Review

Structure and function of the uncoupling protein from brown adipose tissue

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Abbreviations: UCP, uncoupling protein; BAT, brown adipose tissue; AAC, ADP/ATP carrier; FA, fatty acid
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1. Introduction

Thermogenesis in brown adipose tissue (BAT) is attributed to uncoupling of respiration which is caused by a specific component located in the inner mitochondrial membrane, the uncoupling protein (UCP-1). This review is confined to the uncoupling protein (UCP-1) from BAT. We summarize the present status of our knowledge of the structure, the characteristics of this membrane protein, the function, the regulation of function, the interaction with ligands, and the structure–function relationships as elucidated by specific reagents and by mutagenesis. Previous reviews [1,2] have also dealt with these subjects but the rapid development in this field asks for a new review. A recent brief survey deals primarily with the work from the Ricquier group [3]. The present article will not treat in detail the physiology of UCP-1 in thermogenesis, the regulation in vivo, the regulation of its expression by temperature and other factors, the regulation of activity by hormones and the signal transduction. Here excellent overviews are available in the literature [2–4]. Recently, genes have been described coding for uncoupling protein isoforms (UCP-2 and UCP-3) which are expressed also in tissues other than the BAT as deduced from their mRNA distribution. These can be linked more to obesity than thermogenesis [5–8]. However, there does not yet exist a biochemistry of these uncoupling proteins to speak of and therefore they will not be treated here.

The biochemistry of UCP-1 started with the isolation of the intact nucleotide binding protein from BAT mitochondria [9,10]. This advance was based on the finding that BAT mitochondria could be recoupled by GDP [11]. Further a GDP binding site on the outer face of the inner membrane was found [12,13]. These results suggested that a nucleotide binding protein was responsible for the uncoupling. Subsequently, by photoaffinity labeling of BAT mitochondria with azido [γ-32P]ATP, a 32 kDa component was identified in SDS gels [14]. Since the ATP binding capability and the molecular mass were similar to the ADP/ATP carrier (AAC), the isolation of this nucleotide binding protein from hamster BAT mitochondria was initiated [9] under the working hypothesis that it has a similar structure as the AAC. Thus, essentially the same isolation methods (e.g., use of non-ionic detergents, hydroxyapatite step) previously developed for the isolation of the first intact transporter, the AAC [15], were successfully applied for the isolation of a protein from BAT mitochondria which retained the nucleotide binding capability [9,10].

In view of its putative uncoupling function we christened this protein ‘uncoupling protein’ [9]. In part of the literature (see, e.g., [2]) the same protein was also called ‘thermogenin’. The primary structure elucidated from the isolated hamster UCP-1 by amino acid sequencing [16] vindicated the working assumption of the similarity to AAC. The sequence similarity of UCP-1 not only with AAC but also with the subsequently sequenced mitochondrial phosphate carrier [17] led us to postulate the existence of a homologous mitochondrial carrier family. This view has been confirmed by the elucidation of further mitochondrial carriers (reviewed in [18–21]). In the meantime the sequences of UCP-1 from numerous mammalian sources are known [22–27]. UCP-1 together with AAC and PiC forms a functional subgroup of carriers linked to energy transfer in mitochondria. It is remarkable that similar proteins deal with the transport of solutes which have an extremely different size, ATP and ADP being about the largest and H+ the smallest transported solute [28].
2. Structure

UCP-1 from hamster consists of 306 amino acids with a calculated molecular mass of 33.2 kDa. Of these there are 28 basic and 19 acidic residues, resulting in an excess of 9 positive charges. An excess of basic residues is also found in other mitochondrial carriers such as the bovine heart ADP/ATP carrier (+19 charges) [17] and phosphate carrier (+12 charges) [17,29]. Presumably some of the positive charges are necessary for proper anchorage to the phospholipid especially cardiolipin environment.

UCP-1 has been isolated using the non-ionic Triton X-100. This procedure yielded functionally intact protein as shown by its nucleotide binding capability [9] and H\(^+\) transport activity in the reconstituted system [30]. In this soluble form, UCP-1 exists in a large micellar structure with Triton X-100 and remnant phospholipids. A homodimer structure (M\(_r\) 66 kDa) encircled by about 160 Triton molecules was evaluated from analytical ultracentrifugation [31]. Evidence for the homodimer structure was also obtained from a ‘half-site reactivity’ nucleotide binding capacity of 16 \(\mu\)mol GDP/g protein [10], and from cross-linking experiments of UCP-1 as the isolated protein [32] and in mitochondria [33]. The similar Triton-protein micellar structures of UCP-1 and of AAC [34] explain the striking passage of these proteins in the intact state through hydroxyapatite. The homodimer structure for UCP-1 is in line with an early developed concept [35] that membrane proteins form preferentially oligomers, in particular dimers with the twofold axis perpendicular to the membrane. This principle was first inferred from the AAC structure [34] and has since been proven to hold for many membrane proteins involved in solute transport and also for the electron transport complexes. Recently the importance of the homodimer structure for transport function was elegantly demonstrated with the mitochondrial phosphate carrier [36], which is a homolog to UCP-1.

Based on the primary structure, a folding model of UCP-1 was derived, which in its simplest form contains six transmembrane \(\alpha\)-helices [16,17]. A structural model employing our present view is given in Fig. 1. The hydrophobicity profile delineates transmembrane helices in UCP-1 only poorly because of the intrahelical position of some charged residues. Here we define tentatively the transmembrane helices as being terminated by the characteristically repetitive charged residues, in particular acidic groups. The simple six transmembrane model first hypothesized for the AAC [37] is to be modified. According to the evidence presented below not only for AAC but also for UCP-1, intermembrane loops protrude from the matrix side into the membrane region. In the original hydrophobicity analysis, even an amphipathic \(\beta\)-chain was predicted based on strikingly alternating amphipathic patterns comprising 12 residues from 36 to 48 on the matrix side [16]. The meaning of this interesting finding remains obscure at present. Circular dichroism spectra (Appel and Klingenberg, unpublished data) and infrared spectroscopy [38] indicate a 40% to 50% \(\alpha\)-helical content of UCP-1 in agreement with at least six transmembrane helices.

A striking feature is the tripartite structure which UCP-1 shares with the other members of the mitochondrial carrier family [17,37]. UCP-1 is divided up into three similar domains containing about 100 amino acid residues. Each domain comprises two transmembrane helices which are separated on the matrix side by a large, about 40 residues long highly hydrophilic stretch. It contains about 12 charged residues, which are arranged in a repetitive manner. A characteristic motif contained in UCP-1 common to the other family members is the sequence PX (−) XX (+) X (+) starting at the end of the first helix in each repeat domain [17,39]. Another typical feature is the occurrence of an arginine within the second helix in each repeat domain. Conspicuously located intrahelical negative residues specific to UCP-1 are an Asp in the first helix and a Glu near the end of the fourth helix. The function of these residues has been addressed by site-directed mutagenesis (see below).

Experimental evidence for the topology of UCP-1 folding indicates that both the C- and N-termini are directed towards the cytosol. Antibodies generated against the N-terminal sequence reacted in mitochondria indicating its protrusion towards the cytosol [40]. In mitochondria but not in submitochondrial particles a cysteine located near the C-terminal of UCP-1 is cross-linked by membrane impermeant Cu-phenanthroline [32]. A series of antibodies were generated against a random population of peptides expressed as MalE fusion protein. Antibodies against...
several peptides located on either the cytosol or the matrix side were collected and the location of these peptides on either side of the membrane was mapped [41]. Thus a fairly complete picture in agreement with the six transmembrane foldings was obtained, which is reflected in Fig. 1.

### 3. Transport by the uncoupling protein

#### 3.1. Mitochondria

After many years of pioneering research by several laboratories on the biochemical background of thermogenesis by BAT, the application of chemi-osmotic reasoning to BAT mitochondria by Nicholls and coworkers [42–45] contributed much to our understanding of the thermogenesis as an uncoupling caused by transport of $H^+$ through the inner mitochondrial membrane. The argument for a high $H^+$ permeability was based on osmotic swelling experiments with BAT mitochondria using $K^+$ acetate [42]. Massive $K^+$ acetate uptake into non-respiring mitochondria is facilitated by valinomycin-catalyzed $K^+$ uptake and the parallel diffusion of acetic acid coupled to the compensatory $H^+$ release through the inner mitochondrial membrane. The direction of $H^+$ transport in this case is opposite to the ‘uncoupling action’. This method was useful in defining a high $H^+$ and anion transport activity in BAT mitochondria. Also with this method the activation of $H^+$ transport by FA [46] and the inhibition by nucleotides were found [47,48]. In further studies only the uncoupling of the respiratory rate was used to
indirectly estimate the H\(^+\) reflux rate into the mito-
dochondria [48] and its dependence on various param-
eters [44, 46, 48–50]. However, the use of mitochon-
dria has its limitations for a detailed elucidation of
the regulatory factors of UCP-1 because of endoge-
 nous uncontrollable influences. For example, the un-
coupled respiratory rate is limited by the hydrogen
input from substrates and not by UCP-1 which can
have an up to 50-fold higher H\(^+\) transport capacity.

3.2. Reconstituted UCP-1

After isolation of UCP-1, it became desirable to
assay its function separately from the complex envi-
ronment in mitochondria and thus to elucidate vari-
ous factors which influence H\(^+\) transport and eventu-
ally the mechanism of the uncoupling action.
Reconstitution for functional studies was slow in
coming after the isolation of UCP-1, but it has
greatly extended our knowledge of kinetic param-
eters of H\(^+\) transport and of activating or inhibiting
factors.

The reconstitution recipe [30, 51–54] requires some
steps which differ from the isolation of UCP-1 for
other purposes and also from the reconstitution of
other mitochondrial carriers. Whereas UCP-1 iso-
lated with Triton X-100 is the most stable form, it
is not suitable for reconstitution since traces of Tri-
ton cannot be removed and cause leakiness of the
vesicles for H\(^+\) and Cl\(^-\). The use of C\(_{10}\)E\(_4\) for
UCP-1 was found to give the best results since it
does not delipidate UCP-1 as strongly as Triton X-
100 and is more easily removed. Among various
phospholipids, purified hen egg phosphatidylcholine
produced the tightest vesicles [30]. A stepwise remov-
al of the detergent with polystyrene beads yields rel-
avely large proteoliposomes with high activity.
With this procedure proteoliposomes can be pre-
pared either for H\(^+\) import or H\(^+\) export, by loading
with a high pH K\(^+\)-phosphate buffer (pH 8 to 9) or a
low pH Na\(^+\)-phosphate buffer (pH 6). H\(^+\) transport
is induced by valinomycin addition which generates a
K\(^+\) diffusion potential either positive outside or in-
side. Reconstitution was performed in at least three
laboratories with some variations of the described
procedures [30, 52, 55]. The H\(^+\) transport can be
measured by a pH electrode or by pH indicators,
such as pyranine [56, 57]. Indirectly H\(^+\) transport
has also been followed by using a fluorescent indica-
tor of the K\(^+\) flux [57]. In a typical experiment the
H\(^+\) flux is initiated by valinomycin until equilibrium
is reached according to the relation
\[
[H_\text{r}^+]/[H_\text{i}^+] = [K_\text{r}^+]/[K_\text{i}^+]
\]
assuming that the membrane is permeant only for
H\(^+\) and K\(^+\). Since Cl\(^-\) and other small anions are
also transported by UCP-1, their presence had to be
avoided in these measurements.

3.3. H\(^+\) transport and fatty acid requirement

H\(^+\) transport is generally accepted to be central to
the uncoupling activity of UCP-1, although there are
large differences in opinion of how UCP-1 facilitates
the H\(^+\) transport. The research on H\(^+\) transport
deals with the mechanism of H\(^+\) transport and
with the parameters governing the H\(^+\) transport rates
especially the requirement for FA, and the regulation
of H\(^+\) transport by purine nucleotides. H\(^+\) transport
by UCP-1 is dependent on the presence of medium to
long chain FA. This was first inferred from studies
with mitochondria [48, 49, 58]. Reconstituted vesicles
permitted a more detailed insight into the structural
requirements of FA for activation [59]. Further, the
dependence on the concentration and on the pH
could be studied in detail [59]. Once traces of endog-
enous FAs are eliminated by bovine serum albumin,
H\(^+\) transport in proteoliposomes becomes absolutely
dependent on the addition of FA [30, 54] whereas in
preparations of BAT mitochondria an active lipolysis
of adherent triglycerides may produce a constant
supply of FA. Further, phospholipase can produce
FA within the mitochondria. BSA may not be able
to catch FA taken up through the intimate contacts
between triglyceride globules and mitochondria.
Therefore even with BSA, the uncoupling activity
cannot be completely suppressed in mitochondria
[48, 60].

The dependence on the amount of palmitate re-
quired for uncoupling in mitochondria was deter-
mined using BSA as a FA buffer [60]. A \(K_m\) of
90 nM for palmitate was reported. However, we con-
sider this system too complex to permit this quanti-
tation. ATP and Mg\(^2+\) additions were required for
the respiration. It is obvious that with several coun-
teracting factors affecting UCP-1 activity, such as
Mg$^{2+}$, ATP, BSA and FA, the system is frail with uncertainties influencing UCP-1 activity. For example, ATP inhibits UCP-1 whereas excess Mg$^{2+}$ abolishes the inhibition. Also ATP, Mg$^{2+}$ interact with BSA and influence the FA binding. The true amounts of free FA may be much higher due to continuous lipolysis in mitochondria which cannot be completely removed by BSA. Thus the value of $K_{m}$ 90 nM for palmitate can be a too low estimate. Further, the uncoupling measurements relied on the indirect measurements of respiratory activity.

In reconstituted vesicles these complications are removed. The H$^+$ transport activity can be measured directly, avoiding the uncertainties of the H$^+$ transport capacity estimated from swelling and respiratory rate stimulation. The H$^+$ transport activity reaches 100 μmol (mg protein)$^{-1}$ min$^{-1}$ at 12°C. This value is 90 times higher than required for uncoupling of respiration as estimated from respiratory uncoupling in BAT mitochondria [48], taking into account that the mitochondria were measured at 25°C and contained 10% UCP-1 by protein weight. In vesicles

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### Table 1

The dependence of H$^+$ transport activation on the structure of fatty acids

<table>
<thead>
<tr>
<th>Fatty acids and derivatives</th>
<th>Number of hydrophobic carbons</th>
<th>Relative H$^+$ uptake rate $\Delta V/V_L$*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Saturated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lauric acid</td>
<td>$C_{12}$</td>
<td>= 1.0 ($V_L$)</td>
</tr>
<tr>
<td>Palmitic acid</td>
<td>$C_{16}$</td>
<td>0.4</td>
</tr>
<tr>
<td>Stearic acid</td>
<td>$C_{18}$</td>
<td>0.1</td>
</tr>
<tr>
<td><strong>Unsaturated</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oleic acid</td>
<td>$C_{18}$</td>
<td>1.02</td>
</tr>
<tr>
<td>Elaidic acid</td>
<td>$C_{18}$</td>
<td>0.85</td>
</tr>
<tr>
<td>$\gamma$-Linoleic acid</td>
<td>$C_{18}$</td>
<td>1.03</td>
</tr>
<tr>
<td>$\gamma$-Linolenic acid</td>
<td>$C_{18}$</td>
<td>0.90</td>
</tr>
<tr>
<td><strong>Palmitoyl esters</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palmitoyl acid methyl ester</td>
<td></td>
<td>0.11</td>
</tr>
<tr>
<td>Palmitoyl-$\alpha$-carnitine chloride</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Palmitoyl coenzyme A</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td><strong>Hydrophilic substituted</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\omega$-Bromo-lauric acid</td>
<td>$C_{12}$</td>
<td>1.31</td>
</tr>
<tr>
<td>$\omega$-Hydroxypalmitic acid</td>
<td>$C_{16}$</td>
<td>0.55</td>
</tr>
<tr>
<td>2-Bromopalmitic acid</td>
<td>$C_{16}$</td>
<td>1.81</td>
</tr>
<tr>
<td>2-Hydroxypalmitic acid</td>
<td>$C_{16}$</td>
<td>0.93</td>
</tr>
<tr>
<td>$\omega$-Carboxypalmitic acid</td>
<td>$C_{16}$</td>
<td>0.37</td>
</tr>
<tr>
<td>12-Azidooleic acid</td>
<td>$C_{18}$</td>
<td>1.03</td>
</tr>
<tr>
<td>12-Doxylstearic acid</td>
<td>$C_{18}$</td>
<td>0.69</td>
</tr>
<tr>
<td>$\omega$-Glucopyranoside palmitic acid</td>
<td>$C_{18}$</td>
<td>0.82</td>
</tr>
<tr>
<td><strong>Fluorescent derivatives</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anthracene-9-carboxylic acid</td>
<td>$C_{15}$</td>
<td>0.02</td>
</tr>
<tr>
<td>4-(3-Pyrenyl)butyric acid</td>
<td>$C_{20}$</td>
<td>0.15</td>
</tr>
<tr>
<td>Diphenylhexatriene (DPH) propionic acid</td>
<td>$C_{21}$</td>
<td>0.13</td>
</tr>
<tr>
<td><strong>Phenyl substituted</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-Phenylbutyric acid</td>
<td>$C_{10}$</td>
<td>0.11</td>
</tr>
<tr>
<td>6-Phenylhexanoic acid</td>
<td>$C_{12}$</td>
<td>0.25</td>
</tr>
<tr>
<td>8-Phenyloctanoic acid</td>
<td>$C_{14}$</td>
<td>0.57</td>
</tr>
<tr>
<td>10-Phenyldecanoic acid</td>
<td>$C_{16}$</td>
<td>1.16</td>
</tr>
</tbody>
</table>

Data obtained from H$^+$ uptake measurements in UCP-1 proteoliposomes ([59] and unpublished data). FA concentrations 125 to 250 μM. *$\Delta V = V(+FA) - V(O)$. $V_L/C_H^+ \approx 50$ (min$^{-1}$ (mg protein)$^{-1}$).
maximum H\(^+\) transport activity is obtained only with surprisingly high amounts of FA, which can reach up to 8 mol% of the phospholipids in the vesicles [54,59]. Although most of the added FA (oleic acid) was shown to be bound to vesicles, the vesicles still remained tight. It must be assumed that for activation of H\(^+\) transport UCP-1 recruits FA primarily from the surrounding lipid phase. The maximum H\(^+\) transport rates exceed by far what is needed for the uncoupling of mitochondria but are highly significant for evaluating the H\(^+\) transport mechanism and its regulation in UCP-1. A physiological significance of high FA requirement is underlined by the finding that very high FA concentrations (up to 20 mM) occur in BAT on activation of lipolysis [61].

Why is there such a high level of UCP-1 of up to 10% of protein present in BAT mitochondria if with its high capacity much less UCP-1 could accomplish the uncoupling? No simple answer can be given at present, but the following arguments may give a reasonable explanation. UCP-1 in mitochondria is inhibited by ATP. At least four parameters influence H\(^+\) transport activity by UCP-1, ATP\(^{4-}\) concentration, FA ‘concentration’, pH and \(\Delta\psi\). With a total concentration of cytosolic ATP = 3.6 mM [62,63] and 2 mM Mg\(^{2+}\), the free ‘ATP\(^{4-}\)’ in brown adipocytes is calculated to be about 35 \(\mu\)M and thus would inhibit UCP-1 at pH 6.8 to more than 95% of its maximum capacity. In the activated state, a decrease in ATP concentration to 0.8 mM [62,63] and an increase of cytosolic pH to > 7.5 would lower ATP binding to 80% due to much lower affinity of ATP for UCP [64]. In perfused liver a strong pH increase was observed when FAs were supplied [65]. The free UCP-1 might have submaximum activity due to limited access of FAs and because of the low \(\Delta\psi\) which results from the balance of uncoupling and electron transport activity.

3.4. Fatty acid specificity

Surprising is the non-specificity towards FA and derivatives within certain limits [48,59,66]. There is a minimum requirement for the chain length of FA (Fig. 2). Whereas C-8 acid (caprylic acid) starts to have a marginal activity, the activity quadruples with C-10 (decanoic acid) and increases another 1.5-fold reaching maximum with C-14 (myristic acid). It decreases with C-16 (palmitic acid) and more strongly with C-18 (stearic acid). The lower activity of C-16 to C-18 is due to the poor aqueous solubility of these saturated FAs. The more soluble oleic acid (\(\Delta C\)-18) is nearly as good an activator as C-14. FA derivatives in which part of the alkyl chain are replaced by a phenyl group, such as phenyl hexanoic and phenyl butyric acids, are still fair activators (Table 1) [59]. Obviously, not an exactly defined structure such as a linear alkyl chain, but more a minimum hydrophobicity balance is essential for activity. Checking the activity with FA derivatives containing hydrophilic substitutions is interesting for mechanistic reasons. \(\omega\)-Hydroxy and \(\omega\)-bromo substitutions still permitted fair to high activities. Even FAs, where highly hydrophilic and large substituents (e.g., carboxyl and gluco- pyranoside) are introduced at the \(\omega\)-position, still permitted a significant stimulation. Substitution at C-12 and C-18 by azido or doxyl (spinlabel) groups did not markedly inhibit the activity ([59] and Table 1). These results indicate a minimum requirement of only sufficient lipid solubility and of a free carboxyl group. At variance with our results, Jezek et al. [66] recently reported no activity with phenyl substituted FA using indirect H\(^+\) transport measurements.
The pH dependence of $H^+$ transport is of great interest because of its mechanistic implications since it should reflect the dissociation of groups involved in $H^+$ transport. As measured in proteoliposomes, $H^+$ transport activity by UCP-1 increases with the pH, reaches a maximum and slowly falls off [30,54]. The pH maximum depends on the chain length of the FA. With short-chain FA such as C-8 and C-10 the pH maximum is low (pH 6 to 6.5) and then increases with the chain length reaching maximum at pH 8.0 with oleic acid (Winkler and Klingenberg, unpublished data). This increase of the pH maximum coincides with the known $pK_a$ upshift of the FA with increasing chain length when bound to membranes [67]. The $pK_a$ upshift is due to the increased hydrophobicity in the environment around the carboxyl group, in addition to other entropic effects. Electrophilic substitutions (e.g., bromo-substitution at the C-2 position) lower the $pK_a$ of the adjacent carboxyl group. Accordingly, $H^+$ transport activated by 2-bromo-palmitic acid had a pH maximum at 6.8 which is distinctly lower than that of the palmitic acid (Winkler and Klingenberg, unpublished data). The threefold higher activity as compared to palmitic acid (Table 1) is partially due to the better solubility of the 2-bromo derivative.

### 3.5. Anion transport

The particular permeability of BAT mitochondria to small anions was first described by Nicholls and Lindberg [42], by measuring the osmotic swelling of mitochondria in the presence of $K^+$-salts and valinomycin. This anion transport is highly unspecific and tolerates also $Br^-$, $NO_3^-$ and several other non-covalent anions. In fact, the transport of anions other than $Cl^-$, e.g., $NO_3^-$, is up to eightfold faster. This anion transport is inhibited by GDP which is the criterion for the involvement of UCP-1. Interestingly, when purified UCP-1 was reconstituted into asolectin ‘giant liposomes’ $Cl^-$ channel properties could be demonstrated in patch-clamp studies [68]. The 75-pS channel could be inhibited by GTP, GDP but not by AMP, in accordance with the nucleotide binding properties of the UCP-1.

Anion transport, particularly $Cl^-$ transport, was measured in reconstituted system using either $^{36}Cl^-$ [30,53] or anion-sensitive fluorescent dyes [30,57]. Also in the reconstituted system $Cl^-$ transport did not require FAs. However, there is controversy on whether FAs inhibit $Cl^-$ transport. Both on the level of mitochondria and in the reconstitution system no competition has been found by the groups of Rial and Nicholls [48] and Winkler and Klingenberg (unpublished data). At variance, Jezeck et al. [57] reported a competition of $Cl^-$ transport by laurate using SPQ, a fluorescent indicator for $Cl^-$ transport. This competition is used as one of the arguments for the tenet that FAs are transported as anion by UCP-1 [57,69]. A reasonable explanation is that the FA-activated $H^+$ transport and the concurrent $Cl^-$ transport competes for the limited capacity of the membrane diffusion potential generated by valinomycin plus $K^+$, which drives both $H^+$ and $Cl^-$ transport in opposite directions. The much more active $H^+$ transport thus suppresses the $Cl^-$ transport, which is lower than the $H^+$ transport activity by a factor of 5 to 20 in the reconstituted system [56,57].

Jezeck and Garlid [70] reported the transport of alkyl ($C_3$ to $C_6$) sulfonates in BAT mitochondria as judged by the swelling assay. However, with increasing chain length (C_6-sulfonate), the apparent swelling became less GDP sensitive. A competition between alkyl sulfonate and GDP was invoked. This is in contradiction to the evidence presented by these authors that the GDP binding site does not overlap with the anion channel [71]. Moreover, the same authors demonstrated that $Cl^-$ transport and GDP inhibition are uncompetitive [57]. Nedergaard and Cannon [72] reported a competition of hexanesulfonate with GDP binding, however, only at very high concentrations with a $K_i$ of 13 mM, which could partially explain the insensitivity to GDP. To some extent, at the high concentrations used of 54 mM [70], alkyl sulfonates can also take a UCP-1-independent pathway and have a detergent type effect on the membrane which mimics the swelling. They also showed that short-chain alkyl sulfonates strongly compete with $Cl^-$ transport and concluded that they are transported even more actively than $Cl^-$ by UCP-1 [70]. A longer chain alkyl sulfonate, $C_{11}$-sulfonate, was shown to inhibit the lauric acid-stimulated $H^+$ transport [69]. Uptake of $C_{11}$-sulfonate was measured indirectly by the stimulation of valinomycin dependent $K^+$ influx.
3.6. Common or different paths for H\(^+\) and Cl\(^-\)

The existence of separate channels for H\(^+\) and Cl\(^-\) transport was claimed by Kopeccky et al. [47] by comparing H\(^+\) transport and Cl\(^-\) uptake in BAT mitochondria. However, the evidence is not convincing as pointed out by Rial et al. [48]. Rial and coworkers also discussed that FAs might induce a conformation change in UCP-1 [48]. Since the Cl\(^-\) transport did not require FAs, this model might imply that H\(^+\) and Cl\(^-\) transport use separate pathways. Therefore the activation by FA through a conformation change of UCP-1 seems unlikely (see below). A further argument for a common channel is the equal sensitivity to nucleotides of both H\(^+\) and Cl\(^-\) transport ([48,71]; Winkler and Klingenberg, unpublished data). A common channel for H\(^+\) and Cl\(^-\) is well defined in halobacterial retinal protein [73]. The selectivity for H\(^+\) and Cl\(^-\) is here modulated through the elimination or introduction of aspartyl groups. One may argue that a channel engineered for H\(^+\) transport would not be able to transport Cl\(^-\). However, one has to consider that the Cl\(^-\) transport rate is much slower and must be regarded as a kind of side reaction of the H\(^+\) channel. Finally one may ask: could proteins as small as UCP-1 accommodate two different transport channels in parallel? This seems to be improbable in view of the larger size of most other transport and channel proteins.

3.7. The influence of \(\Delta \psi\) on H\(^+\) transport

In line with the uncoupling function, H\(^+\) transport in BAT mitochondria is driven by the membrane potential. In the proteoliposomes a clear linear relation between the rate of H\(^+\) transport and \(\Delta \psi\) generated by the K\(^+\) gradient was demonstrated [30,54]. Also in BAT mitochondria a linear relation between \(\Delta \psi\) and FA stimulated respiratory rates, as a measure of uncoupling by H\(^+\) recycling, was reported [74]. However, the point was made that in the presence of GDP the decreased H\(^+\) conductance at low \(\Delta \psi\) (\(\leq 150\) mV) is transformed into a high conductance at elevated membrane potentials. As a result the H\(^+\) conductance follows a non-linear or ‘non-ohmic’ relation. Previously such a relation has been measured in liver mitochondria with a breakpoint to high conductance at about 180 mV [43]. This phenomenon was also described as ‘slippage’. In BAT mitochondria the threshold for the appearance of high conductance is shifted by FAs down to about 130 mV. In this terminology, FAs increase slippage more in BAT than in liver mitochondria. Since this FA-induced shift is seen only in mitochondria from BAT but not from liver, it was argued that FA can activate even GDP-saturated UCP-1. In essence, the high \(\Delta \psi\) has to override the inhibition by GDP in cooperation with the FA. How this is mechanistically accomplished remains unknown. The physiological role of this effect in BAT will be questioned below.

3.8. Mechanism of H\(^+\) transport by UCP-1 and the role of fatty acids

In a way, UCP-1 is the simplest H\(^+\) transporter known. It does not require a complex ATPase driven H\(^+\) transport machinery, it does not involve a H\(^+\)-substrate cotransport and is not, for example, a H\(^+\) exchanger against Na\(^+\) or Ca\(^{2+}\). H\(^+\) transport in UCP-1 is simply driven by the membrane potential. In a sense UCP is more a carrier than a channel for H\(^+\); it requires additional catalysts for its transport and is structurally related to mitochondrial solute carriers. H\(^+\) transport in general has been treated extensively in the literature both from a theoretical and experimental point of view. Theoretically, H\(^+\) transport is fascinating because of the very low H\(^+\) concentrations at normal pH and the dependence of H\(^+\) transport on chains of H\(_2\)O molecules along which H\(^+\) are conducted. The installation of H\(^+\) donor/acceptor groups within a channel in addition to the H\(_2\)O molecules is crucial for providing a sufficient H\(^+\) transport capacity. Further, H\(^+\) donor/acceptor groups enable unidirectional H\(^+\) transport by providing a pK\(_a\) gradient. Under the influence of \(\Delta \psi\) the pK\(_a\) may be changed to generate this gradient along the translocation channel. In bacteriorhodopsin [75,76] and cytochrome oxidase [77], H\(^+\) donor/acceptor groups have been identified by site-directed mutagenesis to be represented by the carboxyl groups of Asp and Glu within the well defined atomic resolution structure. The identification of potential H\(^+\) acceptor/donor groups in UCP-1 by mutagenesis will be discussed below.

Within the realm of this general picture, H\(^+\) transport in UCP-1 is distinguished by its requirement of
FA. This absolute requirement gives FA a role as cofactor for H⁺ transport in UCP-1. According to our working hypothesis [59], FAs provide carboxyl groups in the translocation channel. They may compensate for the absence of resident H⁺ donor/acceptor groups at some critical positions within the H⁺ translocation channel. A somewhat similar model (Fig. 3A) has been discussed by Rial et al. [48] as a possible alternative to their conformational model. Here the carboxyl groups of the FAs oscillate along the translocation channel carrying the H⁺. This model does not involve the possible intervention of resident carboxyl groups provided by Asp or Glu in the translocation channel. According to our model (Fig. 3C) [59,78], in addition to the resident H⁺ donor/acceptor groups, the FA may fill one or more gaps in the translocation channel. In fact, site-directed mutagenesis provides evidence for the existence of resident H⁺ donor/acceptor groups [79] (see below). This construction of the H⁺ transport machinery in UCP-1 permits the regulation of the uncoupling activity by changes of FA ‘concentrations’. The low affinity as evidenced by the high amounts of FA required for maximum stimulation [54,59] enables regulation over a wide range of FA concentration. In other words, FAs are not sticking to UCP-1 and can allow a rapid return of UCP-1 into the inactive state. In this context it should be noted that FA binding to UCP-1 was claimed to be demonstrated by using spinlabeled (doxylstearic acid) FA [80]. However, the line broadening in these ESR spectra is not uniquely due to a specific binding to UCP-1 but may be caused by adsorption to protein detergent interfaces. With ω-azido lauric acid, inhibition of H⁺ and Cl⁻ transport was also reported [81]. On photoactivation of the azido-FA, radioactive labeling in mitochondria, preferentially of UCP-1, was shown. However, no data on the amount of incorporated azido lauric acid were given. One has to consider unspecific covalent incorporation from the excess of highly reactive nitrene derivative of lauric acid generated in the membrane [82].

A model for the mechanism of FA function representing our present view is given in Fig. 4. It is assumed that FAs mainly act from the cytosolic side of the membrane since ω-glucopyranoside palmitic acid is anchored to the outer surface. The FA carboxyl can penetrate from the interface of the protein to a H⁺ acceptor group. This area may be comparatively unspecific, i.e., the protein provides a favorable, partially hydrophilic environment for a movement of the
carboxyl towards the H⁺ acceptor. The final H⁺ donors are His-145 and His-147 which serve to push H⁺ into matrix [79]. In this ‘lower’ section, the H⁺ path is within the protein and thus here the control by the nucleotide induced conformation change is located. This model is in line with the structural model (Fig. 1) with three loops extending from the matrix into the membrane. These may provide the path for H⁺ and also a compact selection filter analogous to the structure recently described for the K⁺ channel [83].

3.9. Variant models of H⁺ transport mechanisms

Mechanisms of FA activation of H⁺ transport by UCP-1 have been discussed also in terms of an activator rather than a cofactor role by Rial and Nicholls [48]. For example, as indicated in Fig. 3A, FA may activate the H⁺ translocating channel by a conformational change. Also a surface membrane potential effect caused by FA was discussed [48]. A quite different role of FA is visualized in the anion transport model of FA activation of UCP-1 (Fig. 3D) [57]. Originally this model had been proposed by Skulachev [84] based on the observation that the FA-induced uncoupling of mitochondria can be partially inhibited by carboxyatractylate, a specific inhibitor of the AAC [85]. The AAC was speculated to transport FA anions and this model was then extended to explain the activation by FA of H⁺ transport in UCP-1. In this model UCP-1 is not an actual H⁺ transporter but a transporter for FA anions. Once FA anions are transported through UCP-1, they pick up or release H⁺ on either side of the membrane and flip-flop as undissociated FAs through the lipid bilayer (Fig. 3D). As a result, H⁺ is transported through the membrane in the opposite direction to the FA anion transport by UCP-1. Recently the model has been modified such that the FA anions are translocated on the interface between UCP-1 and the membrane [69]. Studying anion transport in BAT mitochondria and in the proteoliposomes, Jezek and Garlid [57] claimed to have verified the FA anion transport in UCP-1 with the demonstration of the alkyl sulfonate transport as model of FA anions.

Since alkyl sulfonates, because of their low pKₐ, cannot flip through the membrane like undissociated FAs, it is reasoned that they perform the anion transport branch via UCP-1 mimicking FA anion transport. However, the experimental evidence is weak as C₁₁-sulfonate is needed for inhibition of H⁺ transport at a 50-fold higher concentration than laurate to activate H⁺ transport. Further, the poor inhibition by external GDP argues that much of the C₁₁-sulfonate did not pass through UCP-1 but was driven through the membrane by the K⁺ gradient in a ternary complex formed with valinomycin and K⁺.

The flip-flop of undissociated FAs is an essential part of the FA anion transport model. Evidence against FA flip-flop playing a role in UCP-1 linked transport comes from the fact that FA with hydrophilic tails, such as ω-carboxyl palmitic acid [59] or ω-glucopyranoside palmitic acid (Table 1) (Winkler and Klingenberg, unpublished data) are also activating H⁺ transport, albeit only to about 50% of the maximum with lauric acid. The strong hydrophilic tail should remain on the surface and thus prevent any diffusion through the membrane.
The limited movement also lowers the activation radius of the FA and explains the reduced activation capability. Recently Jezek et al. [66,86] compared the ability of various FA derivatives to ‘flip-flop’ with their ability to activate $\text{H}^+$ transport, and concluded a parallelism between both activities in support of their FA anion transport model. The inactive derivatives encompassed phenyl substituted FA which, however, have been shown to be active in our hands (Table 1). Surprisingly, the $\omega$-hydroxyl or amino-substituted lauric acids are reported to be inactive whereas the $\omega$-bromo derivative is highly active.

4. Nucleotide binding

4.1. Equilibrium methods and nucleotide binding specificity

Nucleotide binding by UCP-1 has been a key in identifying [12,14] and isolating this $\text{H}^+$ transporting protein [9,10]. It has also provided a means for asaying the functional intactness of UCP-1 [87] and understanding how the $\text{H}^+$ transport activity is regulated [1,47,64,88,89]. Nucleotide binding has been measured to mitochondria [90,12,91] and extensively to the isolated protein [10,64,88,89,92] and to UCP-1 reconstituted into liposomes [30,54,89]. Measurements of nucleotide binding to the mitochondria were limited by enzymatic degradation of the adenine nucleotides [93,91] and therefore guanine nucleotides, particularly GDP, were used in the binding studies. Binding measurements to the isolated protein used equilibrium dialysis [9,10], and a specifically developed anion exchange method [87] based on the rapid removal of free nucleotide by the anion exchanger. Critical to the latter method is the slow dissociation of the tight UCP-1-nucleotide complex.

UCP-1 accepts a broad range of nucleotides but with strong preference to the purine nucleotides [10,64]. GTP, ATP, GDP and ADP bind with high affinities ($K_d = 0.3$ to $3 \, \mu\text{M}$ at pH 6.7). Guanine nucleotides exhibit somewhat higher affinities than the adenine nucleotides. The monophosphates GMP and AMP are poor ligands which bind with over 50-fold lower affinities. The interaction with purine nucleotides is a common denominator among UCP-1 and AAC. However, there are several important and interesting differences, which are also relevant for understanding carrier function in general [28,94]. Whereas UCP-1 binds both guanine and adenine nucleotides, transport by AAC is highly specific for ADP and ATP. Some interaction with guanine nucleotides was observed with NMR [95]. Monophosphate nucleotides interact very weakly with both carriers. In UCP-1 nucleotide binding causes inhibition of carrier activity, while in AAC ADP or ATP activates the carrier into the transport state. The binding affinity is much higher in UCP-1 than in AAC. Nucleotides bind only from the c-side in UCP-1 but from both sides in AAC. Protease resistance experiments showed that nucleotides render the UCP-1 into a more rigid state [33,89], while these ligands labilize the AAC structure [96]. From the ‘induced transition-fit’ theory for carriers [28] it follows that the intrinsic interaction force can be lower in UCP-1 than in AAC because it only transforms the protein into the inhibited state, whereas in AAC a higher intrinsic binding energy is required for the activation of the large conformational changes from the ‘c-’ to ‘m’-state transition. Vice versa, a higher intrinsic interaction energy can be deduced from the high specificity for adenine nucleotides in AAC as compared to the tolerance for adenine and guanine nucleotides in UCP-1.

Fluorescent 2’-O-dansyl nucleotides have enabled both equilibrium and kinetic binding studies [88]. These derivatives of NDP and NTP bind with similar affinities as their parent nucleotides to UCP-1 with 10- to 30-fold fluorescence increase. Remarkably, although AMP binds weakly to UCP-1, dansylation enhanced its affinity by nearly 50-fold, thus enabling dansyl-AMP binding measurements. The fluorescent nucleotides were applied to measure binding to isolated UCP-1 [88], to UCP-1 reconstituted into phospholipid vesicles [89] and to BAT mitochondria [91,97]. Dimethylaminonaphthoyl (DAN) nucleotides, which were previously applied to study the ADP/ATP carrier [98], bound also with enhanced fluorescence to UCP-1 [88,99]. However, NMR analysis showed a mixture of 60% 3’-O-DAN and 40% 2’-O-DAN substitutions, whereas there is 100% 2’-O-dansyl substitution [88]. The DAN-nucleotide analogs may therefore not be suitable for kinetic studies.
4.2. One or two nucleotides bound per UCP-1 dimer?

A binding capacity of 16 μmoles GDP/g protein was measured by equilibrium dialysis [9], corresponding to a stoichiometry of 1 nucleotide/UCP-1 dimer. In all further binding studies in our laboratory the binding never exceeded this value, which is critical to the question whether the stoichiometry is 1 or 2 nucleotides bound per UCP-1 dimer. There is admittedly some uncertainty on the UCP-1 content in these determinations. The report by Feil and Rafael [93] that the stoichiometry was 2 GDP/UCP-1 dimer was based on immunological quantitation of UCP-1 content, a method which is very difficult to calibrate reliably. Measurement of nucleotide binding was also attempted by quenching of the protein tryptophan fluorescence [100]. The binding stoichiometry of 2 GDP/UCP-1 dimer deduced from these studies is flawed by guanine nucleotide and Triton absorbance at 290 nm which causes the ‘inner-filter effect’. The maximum quenching is actually rather small (2%) with UCP-1 isolated in reduced Triton (Huang, unpublished data). With ADP and ATP this interference is absent. Titration with ADP and ATP of UCP-1 isolated in non-UV absorbing non-ionic detergent emulphogen arrived at 1 ADP/UCP-1 dimer (Klingenberg and Appel, unpublished data). The binding of only one nucleotide per UCP-1 dimer may indicate an anti-cooperative effect of the two subunits in the UCP-1 dimer, i.e., binding of one nucleotide molecule to one monomer inhibits the binding to the counterpart either simply for sterical reasons or more probably by inducing a non-binding state on the other subunit [101,102]. Alternatively, a single nucleotide binding site is constructed near the interface of both subunits.

4.3. pH dependence of binding

One of the most remarkable features of nucleotide binding to UCP-1 is its strong pH dependence, which was first noted in mitochondria [90] and then studied in great detail with isolated UCP-1 [10,64,88]. A similar pH dependence was reported in urea-treated membrane vesicles by Rafael and coworkers [103]. In the broad pH range studied by these authors, the dependence largely agrees with the previous observations [10,64,88], except for the decrease in pK_a for GDP and ADP at pH < 4.5. The decrease in pK_a could not be explained by our pH dependence model [64,88], but rather could have resulted from instabilities under these extreme conditions (low pH and 37°C).

Both NTP and NDP exhibit a strong pH dependence but with well defined differences (Fig. 5). 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 (∆pK_D/∆pH) change from ~−0.2 to −1. Above pH 7.0 to 7.2, the slope (∆pK_D/∆pH) becomes steeper at −2 with NTP but not with NDP. The pH dependence was interpreted by an interplay of H⁺ dissociations at the nucleotide terminal phosphate (pK_a = 6.5) and at two constituent resi-

![Fig. 5. pH dependence of nucleotide binding to UCP-1 exemplified with ADP and ATP (data taken from [64]). Measurements with purified soluble UCP-1 by the anion exchanger method. The line drawn through the measured points is based on a model (a) accounting for the pK_a = 6.8 of ADP-H² and ATP-H³ dissociation, (b) assuming 100- and 50-fold higher affinity of ADP-H² and ATP-H³ to UCP-1, (c) assuming regulation by a carboxyl group with pK_a = 3.8 in UCP-1 which allows binding of ADP and ATP only in the undissociated form (R-CO₂H), and (d) regulation by a His for the binding exclusively of the nucleotide triphosphates ATP (GTP), by postulating that binding requires HisH⁺ (pK_a = 7.2).]
idues with $pK_a = 3.8$ and 7.2. UCP-1 was proposed to exist in a binding and a non-binding state [64,88], depending on the protonation of a putative Glu/Asp residue (now identified as Glu-190) (Fig. 6). In the non-binding state, this group forms an internal ion pair which bars the entrance to the binding pocket for the nucleotide phosphate moiety. On protonation of this strategically located Glu/Asp the nucleotide binding site opens and is ready to accept the ligand. The terminal phosphate ($pK_a = 6.5$) of nucleotide imposes a pH dependence by differential binding of the protonated forms NTPH$^{3-}$ and NDPH$^{2-}$ versus the deprotonated forms NTP$^{4-}$ and NDP$^{3-}$. Best fits of binding data revealed a 50-fold stronger affinity for NTP$^4$ over NTPH$^{3-}$ and a 100-fold stronger affinity for NDP$^{3-}$ over NDPH$^{2-}$. Thus, although the population of the stronger binding species NTP$^4$ and NDP$^{3-}$ increased with increasing pH, UCP-1 is transformed progressively into the non-binding state due to deprotonation of the essential Glu/Asp. These counteracting effects result in a rather flat pH dependence at pH $< 6.5$.

At higher pH, nucleotide binding is limited by the availability of UCP-1 that is in the binding state. This superposition explains the observed slope of $\Delta pK_a/\Delta pH = -1$. A second residue in the protein with $pK_a = 7.2$ further contributes to the pH dependence for NTP binding only. Upon protonation of this putative histidine residue, the binding center is assumed to be deepened which then can accommodate the triphosphate moiety for NTP binding [88]. Thus, superimposed to the protonation of Glu/Asp, the affinity for NTP declines with a slope of $-2$ on the $pK_a/pH$ plot. This model was vindicated by the identification of these two participating residues by chemical modification [104] and by mutagenesis [56,79].

4.4. Kinetics of binding

Kinetics of nucleotide binding were measured with fluorescent 2'-O-dansyl nucleotides by stopped-flow fluorimetry [88]. Initial association rates were measured with $k_{on} = 4 \times 10^5$ $M^{-1}$ s$^{-1}$ (pH = 6.3, 25°C) and were largely independent of the nucleotide species. The strong pH dependence of the association rates with a slope $\Delta pK_a/\Delta pH = +1$ suggest that protonation of UCP-1 had to occur prior to nucleotide bind-

ing, consistent with the role of the putative Glu/Asp as a binding–non-binding switch. Dissociation kinetics were followed by the decrease in the fluorescence due to dissociation of the UCP-dansyl nucleotide complex on addition of a large excess of ATP. The dissociation rates were low and strongly dependent on nucleotide. A drastic difference in the pH dependence of the dissociation rates between dansyl-NTP and dansyl-NDP was observed. The $p_{off}/pH$ plot for NDP was fairly flat while the plot for NTP gave a slope of $-1$, in line with the results for the $pK_a/pH$ dependence. Such a pH dependence can be explained by the two-stage binding model, in which this putative His is involved only in the binding of NTP [88] (Fig. 6). Within this model, the nucleotide is assumed to bind rapidly to form a loose UCP-nucleotide complex, which undergoes a slow conformational change into a tight complex. NTP binding requires exclusively the protonated histidine (HisH$^+$). The dissociation of NTP is preceded by the deprotonation of this HisH$^+$ with a concomitant conformation transition back to the loose complex where it can dissociate rapidly from UCP-1. During the dissociation of NDP, UCP-1 returns directly to the loose state without deprotonation of the HisH$^+$.

4.5. The two-stage (loose–tight) nucleotide binding model

The anion exchange method yielded binding capacities consistently lower than the fluorescence titration. The binding capacity was 10% lower for dansyl-GTP and even 50% for dansyl-ATP [88]. Two complexes, one loose and one tight, were proposed to account for this discrepancy (Fig. 6). It is further assumed that the fluorescence method measured the total binding comprising the two complexes, whereas the anion exchange method determined only the tight complex due to a rapid dissociation of the loose complex during passage through the Dowex column. In accordance, a concomitant conformation change could be inferred from the sensitivity towards tryptic digestion [33,89]. Cleavage by trypsin at Lys-292 of the cytosolic protruding C-terminal stretch was differentially inhibited by various nucleotides. Nucleotides that bound tightly and inhibited the transport protected more strongly than loose-binding non-in-
hibitory derivatives such as DAN-ATP, DAN-ADP, and dansyl-AMP [89].

Additional experimental evidence in support of the two-stage mechanism recently came from kinetic binding data [92]. The experiments showed a burst of initial binding, reflecting formation of the loose complex. The subsequent slow phase represented the gradual formation of the tight complex. The binding data could be best fitted with equations derived from the two-stage binding mechanism [92].

The individual parameters (Table 2) for the two-stage binding have been defined [88,89,92]. The initial association rate is fast \((4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1})\), as measured for the dansyl nucleotides. The conformational transition was much slower for NTP than for NDP. Both the forward \(k_{+1}\) and backward \(k_{-1}\) transition rates were strongly dependent on pH and temperature. Apparently increasingly precise ligand recognition occurs at the slow conformational transition step accompanied by maximum interactions between the protein and nucleotide. The intrinsic dissociation constant \(K_d\) for the loose complex (UCP-N) is about 2 \(\mu\)M. The overall \(K_D\) varies and depends on the nucleotide species. A large difference was demonstrated in the equilibrium distribution of the tight and loose complexes. Here \(K'_c = k_{+1}/k_{-1}\) defines the ratio of tight complex (UCP*\(\text{N}\)) to loose complex (UCP-N) at equilibrium. GTP, ATP, GDP and dansyl-GTP form \(>90\%\) and ADP, dansyl-ATP and dansyl-ADP form \(40\%\) to \(70\%\) tight complex. In contrast, dansyl-AMP and DAN-ATP form little \((<20\%)\) tight complex. As a result, dansyl-AMP could reactivates the \(H^+\) transport activity in reconstituted UCP-1 which had been inhibited by ATP, obviously by transferring UCP-1 into the loose transport-active state.

Fig. 6. Two-stage (loose-tight) nucleotide binding model based on kinetic, equilibrium and pH dependence data. The UCP-1 is assumed to exist in binding/non-binding states depending on the protonation at E190. A neighboring carboxyl group [125] is proposed to pull the protonated His (His\(H^+\)) whereby the binding center is deepened. In this state both NTP and NDP can bind, whereas only NDP can bind when the His is in the neutral form. The binding proceeds with a rapid formation of the loose complex and a slow conformational change into the tight complex.
4.6. Influence of ions and temperature

Mg$^{2+}$ was shown to decrease the apparent affinity of nucleotide binding [64]. More detailed studies [91,103] revealed that Mg-ATP bound to UCP-1 with negligible affinity. Rial et al. [48,90] reported only a 10-fold lower affinity of ATP in the presence of 2 mM Mg$^{2+}$. Since Mg$^{2+}$ is present in the cytosol in millimolar concentrations, the free ATP as an effective regulator should represent only a small fraction of the total cytosolic ATP.

Anions were shown to compete with nucleotide for binding to UCP-1 [10,88]. The potency of interaction is: pyrophosphate ($K_a = 0.16$ mM) > sulfate (1.0 mM) > phosphate (4.4 mM) > maleate (8.0) > chloride (9.3 mM) >> acetate. Acetate displayed nearly no inhibitory effect, probably due to a more dispersed negative charge and therefore acetate is recommended whenever a salt has to be present in the binding measurements. The equilibrium binding of dansyl-nucleotides to UCP-1 showed little temperature dependence [88]. The high affinity was ascribed to a remarkably high entropy change ($\Delta S = 136$ J deg$^{-1}$ mol$^{-1}$) that was indicative of strong ionic interactions associated with removal of H$_2$O molecules from the binding site. The kinetics were strongly temperature dependent, requiring a rather high ($\sim 60$ kJ/mol) activation energy for both the binding and dissociation of dansyl-ATP.

4.7. The masking/unmasking phenomena

The level of UCP-1 in BAT is strongly regulated in accordance with the thermogenic requirements. Changes in UCP-1 content on warm- or cold-adaptation of laboratory animals have been well documented [105–107]. In all experiments, these authors observed a correlation between the UCP-1 content in BAT mitochondria and the thermogenic capacity using GDP binding as an index.

An acute change of the environmental temperature produced a discrepancy between the GDP binding sites and UCP-1 content. When rats raised at 27°C were transferred to 4°C for only 20 min, GDP binding to the isolated mitochondria was found to increase 1.3-fold [108], although no de novo synthesis of UCP-1 had occurred under these conditions [105–107]. These findings suggested that the nucleotide binding sites in BAT mitochondria could be ‘unmasked’ to cope with the acute temperature change. Acute unmasking of binding sites could also be achieved with norepinephrine and $\beta$-adrenoreceptor agonists [109–112]. Alternatively, the binding sites could be ‘masked’ on warm adaptation of the animals [107].

Various hypotheses for the unmasking have been discussed previously by Nicholls and Locke [4] and by Cannon and Nedergaard [2]. Changes in FA concentrations or in pH as factors were rejected because they cannot explain the remarkable changes in GDP binding. Mitochondrial swelling as reported by Desautels and Himms-Hagen [113] resulted in some marginal changes in GDP-binding [106], but could not be confirmed by others [108]. Similarly, the difference in GDP binding to UCP-1 proteins isolated from warm- and cold-adapted rats reported by Swick and Swick [108] and Feil and Rafael [93] could not be reproduced in the our laboratory (unpublished data). Here we will briefly discuss the endogenous ATP hypothesis advanced in our laboratory [91].

Evidence for the masking of UCP-1 by endogenous ATP in isolated BAT mitochondria is based on two observations [91]. First, the dissociation of ATP but not ADP was extremely slow from mito-

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$K_D$ and $K_a$ are the overall dissociation constant and the dissociation constant of the loose complex, respectively. $K'_s$ is the ratio of $k_{a1}/k_{a-1}$ evaluated for the two-stage binding model $UCP + N^{\equiv}=UCP\cdot N^{\equiv}=UCP\cdot N$. Data measured at pH 6.7 [88,89,92].
chondria \( (k_{off} = 2 \times 10^{-3} \text{s}^{-1}) \), as shown by chase experiment and by successive washing at room temperature. Thus endogenous ATP can remain bound to the isolated mitochondria. Second, binding measurements in various laboratories were performed under non-equilibrium conditions, i.e., a too short incubation time \([48,90,105,114]\) that did not allow equilibration of externally added ADP or GDP with endogenous prebound ATP. The biphasic Scatchard plots have been interpreted \([91]\) to reflect the non-equilibrium state where the ‘high affinity’ sites represent fast binding of GDP to the free UCP-1 sites in mitochondria, and the ‘low affinity’ site is due to competition with the bound ATP. Only on removal of the prebound ATP with an anion exchanger and prolonged incubation (60 min) did the Scatchard plots become linear with the same nucleotide specificity and similar pH dependence as reported for the isolated protein. The non-linearity can be reintroduced by loading the mitochondria with low concentrations of ATP (1 to 10 \( \mu \text{M} \)).

Apparently BAT mitochondria isolated from
acutely warm-adapted hamsters retained more endogenous ATP than did the cold control, as shown by the lower non-linear binding [91]. On Dowex treatment, both mitochondria yielded the same GTP binding affinity and capacity, suggesting that ATP was masking the binding sites. Furthermore, the observed H$^+$ transport activity correlated linearly with GDP binding (5 min incubation) in mitochondria loaded with a wide range of ATP concentrations by Dowex treatment or ATP additions. Thus the previous conclusion that GDP binding is an index for the thermogenic capacity of the mitochondria [115] can be adequately explained by endogenous ATP masking of the UCP-1 sites.

A mechanism for the masking/unmasking phenomenon incorporating the ATP hypothesis and biochemical findings is illustrated in Fig. 7. In the resting state at 28°C, the mitochondria are coupled since UCP-1 is largely inhibited by endogenous ATP. The basal free FA concentrations are low [116]. A low basal thermogenic activity is maintained by the small number of free UCP-1 molecules. Upon acute cold-adaptation at 4°C or norepinephrine stimulation, FAs are released [61,116] which can both activate UCP-1 and deplete endogenous ATP by FA activation where ATP is converted to AMP. An acute and large drop in endogenous ATP concentration was reported [62,63,117,118], which would result in dissociation of prebound ATP from UCP-1, thus unmasking the binding sites. The mitochondria become uncoupled and produced less ATP. In this state most of the ATP is supplied by substrate-level phosphorylation [119], but mainly consumed for FA activation. The combined activation of UCP-1 by FA and by unmasking of the binding sites lead to a high mitochondrial conductance as observed by Nicholls group [4]. The drop in FA concentration and subsequent increase in ATP concentration [119,120] initially provided by substrate-level phosphorylation will restore the mitochondria into the coupled state.

5. Effects of amino acid reagents on nucleotide binding and transport

A variety of amino acid reagents have been used to elucidate the involvement of specific amino acids in nucleotide binding and H$^+$/Cl$^-$ transport of UCP-1. These studies have been performed on whole mitochondria, on isolated and reconstituted UCP-1 (Table 3). After isolation of UCP-1, an obvious task was probing the nucleotide binding site with amino acid reagents. A weak inhibition of GDP binding was observed with phenylglyoxal and a strong one with the aggressive lysine reagent trinitrobenzene sulfonate [10]. Most of these studies have been performed on mitochondria where nucleotide binding and H$^+$/Cl$^-$ transport were measured by the swelling methods [121,122] or also directly [71]. There were strong variations in the results depending on experimental conditions and also whether the exposure of reagents were more radical. For example, among Cys reagents the more aggressive tetranitromethane inhibited GDP binding quite strongly whereas N-ethylmaleimide (NEM) had an effect only at very high concentrations [122]. No or only marginal inhibition of H$^+$ transport was observed by various groups

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Target</th>
<th>Effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenylglyoxal</td>
<td>Arg</td>
<td>Weak inhibition of GDP binding</td>
<td>[10]</td>
</tr>
<tr>
<td>Trinitrobenzene sulfonate TNBS</td>
<td>Lys (Cys)</td>
<td>Strong inhibition of GDP binding</td>
<td>[10]</td>
</tr>
<tr>
<td>Diazobenzene sulfonate DABS</td>
<td>Lys, Cys</td>
<td>Inhibition of GDP binding; covalent incorporation of DABS</td>
<td>[71]</td>
</tr>
<tr>
<td>Tetranitromethane NEM</td>
<td>Cys</td>
<td>Differential effect on inhibition of H$^+$/Cl$^-$ transport of GDP</td>
<td>[122]</td>
</tr>
<tr>
<td>Butanedione</td>
<td>Arg</td>
<td>Inhibition of GDP, abolition of H$^+$ transport inhibition</td>
<td>[123]</td>
</tr>
<tr>
<td>Photooxidation</td>
<td>His</td>
<td>Inhibition of GDP binding</td>
<td>[10]</td>
</tr>
<tr>
<td>Diethylpyrocarbonate</td>
<td>His</td>
<td>Inhibition of GTP but not of GDP binding; specific target H214</td>
<td>[125]</td>
</tr>
<tr>
<td>Mersalyl (mercurials)</td>
<td>Cys</td>
<td>Inhibition of H$^+$ transport</td>
<td>[124]</td>
</tr>
<tr>
<td>Hydrophobic NEM derivatives</td>
<td>Cys</td>
<td>Activation of H$^+$/Cl$^-$ transport in UCP-1; Inhibition of GDP binding</td>
<td>[122]</td>
</tr>
<tr>
<td>Woodward reagent K (WRK)</td>
<td>ASP, Glu</td>
<td>Specific target E190; abolishes GTP, GDP binding</td>
<td>[104]</td>
</tr>
</tbody>
</table>
[10,71,123,121,122], whereas Jezek [124] reported inhibition with mersalyl. Possibly in this case a generalized damage to UCP-1 occurred. More effective was another arginine reagent butanedione in the inhibition of GDP binding [123]. The most specific inhibition of nucleotide binding was caused by the carboxyl modifying Woodward reagent K (WRK) [104]. Here in the isolated UCP-1 only a twofold molar excess of WRK to UCP-1 was sufficient to completely block GTP and GDP binding. Further, with WRK a specific incorporation only into one residue E190 was elucidated. WRK binding was visualized to be promoted by the sulfonate group of WRK in accordance with the particular affinity of the nucleotide pocket for sulfate. With this finding and further by mutagenesis, E190 was identified to be the predicted gatekeeper to the phosphate moiety binding pocket [56]. The His reagent diethylpyrocarbonate (DEPC) was also shown to have a highly specific target [125]. According to the prediction that this His is involved in GTP binding but not in GDP binding, DEPC inhibited GTP but not GDP binding. DEPC did not inhibit H\(^+\) or Cl\(^-\) transport and therefore, H145 and H147 are excluded as targets for DEPC, since this His pair is essential for H\(^+\) transport. Only the well conserved H214 is left as a DEPC target. Obviously, H214 has a more favorable environment for DEPC to alkylate the imidazole moiety than the other histidines.

It can be generalized that the nucleotide binding site is much more sensitive and more easily affected by amino acid reagent than H\(^+\) or Cl\(^-\) transport. The latter are insensitive to most reagents unless they are used under damaging conditions. On this line, first Kopecky et al. [71], using diazobenzene sulfonate (DABS) on mitochondria, and then Katyar and Shrago [123], using butanedione in reconstituted UCP-1, concluded from the inhibition of GDP binding and the insensitivity of H\(^+\) transport that the nucleotide does not bind at the transport site. This was shown also with WRK [104] and DEPC [125] which did not affect any transport but completely abolished nucleotide binding. The non-identity of the nucleotide binding site with the transport site is also evident from the fact that nucleotide derivatives, such as DAN-ATP and dansyl-AMP, bind to UCP-1 but do not inhibit H\(^+\) transport [33,88]. A minor effect of FAs on MgATP binding was reported by Rial et al. [48]. However, as these authors also pointed out, FAs do not act by displacing nucleotides from UCP-1. A further argument is that in our hands no competition has been observed (unpublished data) between nucleotides and FAs.

6. Heterologous expression of UCP-1

For various reasons, in particular for mutagenesis, heterologous expression of UCP-1 was attempted and performed in various laboratories. The research group of Ricquier tried to express UCP-1 in various mammalian cell lines [126,127]. In Chinese hamster ovary cells the expression of UCP-1 was detected in the mitochondrial fraction, suggesting that UCP-1 was inserted into the mitochondria in these cell lines [126]. However, the level of expression was low and unsuitable for isolation of UCP-1 and characterization of its function. Attempts to express UCP-1 in *Xenopus* oocytes failed to produce viable UCP-1 [127]. UCP-1 was also expressed in *Escherichia coli* using galactose or temperature-sensitive promoters (Bienengraeber and Klingenberg, unpublished data). In both cases high yields of UCP-1 were obtained in inclusion bodies, as found with other mitochondrial carriers expressed in *E. coli* [128–131]. The inclusion bodies contained about 70\% UCP-1. The protein could be solubilized by sarcosyl and reconstituted into vesicles, however no H\(^+\) transport activity could be regained (unpublished data).

The most successful and widely used system for UCP-1 expression so far is the *Saccharomyces cerevisiae*. The start codon in the UCP-1 gene was changed to conform with the yeast consensus sequence ATA ATG for obtaining good expression levels [132]. By removing the 5’ and 3’ non-coding sequences, expression levels of 24 \(\mu\)g UCP-1/mg mitochondrial proteins were obtained [133]. Garlid’s group [134] claimed a high expression level of 70 to 100 \(\mu\)g UCP-1/mg mitochondrial protein, after changing the sequence around the start codon to resemble the GAPDH gene. However this claim is in contradiction to the GTP binding capacity measured by the same authors which corresponds to only 26 \(\mu\)g UCP-1 per mg mitochondrial protein. Unless UCP-1 is quantified by immunoassay, GTP binding
capacity should be a good measure of UCP-1 content provided precautions are taken for unmasking UCP-1 from endogenous ATP [91].

The growth of the yeast transformants is somewhat or severely retarded after introduction of UCP-1, according to the strength of promoting UCP-1 expression [132]. These results suggest that UCP-1 in situ does some uncoupling and thus affects cell growth. However, the fact that yeast cells grow fairly well although UCP-1 is expressed indicates that the uncoupling action of UCP-1 is inhibited to a large extent by endogenous ATP. This presumption is supported by the results of Bouillaud et al. [135] that a UCP-1 mutant which does not bind nucleotides has a higher uncoupling effect on the yeast cells, as measured by flow cytometry.

From these yeast cells, mitochondria containing UCP-1 were isolated in which the uncoupling properties were assessed by respiratory control, its response to activation by FAs and inhibition by nucleotides [56,133,135]. In general, the stimulation by FAs was lower than in mitochondria from BAT. The uncoupling by UCP-1 in the yeast mitochondria was further confirmed by measurements of the intracellular mitochondrial membrane potential. H⁺ and Cl⁻ transport were assayed by mitochondrial swelling in K⁺ acetate and KCl. Phosphate had to be included to inhibit the ‘yeast uncoupling pathway’ [133]. Under these conditions GDP-sensitive swelling was observed, which could be activated by palmitate. The Cl⁻ transport dependent swelling was inhibited by GDP and not activated by palmitate similarly to what is the case in BAT mitochondria. All these observations suggest that the uncoupling and regulatory properties are preserved in UCP-1 expressed in yeast mitochondria. Measurement of GDP binding to these mitochondria revealed high and low affinity binding sites [133], similar to those observed in BAT mitochondria [48,90].

From these yeast mitochondria UCP-1 has been isolated and partially purified [56,79,134] with the hydroxyapatite procedure. The binding of dansyl-GTP and [14C]GTP to the isolated UCP-1 exhibited affinity and pH dependence [56] similar to the natural hamster UCP-1 [64,88]. After reconstitution into proteoliposomes the H⁺ transport activity was determined using pyranine as fluorescent pH probe [56], or indirectly by the K⁺ counterflow with a fluorescent cent K⁺ probe [69]. H⁺ transport rates estimated with the latter method may be too low because of other ion movements driven by K⁺. Cl⁻ transport activity was measured using Cl⁻ specific fluorescent probes [56,57].

6.1. Mapping the nucleotide binding site

Mapping the nucleotide binding site in UCP-1 with covalently interacting nucleotide derivatives should have a good chance of success as compared to the same approach in the AAC for two reasons. First, the affinity towards nucleotides is much higher in UCP-1 than in AAC [136,137]; second, the ligand tolerance for binding in UCP-1 is higher and thus UCP-1 can accept nucleotides substituted with reactive groups for covalent modification [138,139]. 8-Azido ATP and still more 2-azido ATP compete with GDP for the binding site at UCP-1. Specific photoaffinity labeling could be differentiated from the usual background by prior blocking of the nucleotide binding site with GTP. Identifying the incorporation site posed considerable difficulties due to the unusually high photolytic sensitivity and partial resistance of UCP-1 towards cleavage by proteases and BrCN [139]. Surprisingly, photoaffinity labeling by 2- and 8-azido ATP in intact BAT mitochondria was found in regions (T259 and T264) which, according to the classical model of 6 transmembrane helical folding, were supposed to be located on the matrix side [138,139]. This seemed to be in contrast to the well-defined access of nucleotides only from the cytosol. A similar paradox was noted previously with the AAC, where 8- and 2-azido ATP labeled a section located on the matrix side in the second domain [136,137]. Both 8- and 2-azido ATP are supposed not to be transported, however in AAC some transport across the membrane cannot be excluded as rigorously as in UCP-1. With 3'-O-(5-fluoro-2,4-dinitrophenyl)-ATP, a third residue, C253, could be covalently labeled, which is also located in the matrix section of the third domain near the photoaffinity labeled T264 [138].

Already in the AAC, labeling of matrix localized lysines with the membrane impermeable reagent pyridoxal phosphate led to the conclusion [140] that part of the matrix section forms a loop protruding into the translocation channel area, thus becoming acces-
sible from the c-side. Whereas in AAC evidence was obtained for an intermembrane loop in the second repeat domain, in UCP-1 the photoaffinity targets were found in the third domain, suggesting that because of the three repeat structure, intermembrane loops may occur in all three domains providing a threefold lining for the translocation channel. Together with the pH sensing residues E190 and H214 identified by mutagenesis [56,125], a total of five residues have been defined to be involved in nucleotide binding. Four of them are localized in the third domain, only E190 resides on the fourth helix in the second repeat domain. The positioning of ATP in UCP-1 suggested by these mapping results is shown in Fig. 8.

6.2. Site-directed mutagenesis

Mutagenesis of UCP-1 has been performed in three different laboratories using the yeast expression system. The work of Bouillaud and Ricquier’s laboratory concentrated on manipulations of a UCP-1 within a putative consensus sequence 261 to 269 with the DNA-binding domain of an estrogen receptor [135,141]. They argued that this region is similar to the homologous region in AAC but that it does not occur in the phosphate carrier. These residues coincide with the matrix or intermembrane loop region in the third domain which was earlier associated with the nucleotide binding site by photoaffinity labeling [138,139]. After deletion of the nine residues (UCP-1Δ9), the growth of the yeast cells became very poor, and a breakdown of the transmembrane potential could be monitored by flow cytometry [135]. This suggests that the intracellular nucleotide binding is abolished by this deletion. Deletion of the three residues F267, K268 and G269 is much less deleterious and the cells still retained considerable growth activity. In the isolated mitochondria, palmitate-activated swelling was measured without response to GDP, suggesting that the three residues may be involved in nucleotide binding. The single mutations F267Y [135] and K268Q [142] did not affect nucleotide binding. Thus, no single residue could be attributed to the nucleotide binding in this region. The large deletions may have caused structural changes which affect nucleotide binding in an unspecific manner without being part of the nucleotide binding site. Single mutations of all seven cysteines in UCP-1 showed that none of the cysteines is essential for activity [133,143]. C304 was implied in FA activation because 2-bromopalmitate severely inhibited the growth of the C304S mutant or several other mutants [144]. It was proposed that this group is involved in binding of FAs which activate H⁺ transport [144]. However, this hypothesis was later retracted since it was shown by Ricquier and Bouillaud [3], that truncated UCP-1 lacking the last 11 hydrophilic residues still retained FA-activated H⁺ transport.

The intrahelical arginines present in the second helix of each repeat domain are a striking signature of the mitochondrial carrier family and therefore not specific to UCP-1. In UCP-1 mutation and neutralization of R276 had little effect on H⁺ transport ac-
tivity but abolished inhibition by GTP [142,145]. Mutation of all three intrahelical repeat arginines in UCP-1 was found not to affect $H^+$ transport, while inhibition by GDP was abolished by all three mutations [142]. No GDP binding to isolated UCP-1 was reported for the R82Q and R182T mutants whereas surprisingly the R276L mutant fully retained nucleotide binding [142]. However, this finding could not be confirmed by work from our laboratory (see below) in which also the R276Q mutant displayed no binding activity (Echtay and Klingenberg, unpublished data).

It is important to note that all three Arg mutants are expressed to a level of only 50% of the wild-type as assayed by quantitative enzyme-linked immuno-sorbent assay (ELISA) (Bienengraeber and Klingenberg, unpublished data). This resembles the much stronger inhibition of expression of the yeast AAC2 caused by these mutations [146–148], indicating that the main function of these arginines typical for the mitochondrial carrier family resides in the biogenesis process. Possibly, with their positive charge the arginines may be required, during the import of UCP-1, for the interaction with one of the outer or inner membrane transport factors. The loss of nucleotide affinity may be caused only indirectly by replacement of the arginines rather than indicating their participation in the binding process.

The rare case where site-directed mutagenesis strikingly confirms a predicted residue function was the identification of the postulated pH-regulating residues for nucleotide binding. As discussed above, based on the pH dependence two $H^+$ dissociating groups in UCP-1 were proposed to be involved in nucleotide binding, a carboxyl group with a low $pK_a$, regulating access of both NDP and NTP, and a His group with a $pK_a \approx 7.2$ regulating only NTP binding [64,88]. With the help of the highly specific Woodward reagent K, the carboxyl group was identified as E190 located within the fourth helix [104]. Mutational neutralization [56] in E190N largely abolished the pH dependence of nucleotide binding and increased the affinity at $pH > 7$, exactly as would be predicted from the role of E190 as a gatekeeper for the phosphate moiety binding niche. Accordingly, the inhibition relief of $H^+$ transport by high pH was abolished. Mutation of H214 within helix 5 and possibly quite close to E190 decreased pH dependence of binding only for NTP but not for NDP [79], in accordance with the prediction that this His is involved in interaction only with the $\gamma$-phosphate. However, different from the prediction, the binding is retained on replacement of the His with Glu in H214N and therefore it was concluded that H214 in the protonated form (His$H^+$) is not interacting with the $\gamma$-phosphate, but rather that in the neutral form it protrudes into the $\gamma$-phosphate region. On lowering the pH, the His$H^+$ is assumed to be retracted by a background negative charge (Fig. 6). Therefore, the substitution of this His by a smaller residue as in H214N does not prevent binding whereas substitution by a larger residue such as Trp in H214W strongly decreases the affinity for NTP but not for NDP [125]. It should be pointed out that mutation of neither E190 nor H214 affects $H^+$ or $Cl^-$ transport, although both are intrahelical $H^+$ dissociating groups. The results also stress that not only the nucleotide binding site but also its regulation by $H^+$ dissociation is segregated from the groups involved in the $H^+$ translocation. It should be noted that E190 and H214 are conserved in UCP-1 from all sources found so far as well as in the UCP-2 [5] and UCP-3 [6,149] isoforms, indicating that they may share a similar pH control of nucleotide binding.

A His pair, H145 and H147, is located in the second domain in the middle of the hydrophilic matrix region. Replacement of the His in H147N decreased $H^+$ transport drastically to only about 20%, while $Cl^-$ transport activity and nucleotide binding were both unaffected [79]. The double mutant H145Q+H147N decreased $H^+$ transport to only 12%. Interestingly, these two His are absent in UCP-2 and UCP-3 and are replaced by one Arg, with the exception of human UCP-3 where the homolog to H145 is retained. Another potential $H^+$ donor/acceptor conserved in all UCP versions is D27 localized in the first transmembrane helix. It is highly characteristic for UCP; at the same position a rare intrahelical Lys is localized as a marker for AAC. This Lys has been found to be essential for AAC transport activity [146]. In UCP-1 mutational neutralization in D27N drastically reduced $H^+$ transport activity to about 20% but did not markedly affect $Cl^-$ transport (Echtay and Klingenberg, unpublished data). Further, GTP binding was still retained to about 60%. By these studies at least two
H⁺ accepting groups have been identified which can be linked to H⁺ transport.

Other acidic and basic residues were converted into neutral residues with varying defects on the GTP binding [56,79,111,135,145]. Summarizing the results from mutational studies on about 30 different charged residues, it can be concluded that the hit rate of affecting functionality is high. In general, the nucleotide binding site is more sensitive to mutations than the H⁺ or Cl⁻ transport. Typical for mitochondrial carriers, UCP-1 within its comparatively small size of 306 residues has little redundancy but a well equilibrated network of charged residues necessary for the uncoupling and regulatory functions. The same experience of an extraordinary high mutational hit rate has been made previously with the AAC [146–148].

7. Outlook

There are two major avenues of interest on UCP-1, the mechanism of H⁺ transport and its physiological role and regulation. Despite the progress reported in this review, there are still large lacunae in both fields. Within the realms of H⁺ transport mechanisms explored in many other systems, UCP-1 excels by its dependence on FAs which are interesting, but difficult to elucidate ligands. Whereas site-directed mutagenesis may assist at the present stage in ruling out alternatives of H⁺ transport models, a more penetrating insight can only come from the knowledge of the three-dimensional structure. Many years of attempts in several laboratories to solve the structure of the homologous AAC have failed so far. Actually, up to now no three-dimensional structure for any transporter is known.

The physiological role of UCP-1 and its regulation in the thermogenic BAT is still a vexing problem which has come again into focus by the discovery of UCP-2 and UCP-3 in other tissues. Regulation of H⁺ transport by FA is obvious for UCP-1 and also inferred from the close relationship of the UCP-2 and UCP-3 expression with the FA levels [150–152]. Although the mechanism of function of UCP-2 and UCP-3 is still unknown, there are good indications in the sequence structure that both are similar to UCP-1. Once their function is also elucidated, a comparison between all UCP-1 will give a positive feedback also for understanding the role of UCP-1.

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References