoA derivatives inhibit ADP transport in isolated mitochondria. FA, being expelled via AAC, prevents one turnover of the ADP/ATP exchange and therefore also prevents the consumption of the corresponding (one unit) of $\Delta\tilde{\mu}_{H^+}$. Since the return of protonated FA consumes simultaneously the same unity of $\Delta\tilde{\mu}_{H^+}$, the result is a transient decrease in the cytosolic ATP/ADP ratio and increase in the matrix ATP/ADP ratio at a constant $\Delta\tilde{\mu}_{H^+}$. Nevertheless, the latter slows down ATP synthesis by a feed-back control. From this example, it can be seen why the specialized uncoupling proteins were phylogenetical-

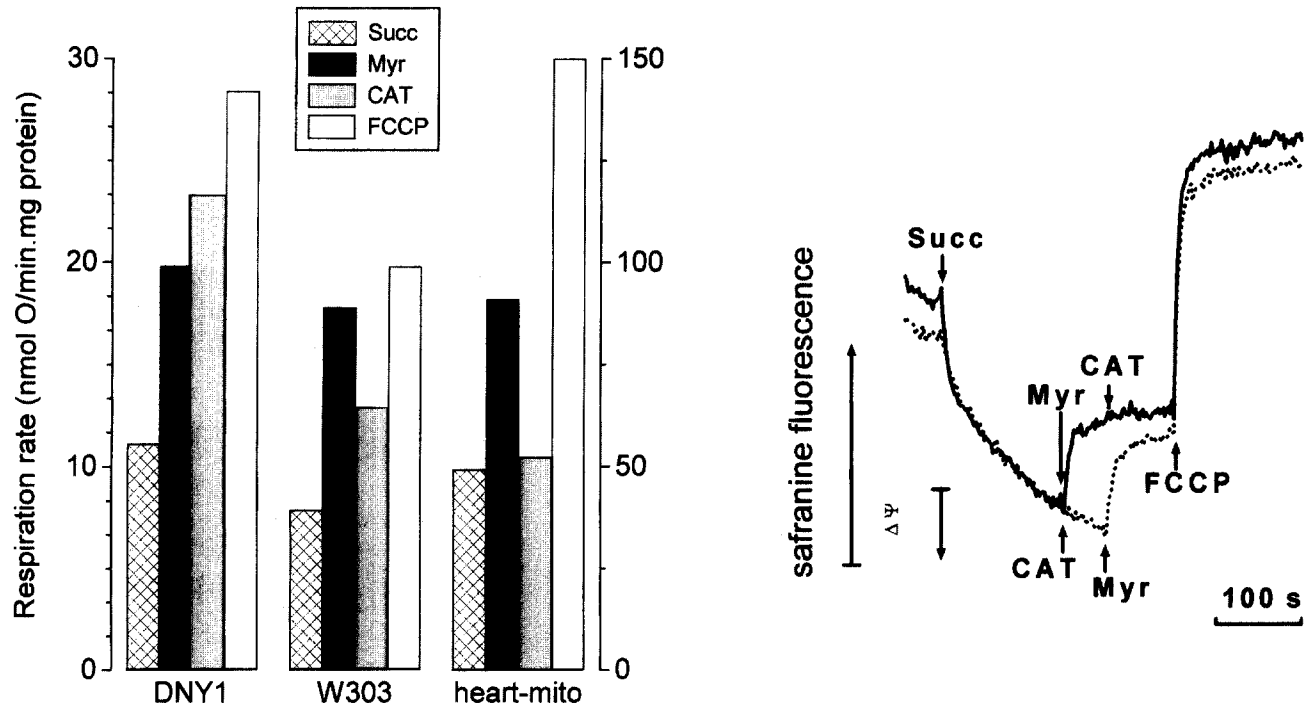

Fig. 4. Lack of carboxyatractyloside effect on fatty acid-induced uncoupling in mitochondria of yeast mutant strain DNY1, having deleted AAC2 gene. Left panel, respiration; right panel, membrane potential monitoring. Mitochondria of the DNY1 yeast strain were isolated using Zymolyase (Seikagaku, Japan) as described in Ref. [28]. Left panel: columns represent typical respiration rates of mitochondria from DNY1 strain, or from control W303 strain (left axis), and from rat heart (right axis), suspended in 650 mM mannitol, 20 mM Tris–MOPS, 0.5 mM Tris–EGTA, 2 mM $MgCl_2$, 10 mM K_2HPO_4, pH 6.8, containing 5 μM rotenone. Right panel: a typical result of membrane potential monitoring with safranine (5 μM) for DNY1 yeast mitochondria in 100 mM KCl, 20 mM Tris–Cl, 5 mM K_2HPO_4, pH 6.8, containing 5 μM rotenone. Further additions were as follows: 5 mM succinate (Succ), 50 μM Na-myristate (Myr), 5 μM carboxyatractyloside (CAT) and 10 μM FCCP.

ly developed to achieve the net uncoupling, mediated by fatty acid cycling, and in a more straightforward way.

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