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Review

Uncoupling proteins: the issues from a biochemist point of view

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Abstract

The functional characteristics of uncoupling proteins (UCP) are reviewed, with the main focus on the results with isolated and reconstituted proteins. UCP1 from brown adipose tissue, the paradigm of the UCP subfamily, is treated in more detail. The issues addressed are the role and mechanism of fatty acids, the nucleotide binding, the regulation by pH and the identification by mutagenesis of residues involved in these functions. The transport and regulatory functions of UCP2 and 3 are reviewed in comparison to UCP1. The inconsistencies of a proposed nucleotide insensitive H^+ transport by these UCPs as concluded from the expression in yeast and *Escherichia coli* are elucidated. In both expression system UCP 2 and 3 are not in or cannot be converted to a functionally native state and thus also for these UCPs a nucleotide regulated H^+ transport is postulated.

Keywords: Uncoupling protein; H^+ transport; Cl^- transport; Fatty acid; Nucleotide binding; Site-directed mutagenesis; Thermogenesis; Brown adipose tissue

1. Introduction

This article will address some pertinent issues of the uncoupling protein (UCP) from brown adipose tissue (BAT) UCP1 and the UCP variants, from our point of view (see reviews [1–4]). Primarily we will discuss the function, regulation and the mechanism of function, as it is known in some detail for UCP1. At the end we will consider the function and regulation of the UCP variants under the tenet that UCP1 from BAT is a paradigm for the other UCP variants.

The leading role of UCP1 in our progress of understanding the function of UCPs is not only a historical but foremost a practical one. The thermogenesis in BAT is such a striking feature that here the existence of an uncoupling mechanism was a dominant feature. This uncoupling was traced to UCP1 which occurred in abundance in BAT mitochondria. Hamster BAT mitochondria contain 2.4 UCP1 dimers/cytochrome aa_3 [5]. The possible reasons for this seemingly excessive of uncoupling power will be discussed below. The practical advantage was the availability of abundant isolated UCP which permitted the isolation [5,6], the elucidation of its sequences [7] and a detailed study of its functional and regulating characteristics [8]. In contrast, in other tissues uncoupling is not a dominant feature and UCPs occur only in small amounts, so far forbidding the iso-

Abbreviations: UCP, uncoupling protein; BAT, brown adipose tissue; AAC, ADP/ATP carrier; FA, fatty acid; NDP, nucleoside diphosphate; NTP, nucleoside triphosphate; Dansyl-nucleotide, 2′-*O*-[5-(dimethylamino)naphthalene-1-sulfonyl] nucleotide; DAN-nucleotide, 3′-(2′)-*O*-[5-(dimethylamino)naphthalene-1-sulfonyl] nucleotide

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lation of other UCPs. Therefore a heterologous expression became mandatory for these UCPs. However, following the successful introduction into *Saccharomyces cerevisiae* of UCP1 [9–12], the expression of the UCP variants proved to be problematic and led to erroneous conclusions concerning their function as we will show below.

2. H^+ transport by UCP1

2.1. The stimulation of H^+ transport by fatty acids (FAs)

The activation of uncoupling by FAs has been one of the most debated issues over the years. There are two different levels of studies on the role of FA; mitochondria and reconstituted UCP1. In mitochondria the stimulation by FA of swelling-related H^+ transport was discovered [13,14], but later its role in regulation of uncoupling was questioned [15,16]. In the reconstituted UCP1 added FA are indispensable for H^+ transport, provided traces of endogenous FA have been carefully removed [17,18]. To what extent the discrepancy between mitochondria and proteoliposomes are explained by the driving membrane potential will be discussed below. UCP1 has a surprisingly wide tolerance towards the structure of the FA molecule. A minimum requirement is sufficient hydrophobicity. The chain length must at least be C_{10} (decanoic acid) [14,17]. A free carboxyl group is indispensable. Unsatured FA (oleic, linolic, linoleic) are all good activators of H^+ transport. Even substitution with phenyl (phenylhexanoic, etc.) allows for some although lower activity [17]. Also an oligoisoprenoid acid (retinoic acid) is a good activator [19]. More surprising is the tolerance towards hydrophilic substitutes. 2Br-palmitic gives the highest activity, but also in the ω-position Br- and OH-substitution are well tolerated. Even larger substitutes such as the spin label doxyl permit activity [17]. Of great interest for the mechanism of FA activation is the substitution by glucopyranoside in the ω-position of palmitic acid, which still produces good activity [3,17].

Parallel with the structural tolerance is the 'low affinity' as reflected in the 'high concentration' of FA (200 μM FA) (lauric, oleic) required for maximum H^+ transport activity in the reconstituted system [17]. Most of the FA are absorbed by the phospholipid vesicles where they can reach an 8% molar ratio related to the phospholipids, without introducing a leakage in the vesicles. Obviously UCP1 recruits FA from the membrane which is there abundantly available.

2.2. H^+ transport activity in mitochondria versus proteoliposomes

The maximal activities measured in reconstituted vesicles saturated with FA exceed by 50–100 times the H^+ transport required for uncoupling in mitochondria but are significant for understanding the H^+ transport by UCP1. In BAT mitochondria the $K_{1/2}$ for FA to activate uncoupling by UCP1 is reported to be at 90 nM [20]. Recently a similar value has been reported for UCP1 expressing yeast mitochondria [15]. Part of the differences to the proteoliposomes can be traced to the different methods used. Originally in mitochondria H^+ transport was measured by swelling with K^+ acetate [13,14]. Then the most widely used method for measuring uncoupling became respiration [20]. Measuring directly H^+ transport with pH electrodes was applied to reconstituted vesicles [17,21,22] and occasionally to mitochondria [23]. Also the fluorescent pH probe pyranine was used for H^+ transport reconstituted vesicles [11]. Indirectly H^+ transport was also measured using a fluorescence probe for the buffer anions [24]. Since respiration is limited by electron transport capacity, the uncoupling activity of UCP determined in reconstituted vesicles is up to 90 times higher and reflects the actual H^+ transport capacity of UCP1. Another major factor decreasing UCP activity in isolated mitochondria is the masking of the nucleotide binding sites by endogenous ATP, unless the mitochondria are treated with anion exchanger to remove the UCP-bound ATP (see below) [25]. A further complication in making conclusions from experiments with isolated mitochondria, is the continuous formation of FA. In the reconstituted system these factors can be much better controlled and thus the dependence on FA, the control by nucleotides and by pH more unequivocally established.

The recent claim that UCP1 is able to transport H^+ without FA is based on UCP1 containing yeast

mitochondria [15]. At first sight this may contrast with results in the reconstituted system where H^+ transport is fully dependent on FA. The explanation may reside in the orders of magnitude different rates of H^+ transport by UCP1 in both systems. The level of H^+ transport activity claimed to be independent of FA is less than 1% of the maximum activity observed with the reconstituted UCP and thus might be well below the level of detection. The recent claim by Matthias et al. [16] that FA are not physiological activators will be discussed below in context with the issue of the intracellular regulation of UCP.

2.3. FA anion cycle versus catalytic (H^+ donor–acceptor) model of FA function

The role of FA in the H^+ transfer has been widely debated. Besides the more extreme views that UCP catalyzes FA anion transfer [26,27] or that FA just provide a 'missing' carboxyl group [17,28] within the relay of H^+ transferring groups along the translocation path, more compromising pictures are possible.

The FA anion model for UCP-catalyzed H^+ transport draws its main support from the use of alkylsulfonate [24,29], which can be regarded as 'permanent' FA anion analogs, since at physiological pH they cannot pick up a H^+. The uptake of short chain alkylsulfonates (C_3 to C_6) into mitochondria as observed by a K^+-valinomycin driven swelling was interpreted to use the formerly established capability of UCP1 to transport anions such as Cl^-, NO_3^- etc. [30]. In this work [24] the same authors also tried to derive the alkylsulfonate transport activity by measuring the competition with Cl^- uptake. Only with the longer chain C_{11}-sulfonate the competitive suppression of the lauric acid-catalyzed H^+ transport was demonstrated. A major problem is the sensitivity towards GDP which in our view is the criterion for a specific UCP involvement. With increasing chain length the uptake of alkylsulfonate (C_6 and C_{11}) becomes more insensitive to GDP. A competition between alkylsulfonate and GDP was invoked but this contrasts to the noncompetitive type of inhibition by GDP, well established for Cl^- and H^+ transport [11,17,31]. Thus a large portion of the effects of the more detergent type C_6 and C_{11} sulfonate does not fulfil the requirement for high nucleotide sensitivity and appears to be not linked to UCP-catalyzed transport. The suppression of H^+ transport by C_{11}-sulfonate can be interpreted as a competition for the FA binding site rather than for the FA anion transport.

There are other types of evidence and arguments against the FA anion cycling through UCP: (a) The rate of Cl^- transport is about an order of magnitude slower than H^+ transport, which is inconsistent with FA anion transport being involved in H^+ transport [11]. (b) Reconstitution of UCP1 expressed in *Escherichia coli* permitted to obtain Cl^- transport but FA could not induce H^+ transport [32]. The reported claim [33] that UCP2 and UCP3 expressed in *E. coli* transport H^+ in the reconstituted vesicles is not in contrast to our results, since the reported activities actually amount to only a few percent of that measured with native UCP1 (see below). This H^+ transport is barely inhibited by high concentration of GDP and thus must be classified as an unspecific FA induced leakage. (c) A mutant has been generated in UCP1 (E167Q) expressed in *S. cerevisiae*, where Cl^- transport capability is lost, but H^+ transport retained [23]. Thus anion transport capability is not a prerequisite for H^+ transport in UCP1. (d) ω-glucopyranoside palmitate with the bulky hydrophilic ω-substitute also activates H^+ transport although it should not be able to flip with the ω-glucopyranoside terminal through the membrane since it remains anchored at the membrane surface [3,17]. (e) Although operating at only a low level, the FA independent H^+ transport reported by Gonzalez-Barroso et al. [15] argues against the FA anion cycling and can be reconciled with the FA cofactor role (see below). (f) The FA anion cycle proposal for UCP originates from some evidence that the ADP/ATP carrier (AAC) in mitochondria facilitates a small FA dependent uncoupling [34,35] which was related the anion transport capability of the AAC. Although this model seems to be widely accepted in the literature, there is no evidence that AAC transports FA anions.

2.4. The cofactor–facilitator mechanism of FA

When compared to other H^+ transporting membrane proteins, UCP1 stands out for apparent simplicity. It has no active H^+ pump such as the well studied bacteriorhodopsin [36,37]. It can be linked to

substrate H^+ cotransporters which also use the membrane potential to drive H^+ transport, only that in UCP the substrate binding had been amputated [28]. Common to all H^+ transporters is the involvement of carboxyl groups which facilitate the H^+ transfer along the chain of water molecules. The transfer of H^+ along this chain requires a coordinated reorientation of water molecules which becomes more difficult with the length of the chain. Interdispersed carboxyl groups fragment this chain and thus accelerate H^+ transfer [38,39]. With their highly variable pK, conditioned by the local environment, carboxyl groups also may facilitate directional H^+ transfer, as driven by $\Delta\psi$. In UCP1 the carboxyl group of FA is proposed to provide this facilitator function instead of, or in addition to resident groups. It is proposed that FA carboxyls cooperate with resident groups [17,28]. Through this construction of the H^+ transport machinery in UCP1 the uncoupling activity of UCP can be regulated by changes of the FA concentration. FA are not tightly bound to UCP since UCP1 can be easily inactivated by removal of FA with BSA [17,21]. For saturating the H^+ transport capacity of UCP1 a high density of FA molecules must exists in the membrane. Probably FA are recruited by UCP1 from the lipid bilayer to which most of the FA are bound.

Another argument for the FA cofactor role comes from the identification of H^+ dissociating groups in UCP1 apparently involved in H^+ transport. By replacing the His pair H145 and H147 with neutral residues, H^+ transport was drastically decreased to only about 10% of wild-type (wt) activity [40]. There was no influence on other functions of UCP. Another potential H^+ donor–acceptor is the conspicuous D27 located in the first transmembrane helix, since in D27N H^+ transport is reduced to 20%, with little decrease of Cl^- transport and nucleotide binding [23]. Neutralization of D210 localized on the cytosolic side also reduced H^+ to 20% of wt, without affecting other functions [23]. The identification of those groups to be involved in H^+ transport suggests that a carboxyl group on the cytosolic and the His pair on the matrix side form a pK gradient which in conjunction with the $\Delta\Psi$ drives H^+ into the matrix. D210 with a lower pK accepts and delivers H^+ into the UCP channel where the FA carboxyl intercepts and presents H^+ to the 'down streams' acceptor D27

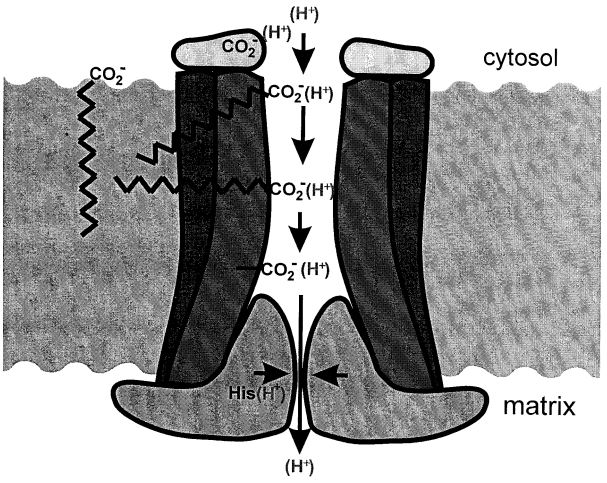

Fig. 1. A model of the mechanism of H^+ transport by UCP1 and the role of FA. The FA is visualized to penetrate from the lipid phase with its carboxyl group into the H^+ translocation path. Here it acts as H^+ donor–acceptor between resident carboxyl groups of UCP1. The H^+ transport path is proposed to consist of a wider aqueous pore and a narrow path lined by the loops protruding from the matrix side. Here inhibition of H^+ transport occurs by closure of the narrow path under influence of nucleotide binding. The resident H^+ transferring groups are from the cytosol, D210, D27, H145 and H147.

or H145+147. In another version, FA carboxyl is the H^+ acceptor at the membrane surface and the undissociated FA (FAH) dives into UCP from the membrane (Fig. 1). Here it delivers H^+ to the acceptor groups. The anionic headgroup of FA returns through the aqueous cytosolic funnel of UCP1 to the membrane surface where it recollects H^+ due its high pK. Thus the energy for stripping H^+ of its large hydration shell is overcome by the H^+ affinity of the membrane-bound FA.

3. Nucleotide binding

3.1. General

Binding of nucleotides is the best defined biochemical function of UCP1. UCP1 shares this ability with numerous other proteins as the most common type of protein–ligand interaction. Nucleotide binding has been the key to identify [41–43] and to isolate [5,6] UCP1. In view of these facts it is surprising that, besides in our group, there has been only marginal research effort on nucleotide binding. As a result, the

nucleotide binding per se is not a controversial issue. However, the physiological relevance of a regulation of uncoupling by nucleotide has been widely questioned by ignoring some of the established facts of nucleotide binding. In the following we will try to elucidate the importance of nucleotide binding studies in defining the function of UCP and the relevance of the intricate fine tuning of nucleotide–UCP interaction for the physiological regulation of uncoupling. Finally we maintain that nucleotide binding and inhibition of H^+ transport is universal to all UCPs.

3.2. UCP1 versus AAC

There are several unusual features governing the nucleotide binding to UCP1, the high pH dependence [44,45], the slow binding rate [46] and the distinction between the loose and tight binding [47]. The strong pH dependence is a striking feature, probably of great importance in regulating nucleotide binding and thus indirectly the H^+-translocating activity of UCP1. This was first noted in mitochondria [42,48] and then studied in great detail with isolated UCP1 [5,44,45,49,50]. Detailed measurements of the binding properties with various nucleotides were accomplished with isolated UCP1. Whereas with labeled nucleotides the binding affinity was measured using the ion exchange method [51], fluorescent nucleotides derivatives were used to measure the kinetics of binding and dissociation and the affinity of other nucleotides by competition with the fluorescent deviations [45]. Both are important for understanding the shift of UCP1 from the inhibited nucleotide occupied to the active free state; as we have discussed above in the analysis of the masking phenomenon.

UCP1 accepts with strong preference the purine ribose di- and tri-nucleotides: GTP, GDP, ATP and ADP ($pK_D = 6$ to 7) whereas the monophosphates GMP and AMP are poor ligands which bind with over 50-fold lower affinities [44]. UCP1 accepts only the free forms of nucleotides not the Mg^{2+} complexes [44,45,52]. The facilitation by Mg^{2+} of phosphate anhydride cleavage is not required in UCP1 and thus nucleotide binding cannot be compared to the vast kingdom of nucleotide–protein interaction in which nucleotide phosphates undergo cleavage or synthesis. They have an entirely different structural motif not found in UCP1. There are several similarities but also interesting differences to the nucleotide interactions with another carrier, the binding member of the mitochondrial carrier families AAC [53–56]. The AAC interacts also only with purine nucleotides but with much higher preference to the adenine over guanine nucleotides and also does not require Mg^{2+} [57]. However, the binding affinity is much lower, $K_D > 10$ μM. In the AAC nucleotides interact from both sides of the membrane, whereas in UCP1 only from the cytosol. In the AAC, ADP and ATP trigger the translocation i.e. they are activators, whereas in UCP they are inhibitors of H^+ and Cl^- transport. In the AAC the loose binding is the substractive difference between a strong binding interactive energy ($-\Delta G_B$) and the large conformation change for the translocation ($+\Delta G_T$), whereas in UCP1 the binding energy is compensated only by a small conformation change of the proteins. In the AAC the best fit of the binding site is induced by ADP and ATP, whereas in UCP the binding site is well prepared to accept nucleotides. The larger tolerance towards nucleotides of UCP indicates that the intrinsic binding energy is lower than in the AAC [45,54,58,59].

3.3. pH dependence

The nucleotide binding to UCP1 reveals some peculiar features which we will highlight here. Most prominent and relevant for regulation is the unique pH dependence. It is the result of a intricate interaction of several ionizing groups with each other and with the nucleotide [44,45]. This network is imposing a different pH dependence on the nucleoside di- and triphosphate binding. It has been highly rewarding to identify in a second phase of research the existence of the postulated residues involved in this pH dependence within the amino acid sequence of UCP1. The pH dependency is illustrated in the pK_D/pH curve [44]. Binding of both nucleoside triphosphate (NTP) and nucleoside diphosphate (NDP) exhibit a pH dependence but with well defined differences. The affinity (pK_D) decreases with pH, with a 'break' in the pK_D/pH curve at around 6.8 to the downside. The slopes ($\Delta pK_D/\Delta pH$) change from -0.2 to -1. Above pH 7.2, the slope becomes steeper at -2 with NTP but not with NDP. The whole curve can be analyzed quantitatively according to a binding

model involving the H^+ dissociation at the nucleotide terminal phosphate (pKa$\approx$6.5) and two H^+ binding groups in UCP1 with p$K\approx$4 and 7.2. On the basis of these observations, it was proposed that a regulatory carboxyl group with a pK$\approx$4 forms an ion pair in the dissociated form with a positive group at the entrance of the phosphate moiety binding cleft. On protonization, the gate opens and the pocket becomes accessible for the phosphate moiety binding. This regulatory carboxyl group was later identified as E190 using Woodward reagent K [60] and site-directed mutagenesis (see Fig. 2) [11].

Further, a second residue, with pK=7.2 proposed to be a His, contributes to the pH dependence for NTP binding only. Upon protonation of this residue, the binding center is assumed to be deepened to accommodate the γ-phosphate moiety of NTP. Thus, superimposed to the protonation of E190, the affinity for NTP declines with a slope of −2 on the pK_D/pH plot. This model was vindicated by the identification of this residue as H214 using site-directed mutagenesis [61] (see below). In the ionized state, H214H^+ is visualized to be withdrawn from the phosphate binding pocket by a background negative residue, probably D209 and D210 to allow insertion of the γ-phosphate of NTP (see Fig. 2). The low pH dependence of only NTP observed upon neutralization of D209 and D210 corresponds to those of the postulated background negative charge that regulate H214 [23]. This adjacent carboxyl pair provides an interesting variant to a single carboxyl group as a background charge. The pair is able to maintain on average at least one negative charge down to or even below pH 4.0. This explains the observation that no additional pK_D for the best fitting of pK_D/pH plots of NTP binding has emerged, which should be expected from the dissociation of the background carboxyl group.

3.4. Two stages (loose and tight) binding

A discrepancy between the rates of binding measured by ^{14}C-nucleotides with the anion exchanger method and by following the fluorescence increase with fluorescent nucleotide derivatives, was finally resolved by assuming a binding in two different binding affinity stages [46,47]. First a rapidly attained loosely binding state and then at an about 10^3 slower transition rate into the tightly binding state. The fluorescence method detects the changes between

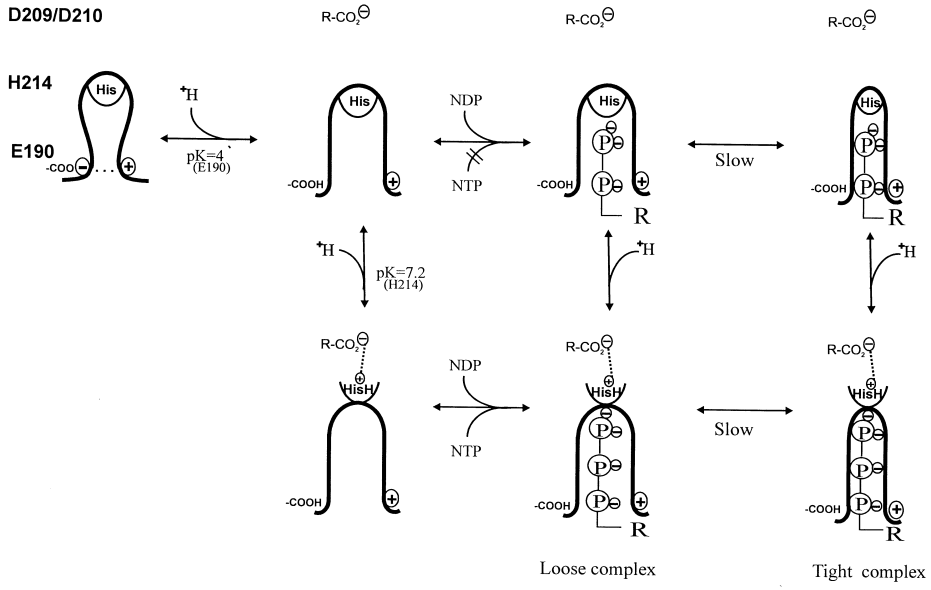

Fig. 2. The nucleotide binding model in UCP1. The regulation of nucleotide binding in the phosphate moiety binding pocket by H^+ acceptor groups. Different pocket size for NDP and triphosphate regulated by the protonization of His-214. In the upper row only NDP binds since His blocks the niche for the γ-phosphate. In the lower row NTP can bind, because His H^+ is retracted by a negative background charge (D209, D210). In both cases the entrance to the binding pocket is controlled by the protonization of Glu-190. Initial binding to the loose complex is fast, followed by a slow transition into the tight complex.

the two states, but the anion exchanger measures only the tightly bound nucleotide. The loose state can be nearly fully arrested with 3′-(2′)-*O*-[5-(dimethylamino)naphthalene-1-sulfonyl] nucleotide (DAN-nucleotide) and partially with 2′-*O*-[5-(dimethylamino)naphthalene-1-sulfonyl] nucleotide (Dansyl-nucleotide) [45].

The tight state correlates with the inhibition of H^+ and Cl^- transport. The four purine nucleotides, ADP, ATP, GDP and GTP all attain tight binding and inhibition, but Dansyl-ATP and Dansyl-ADP attain partial, and DANATP no inhibition although they can saturate the binding site of UCP1 [45]. The slow transition into the tight state indicates a major conformational change. A structural tightening as reflected in the tryptic cleavage of the C-terminal peptide correlates well with the ability to inhibit H^+ transport [45,58]. The very slow rate of transition into the tight binding state is an unusual feature of UCP1. It may reflect a complicated rearrangement within UCP1 which requires the coordination between the two subunits within the cooperatively operating dimer. Yet, the slow transition between the active and inactive states should be sufficiently fast for the onset of thermogenesis.

3.5. *The masking–unmasking phenomenon of UCP1 in BAT mitochondria*

Nucleotide binding to UCP1 has been measured first on mitochondria and by using the covalent label 8-azido-^{32}P-ATP the existence of a nucleotide binding protein was identified [42]. It was observed in some laboratories that the binding capacity of the mitochondria varied with short term changes of the temperature exposure [8,62,63]. On the transfer of rats from 27 to 4°C for only 20 min, GDP binding increased 1.3-fold [64]. Reversibly on short term warm adaptation the binding was masked. Since no de novo synthesis occurred in this short time the results suggested an 'unmasking' of binding sites. This acute unmasking could also be achieved with norepinephrine and β-adrenergic receptor agonists [65–68]. The effect has raised broad interest and speculation about its nature.

The unmasking phenomenon has distorted the binding characteristic of UCP1 in mitochondria [13,48,69,70]. Thus a nonlinear mass action plot indicated the existence of high and low affinity binding sites. The pH dependence of binding is flattened as compared to the isolated UCP1 [44,45]. All these phenomena can be explained by evidence that UCP1 in mitochondria is partially occupied by endogenous ATP [25]. As shown by measurements of nucleotides binding with isolated UCP1, ATP binds tightly to UCP1 and dissociates extremely slowly, whereas ADP dissociates somewhat faster [45]. In the isolated mitochondria UCP1 could be completely unmasked by treatment with Dowex at high pH [25]. In those mitochondria a high binding capacity with only high affinity, i.e. a linear mass action plot was measured. Further the pH dependency increased to the level of the isolated protein. The nonlinearity i.e. high and low affinity binding could now be explained as an artifact of a brief incubation with the nucleotide, since the binding becomes retarded by the bound ATP [25]. The retardation is stronger at low pH and therefore the affinity and binding will be more distorted at lower pH, and as a result the pH dependence is partially masked.

Depending on the energy state of the BAT, after isolation of the mitochondria UCP1 will be more or less occupied by ATP. In the cold, due to β-adrenergic stimulation of FA release and subsequent FA activation, the ATP level is decreased and UCP1 binding sites are unmasked. This 'unmasking' of the masking–unmasking phenomenon has important consequences for understanding the regulation of uncoupling. The variations of the masking by endogenous ATP shows that in BAT cells UCP1 is actually regulated by ATP binding. It further shows that the binding characteristics established for isolated UCP1 are valid for UCP1 in situ. The intricate control of binding affinity by pH, the difference between ADP and ATP binding are all highly relevant for the in vivo regulation of UCP1. However, in a strange development, these most obvious types of regulation of UCP1 has been ignored, as we will discuss below.

4. Site-directed mutagenesis of UCP1

Replacement of residues, in particular of charged amino acids, by neutralization of the charges, have been very fruitful in UCP1 (see Fig. 3). In particular the unique pH dependency of nucleotide binding has

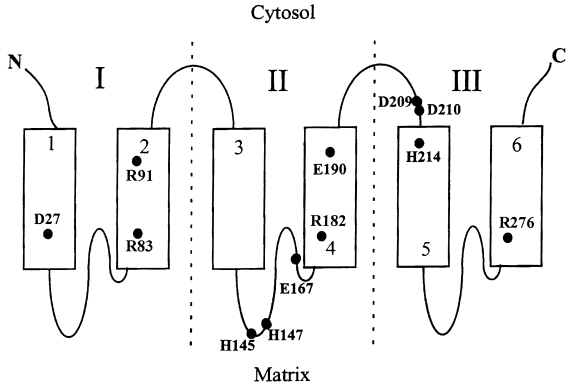

Fig. 3. Localization of the mutagenized residues (discussed in the text) in hamster UCP1. In all cases the charged residues were replaced by neutral residues.

been a source for associating several residues with a defined function [11,23,61]. A survey of the residues mutagenized in our laboratory is given in Table 1. The presentation is simplified in giving only an indication which of the four functions are affected. Under 'binding' a compounded evaluation of the K_D and rate measurement is given. The effect of these mutations is significant for several contested issues; the mechanism of H^+ transport, i.e. the involvement of internal H^+ transfer groups, the relation of H^+ and Cl^- transport, i.e. the question of whether anion transport ability is necessary for H^+ transport and the role of intrahelical arginines. Uncontested are the issues, elaborated here by mutagenesis, that the nucleotide binding site is not overlapping with the H^+ and Cl^- transport channel, and the machinery of pH control of nucleotide binding.

A striking residue common to all known members of the UCP subfamily is the intrahelical D27 not found in other mitochondrial carriers. Interestingly, in the AAC at the homologous position a lysine is located and conserved in all AAC known so far [71,72]. Neutralization of D27 inhibits partially H^+ transport but not Cl^- transport and nucleotide binding [23]. The same pattern but to a different level, i.e. inhibition of only H^+ transport, is observed on mutagenic neutralization of the His pair H145 and H147 located at the matrix side in the hydrophilic loop of the second repeat domain [40]. This His pair occurs only in UCP1, whereas in UCP2 both histidines are missing and H145 is in a homologous position replaced by arginine and in UCP3 only the homolog to H145 is retained. We therefore suggested that in UCP2 and 3 H^+ transport is absent or different, possibly with a modified FA interaction. In BAT cells UCP1 can be assumed to operate under much lower $\Delta\psi$ than UCP2 and 3 in their respective cells, because BAT is geared for thermogenesis. The His pair may be required to enable efficient delivery of

Table 1
Summary of the results of some charge residues mutated in hamster UCP1

UCP1 mutants	transport activity		nucleotide	
	H^+ inhibition	Cl^- inhibition	binding	pH dependence decreased for
D27N	+	−	−	
D27E	−	−	−	
E190Q	−	−	−	NDP, NTP
H145Q	+	−	−	
H147N	+	−	−	
H145Q+H147N	+	−	−	
H145R+H147R	+	−	−	
H214N	−	−	−	NTP
H214W	−	−	−	NTP
D209N	−	−	−	NTP
D210N	+	−	−	NTP
D209N+D210N	+	−	−	NTP
E167Q	−	+	−	
R83I	−	+	+	
R91T	−	+	−	NDP, NTP
R182Q	−	−	+	
R276Q	−	−	+	

+, affected by mutation; −, NOT affected by mutation (same as wt).

H^+ into the matrix because of the low $\Delta\psi$ under which UCP1 works in BAT. An attractive mechanism would be, that on H^+ uptake the $HisH^+$ are repulsed more into the matrix where they deliver H^+ and than flip back as His for another H^+ delivery cycle. On the cytosolic side D210 presents a carboxylic group apparently important for H^+ transport [23]. As shown above it is also involved in the pH control of NTP binding. In an interesting proposition D210 would be the link between H^+ uptake and activation of UCP1 by triggering release of NTP. In conclusion, the existence of H^+ donor–acceptor groups in UCP1 apparently involved in H^+ transport are in line with a H^+ transport mechanism in which FA are cooperating with these groups as a H^+ donor–acceptor.

The independence of H^+ and Cl^- transport is substantiated by those mutations, where Cl^- transport is inactivated but H^+ transport is retained. These are E167Q in the second domain and the two arginines R83I and R91T both within the second helix. The mutants show that the ability to transport anions is not a prerequisite for the FA dependent H^+ transport, as implied in the FA anion cycle model of H^+ transport.

Mutations which affected only the pH dependency of nucleotide binding were most rewarding. As mentioned above, they elegantly confirmed the postulates derived from the pH dependence of nucleotide binding. The gate keeping carboxyl groups controlling both NDP and NTP binding was identified as E190 [11]. In E190Q the pH dependence was much less pronounced, such that at $pH > 7$ the affinity increased over the wt. The predicted His group, controlling NTP binding only, was also identified as H214 [61]. In H214N, the pH dependency of binding was decreased only for NTP and not for NDP. But, different from the prediction, affinity was not increased. Thus we reinterpreted the pH control mechanism of H214 by assuming that the positive charge of H214 is not used for binding the γ-phosphate of NTP but is used to retract $H214H^+$ from the binding pocket by a negative background charge (D209, D210) to make space for the γ-phosphate. This model was supported by the H214W mutant with a more bulky substituent, where the affinity is strongly decreased for NTP only [61].

Mutational neutralization of intrahelical arginines all resulted in loss of binding (as in R83I, R182Q and R276Q) or decreased binding affinity (as in R91T) whereas H^+ transport was not affected (manuscript in preparation). Our observation of the loss of binding in R83I and R182Q are in agreement, whereas the results with the last intrahelical R276 are at variance with those described by Modriansky et al. [73]. They report the paradoxical case that in R276Q the nucleotide binding is retained but the inhibition of H^+ transport abolished.

5. Intracellular regulation of UCP1

The physiological regulation of UCP is still a matter of uncertainty and divergent discussion. There is only fragmentary knowledge of the conditions under which UCP1 operates in BAT. We have maintained that the regulatory properties of UCP1 studied with the isolated protein and in reconstituted proteoliposomes have a physiological potential [3,74]. Nevertheless the control by nucleotide has been dismissed as not relevant for the physiological regulation of UCP1 because, due to the high binding affinity and the high cytosolic nucleotide concentration UCP1, should be constantly blocked. At the same time the pH control of nucleotide binding is ignored or dismissed. We ask emphatically, why would UCP1 have been equipped with an elaborate system of inhibition of uncoupling by nucleotides and of controlling this nucleotide binding by pH, if not for important physiological reasons.

First Nicholls postulated that uncoupling by UCP1 is regulated only by FA but not by nucleotides [13]. The H^+ conductivity–$\Delta\psi$ relationship in BAT mitochondria in the presence of GDP is nonlinear and shifted to a lower threshold value by FA. The downshift by FA is stronger in BAT than in liver mitochondria and thus a participation of the GDP-saturated UCP1 was invoked. However, with reconstituted UCP1 H^+ transport could not be markedly activated by FA in the presence of GDP, in particular, when vesicles were loaded inside and outside with GTP [17]. There is also no competition of FA with nucleotide binding. We therefore interpret the effect of FA in mitochondria as a H^+ leak not linked to UCP1, which is enhanced in BAT mitochondria for some unknown reason. We argue that the UCP1-

linked uncoupling is only possible on release of nucleotide binding where the H^+ transport has a linear dependence on $\Delta\psi$. More recently the same authors argued that even FA may have no regulatory influence, based on experiments with UCP1 expressing yeast mitochondria [15]. Here even in the absence of FA a small GDP sensitive uncoupling was observed. The authors state that the FA independent uncoupling can only be observed in mitochondria with high $\Delta\psi$ but not in reconstituted vesicles with a lower steady state $\Delta\psi$. Although these results are significant for interpreting the mechanism of FA activation, they are not relevant for the in vivo uncoupling.

Recently we have performed experiments to understand the in vivo regulation of UCP1 expressed in yeast cells. By comparing wt UCP1 with the intrahelical arginine mutants of UCP1, we were able to elucidate the influence of nucleotide binding. The cell growth on lactate was taken as a measure for the uncoupling. Cell growth was inhibited in the arginine mutants where nucleotide binding is abolished, but only when FA (2Br-palmitate) were present (manuscript in preparation). The results show the importance of nucleotide inhibition and the necessity for abolishing nucleotide binding for uncoupling. They further show that FA are necessary for the uncoupling in vivo. We feel that the response of UCP1 in yeast cells is a valid although limited model for the regulation in BAT.

By comparing BAT mitochondria from wt with UCP1-ablated mice, Matthias et al. [16] arrived also at the conclusion that FA do not control uncoupling by UCP1. Again this work is based on the assumption that in vivo UCP1 is always saturated with nucleotides. Thus it is not surprising, that they find equal uncoupling by FA in wt and UCP1-deficient mitochondria. However, they suggest the existence of another intracellular factor which stimulates uncoupling in BAT, which is missing in isolated mitochondria. They had earlier suggested that palmityl CoA might be this activator by displacing the nucleotide from UCP1 [75].

The major issue in these divergent views is the regulation by nucleotides. The assumption that the 'physiological' state in vivo is the UCP1 nucleotide complex is unfounded on closer scrutiny. Only the concentration of 'free' ATP, and not of the Mg^{2+}–ATP complex is relevant for the binding to UCP1. For example with 5 mM ATP and 5 mM free Mg^{2+}, there is only $\approx$0.1 mM free ATP. The release of nucleotides by pH increase requires comments. Originally only an unimpressive decrease of GDP binding to BAT mitochondria with pH was noted and this contributed to ignore the physiological role of the pH in regulating UCP1. However, the binding to mitochondria is distorted by the masking phenomenon, as explained above. The detailed binding studies with isolated UCP revealed a much steeper pH control than in mitochondria [25]. Most impressively the affinity for ATP decreases above pH 7.2 still steeper, so that with $\Delta pH = 0.1$ the affinity is reduced nearly 10-fold [44,45]. At the same time the concentration of free ATP (ATP^{4-} and $ATPH^{3-}$) decreases in favor of an increased Mg^{2+}–ATP complex.

The ATP concentration is lowered by the norepinephrine-induced lipolysis and ensuing FA activation both in the cytosol and intramembrane space (see Fig. 4). The small cytosolic volume favors ATP depletion [76,77]. Moreover the intramembrane thiokinase may further contribute to ATP decrease near UCP1. The generated AMP is unable to exchange with intramitochondrial ADP and ATP. Also the limited passage of ATP through porin of the outer membrane may slow a replenishment from the cytosol. In this context again the early discussed role of palmityl CoA might be considered as a nucleotide displacing agent [75]. Strielemann and Shrago [78] were able to partially activate nucleotide inhibited Cl^- transport in BAT mitochondria as a model for H^+ transport. These results require confirmation with direct measurement of H^+ transport.

How can the pH increase be accomplished? Early by Chinet et al. [79] an alkalinization of the cytosol in BAT was noted in connection with the thermogenetic activation of BAT. Possibly the pH increases by a pH controlling mechanism at the plasma membrane, e.g. Na^+–H^+ exchanger in a relay to the norepinephrine signal. An additional contribution may be the uptake of the freshly generated FA into the mitochondria matrix which consumes cytosolic H^+. At pH 8.0 for ATP, a $K_D = 30$ μM has been determined [44,45]. Thus at 30 μM ATP half of the UCP1 would be free. Even this partial uncoupling capacity is in large excess of the H^+ production by the respiratory chain. Thus with a low $\Delta\psi$ and low FA con-

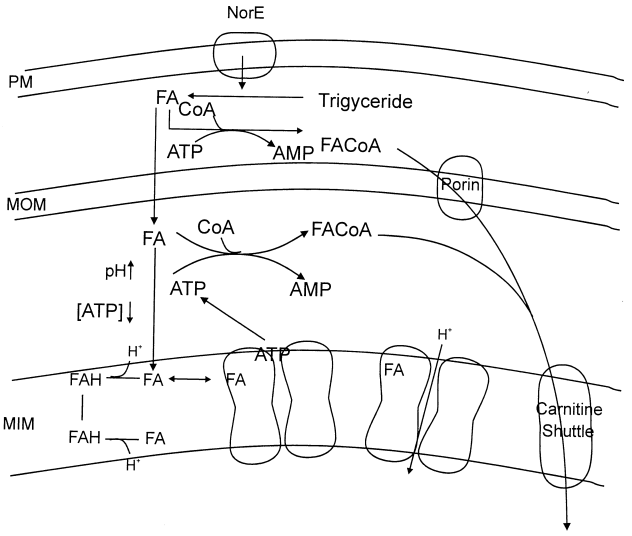

Fig. 4. The intracellular activation of UCP1. The role of FA and nucleotides in the activation process. NorE = norepinephrine, PM = plasma membrane, MOM = mitochondrial outer membrane, MIM = mitochondrial inner membrane (see text for details).

centration and only partial nucleotide relief, the H^+ conduction of UCP1 can still match the in vivo uncoupling requirements. We conclude that the in vivo regulation of UCP1 activity by nucleotides and by FA is feasible and logical. No shift by FA of the nonlinear response to $\Delta\psi$ and no additional cytosolic activating factor has to be involved, which were invoked under the presumption that UCP1 is permanently blocked by nucleotides [13,16].

In this context the question arises, as to why the BAT mitochondria are equipped with such an apparent excess of UCP1, amounting to 2.5 UCP1 dimers per cytochrome aa_3 complex [5]. This corresponds to a ratio of one active UCP1 per H^+ pumping electron transport complex. Obviously there must be important reasons why the in vivo activity of UCP1 is much below its H^+ transport capacity. Uncoupling is an extremely dangerous activity and must be dealt with delicately in order to maintain cell vitality. This may be achieved only by a subtle activation of H^+ transport where UCP1 works only to a few percent of capacity. Thus a given uncoupling requires more UCP1 molecules operating at low capacity. As a result an even distribution of uncoupling, i.e. of $\Delta\psi$ decrease over the cristae membrane is achieved due to the possible association of one UCP1 with each H^+ pumping electron transport complex. With less UCP1 molecules uncoupling would be more punctual and cause drastic local decreases of $\Delta\psi$ around UCP1 which may be unfavorable for the membrane stability.

6. UCP variants, UCP2 and UCP3

The similarities of the amino acid sequence of UCP2 and UCP3 with UCP1 suggested that these proteins have an uncoupling function. We noted that residues shown to be involved in nucleotide binding and pH regulation of binding are conserved in UCP2 and 3, whereas those important of H^+ transport in UCP1 are only partially retained in UCP2 and UCP3 [80–84]. This leads us to postulate that any uncoupling in UCP2 and 3 must be also fully inhibited by nucleotides as in UCP1. An uncoupling function seemed to be confirmed by expressing these proteins in yeast cells and measuring the response to membrane potential probes by the FACS method or by O_2 consumption [80,84–92]. Surprisingly in yeast cells expression of UCP2 and UCP3 but not of UCP1 seemed to uncouple. How could this discrepancy between UCP1 and UCP2 and 3 be explained? An uncertainty with the FACS method is the inability to differentiate between uncoupling and a decrease of the mitochondria content. The dilemma was exacerbated by two developments: (a) The numerous studies on the regulation of expression of UCP2 and UCP3 in various organs [89,90,93]. Divergent results were obtained on the induction of UCP2 and UCP3 expression by cold exposure, food limitation, exercise, leptin, thyroxine etc. which seemed not to be fully consistent with an uncoupling function of UCP3 and suggested other roles such as FA import, etc. (see reviews [89,90]). (b) The studies with isolated mitochondria from yeast added to the confusion. By measuring respiration of mitochondria from UCP3 expressing yeast cells, several groups find an increased uncoupling which is insensitive to inhibition by nucleotides. FA, if at all, only very poorly stimulates respiration of mitochondria both from UCP2 and UCP3 expressing yeast cells. The authors conclude that the physiological regulation of uncoupling by UCP3 due to the lack of inhibition by nucleotides is different from UCP1 [86,88,91,92].

In contrast, we concluded that the uncoupling ef-

fects found in yeast mitochondria do not reflect the normal function of UCP3 in skeletal muscle. Our studies with UCP3 expressing yeast cells showed strongly decreased oxidative phosphorylation as compared to UCP1 expressing cells. In UCP3 containing yeast mitochondria the fluorescent probe indicated a low $\Delta\Psi$ which responded poorly to FA and nucleotides in contrast to the UCP1 containing yeast mitochondria. Also FA dependent H^+ transport in mitochondria was poorer in UCP3 than in UCP1 containing mitochondria. The results were rationalized by assuming that different from UCP1, UCP3 incorporated into the yeast mitochondria does not obtain the native function. It exists largely in a deranged conformation which forms in the inner membrane an unregulated pore for a FA independent H^+ transport.

This conclusion is consistent with the following facts. (a) In UCP3 most of those residues are present which have been identified in UCP1 to participate in the nucleotide binding and in the regulation of binding. (b) The short form UCP3s facilitate unregulated H^+-transport as UCP3. The amputated protein with only five helices should be barely able to form a stable functionally competent conformation and thus exist in a deranged folding. (c) The unregulated uncoupling is highly variable, as observed by comparing the uncoupling of human and murine UCP3 in yeast cells [95]. A subtle difference in the amino acid composition may influence an unstable deranged conformation more than a definitive native conformation. (d) Both UCP3 and UCP1 expressed in inclusion bodies of *E. coli* and reconstituted into vesicles are able to catalyze Cl^- but not H^+-transport [32]. Cl^- transport is inhibited by nucleotides with a $K_I < 1$ μM both for UCP3 and UCP1. The absence of H^+-transport is attributed to the lack of a cofactor in the reconstitution from the inclusion bodies. The H^+-transport activity reported recently by Jaburek et al. [33] does not contradict our results, since the activity is about 30 times lower than with native UCP1. In our studies we have therefore dismissed the same observation as unspecific H^+ leakage in the reconstituted vesicles. Hinz et al. [86] argue that the conformation of UCP3 in reconstituted system has an artificially increased affinity for nucleotides. Such a reasoning would be opposite to the generally accepted criteria that a more native folding

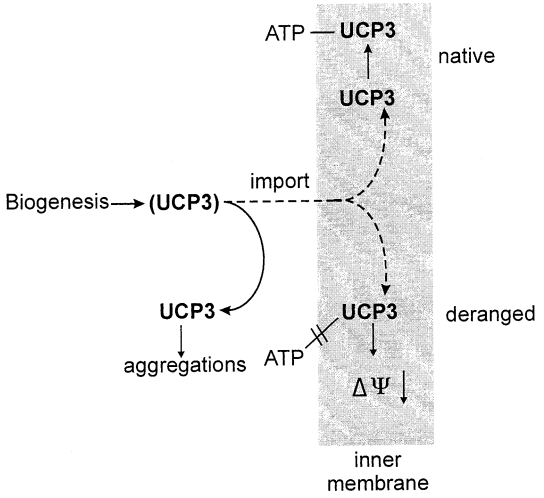

Fig. 5. The state of human UCP3 expressed in yeast. Initially some UCP3 is imported into the inner membrane of yeast mitochondria. A part is folded into the native state and blocked by ATP, similar to UCP1. Another possibly larger portion is, after insertion in the inner membrane, in a deranged conformation which cannot bind ATP. It causes uncontrolled H^+ leakage and thus largely uncouples the mitochondria in the yeast cells. As a result the low $\Delta\psi$ prevents uptake of further synthesized UCP3 so that the majority ($>90\%$) of UCP3 remains outside the mitochondria in an aggregated state.

of a protein creates a function such as a specific nucleotide binding site. (e) The work of Boss et al. [94] shows that overexpression of UCP3 in myoblasts produces a much smaller downshift in FACS than in yeast cells. Obviously on expression in natural mitochondria, UCP3 is in a native state where its uncoupling capacity is largely inhibited, i.e. regulated by internal nucleotides and FA. (f) Finally evidence from our laboratory (in preparation) shows that most of UCP3 expressed in yeast is not incorporated into mitochondria, but exists in inclusion body type aggregates. Apparently UCP3 is largely rejected from the yeast mitochondria because of the unregulated uncoupling by low amounts of incorporated but deranged UCP3, which inhibits the further UCP3 uptake (see Fig. 5).

Some clarifications are required concerning the recent report of a H^+ transport by UCP2 and 3 reconstituted from inclusion bodies expressed in *E. coli* [33]. The maximum H^+ transport rates are given as 10–30 μmol/(min mg) protein. Unfortunately the authors do not specify the temperature although this is critical for rate measurements. We assume room tem-

perature (25°C) whereas our measurements rates are at 10°C. Consistently we have measured H^+ transport in UCP proteoliposomes at reduced temperature, because at room temperature the high rates could not be resolved. Since with the 15°C temperature difference the rate changes about three-fold, and our H^+ transport rate was 6 μmol/(min mg) which would be 18 at room temperature and thus similar to the reported rate UCP2 and 3. This basic rate was not inhibited by 100 μM ATP and therefore must be classified as an unspecific leakage. The observed H^+ transport may be due to the ternary FA–valinomycin–K^+ complex diffusion induced by the K^+ gradient. With UCP some detergent is introduced to the proteoliposome which may enhance the ternary complex diffusion. Further an inhibition by 1 mM ATP is unphysiological, since in the cell occur only between 10 and 100 μM free ATP (not complexed with Mg^{2+}). A striking inconsistency in this work was to compare reconstituted UCP1 from mitochondria with UCP2 and 3 from *E. coli* when stressing that UCP1 is much more sensitive to nucleotides than UCP2 and UCP3. UCP1 reconstituted from *E. coli* has the same inability as UCP3 of regulated H^+ transport activity [32].

We may conclude that at present there is no evidence that UCP2 and 3 do not follow the fundamental transport and regulatory functions of the paradigm UCP1. The expression in the yeast system is misleading because of deranged expression of UCP2 and 3. The expression in *E. coli* leads to an incomplete reconstitution as shown by the UCP1. Therefore the evidence for a nucleotide insensitive H^+ transport of UCP2 and 3 is inconclusive.

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