Uncoupling Protein—A Useful Energy Dissipator[1]

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The structure/ function relationship in the uncoupling proteins (UCP) is reviewed, stressing UCP from brown adipose tissue (UCP1) since, so far, nearly no biochemistry is known for the UCP variants UCP2, UCP3, and UCP4. The transport for H^+ and Cl^- and its dependence on fatty acids in reconstituted vesicles is described. The inhibition and binding of nucleotides to UCP1, in particular, the pH dependence and two-stage binding are analyzed. A model for the role of fatty acid in H^+ transport is shown. The role of specific residues in UCP1 is analyzed by directed mutagenesis in a yeast expression system. The different regulation by the cellular energy potential of UCP1 versus UCP3 is discussed.

KEY WORDS: UCP; adipose tissue; fatty acids; nucleotides.

INTRODUCTION

The present review will state our present knowledge of the uncoupling protein, stressing the results from our laboratory. Besides UCP1 from brown adipose tissue (BAT), some results on UCP3 will also be included. A broad review on the "Structure and Function of the Uncoupling Protein from Brown Adipose Tissue" was recently published (Klingenberg and Huang, 1999.)

The classical uncoupling protein (UCP1) has been associated with the thermogenesis of brown adipose tissue and was found to be exclusively confined to this tissue. Only few laboratories were engaged in the research on UCP1. The recent discovery of the existence of at least three additional proteins similar to UCP1 has increased interest in this field. However, despite major efforts to understand the function of these new uncoupling proteins (UCP2, UCP3, and UCP4), we know quite little of their functions, for reasons which will be discussed below. In contrast,

more than 20 years research on UCP1 has provided quite penetrating insight into its function and its regulation. Remarkable advances have been obtained, more recently, on the structure–function relationships in UCP1. For this reason, the present brief account of the biochemistry of uncoupling proteins will have to concentrate primarily on UCP1.

In brown adipose tissue (BAT), heat generation is not a byproduct of metabolism but its main function. The main component responsible for this heat generation is UCP1, which short-circuits the protons generated by the respiratory chain. Thus, oxidative phosphorylation is bypassed and the combustion energy degraded or dissipated into heat, similar to the way an uncoupler acts *in vitro* on mitochondria. Therefore, we christened the major component responsible for the heat generation "uncoupling protein," although the term "thermogenin" has also been used. Several features of the uncoupling function and its regulation were first uncovered in mitochondria from BAT (Nicholls, 1979), but a more quantitative and precise elucidation became possible only after isolation and reconstitution of UCP1. At this stage, structural studies on UCP also commenced, which in recent years, arrived at an elucidation of the structure–function relationships. Since research on UCP1 in our laboratory started with the isolation of the intact UCP1

Lin and Klingenber, 1980, 1982, we will concentrate on the results of UCP1 research, with some reference to corresponding functions determined on mitochondria.

STRUCTURE

UCP is a member of the mitochondrial carrier family. Upon amino acid sequencing, a surprising similarity to the AAC was found (Aquila *et al.*, 1985) and, with the subsequent sequencing of the mitochondrial phosphate carrier the existence of a mitochondrial carrier family was deduced (Aquila *et al.*, 1987). The main structural features identified thus far are briefly summarized: UCP1 has six transmembrane helices with both the C- and N-terminal facing the cytosolic side (Fig. 1). Evidence for this topology comes from

antibodies against the N-terminal (Miroux *et al.*, 1992) and crosslinking of a C-terminal cysteine by Cu^{2+} phenanthroline only from the outside (Klingenberg and Appel, 1988). The sequence is divided into three similar domains: three repeats containing about 100 residues each with two transmembrane helixes. The two helixes are separated by an average of 40-residue long, highly hydrophilic sequences located at the matrix side. Among these stretches the arrangement of charged residues has striking similarities and also provide the frequently quoted PAXX + X+ motif (Palmier *et al.*, 1992), characteristic for the mitochondrial carrier family. Based on various types of evidence first obtained in the AAC (Bogner *et al.*, 1986; Mayinger *et al.*, 1989) and then also for the UCP, within the about 40-residue long matrix, a section of about 18 residues is suggested to form a loop into the membrane
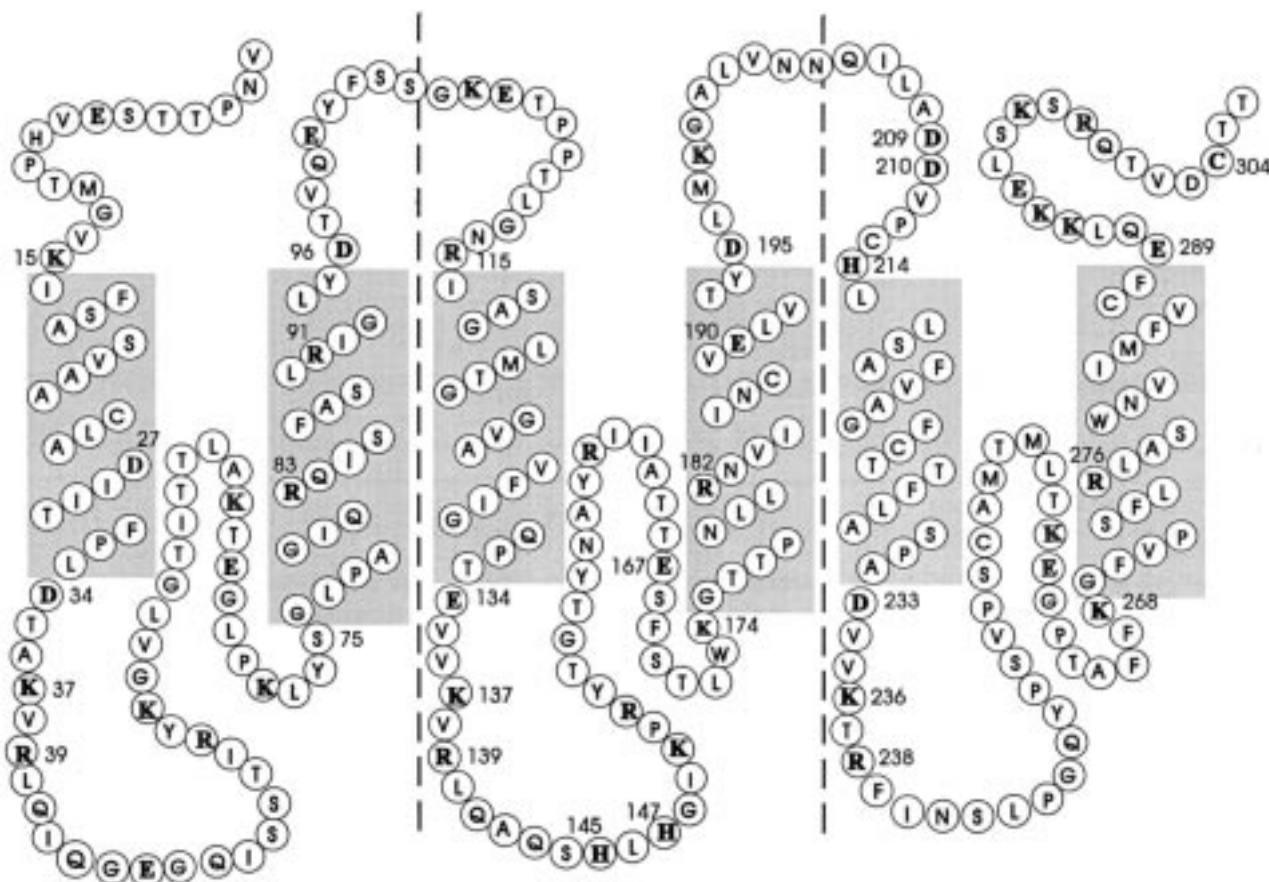

Fig. 1. Folding diagram of UCP1 from hamster brown adipose tissue. The transmembrane organization is derived from topological studies and from assigning charged residues to delimit the transmembrane helices. The total structure is composed of three similar repeat domains. Within each domain an intermembrane loop is assumed to protrude into the membrane space. The charged residues are marked with bold letters.

region (Winkler and Klingenberg, 1992). These loops are visualized to line the translocation path and play a critical role in the actual translocation event. As will be discussed below, the matrix-directed section is probably involved in the specific selection and translocation of H^+, whereas the cytosol-directed section of the translocation channel is suggested to form an aqueous pore.

TRANSPORT BY UCP

H^+ Transport

The uncoupling function and concomitant thermogenesis in BAT is generally accepted to be caused by H^+ transport through the inner mitochondrial membrane. Although this is clear for UCP1, a distinct H^+ transport function for the isoforms UCP2 and UCP3 has not yet been demonstrated. The correlation of the uncoupling in BAT mitochondria to UCP1 was unraveled via the inhibition of uncoupling by purine nucleotides (Rafael et al., 1969). In particular, GDP was used for inhibition and for binding because it did not interfere with the oxidative phosphorylation system, although the natural inhibitor is probably ATP because of its cellular abundance. The first identification of the GDP binding protein was by photoaffinity labeling with 8-azido-ATP in SDS gels (Heaton et al., 1987). The molecular weight of around 30 kD very similar to that of the ADP/ATP carrier, and the capability to bind purine nucleotides triggered the isolation of UCP, according to similar methods previously introduced for the isolation of the ADP/ATP carrier (Lin and Klingenberg, 1980, 1982). With Triton X-100, a stable UCP1 was isolated. It forms a dimer surrounded by about 170 detergent molecules. UCP1 in this form has served primarily to elucidate the binding of nucleotides (Lin et al., 1980).

For measuring the transport properties of UCP in the reconstituted vesicles, another detergent, $C_{10}E_5$ was introduced in which UCP1 is less stable but which permits the generation of H^+ and Cl^- impermeant stable reconstituted phospholipid vesicles (Klingenberg and Winkler, 1985; Winkler and Klingenberg, 1992; Strielmen et al., 1985). The capability of binding nucleotides and the inhibition of transport by nucleotides was a strict criterion to differentiate UCP-dependent ion fluxes from other background fluxes. A reconstituted system affords the best conditions to directly measure H^+ transport by UCP and to identify

the parameters governing the H^+ transport activity. In mitochondria, measurements of UCP transport activities relied on the swelling with appropriate salts (Nicholls and Lindberg, 1973; Locke and Nicholl, 1981; Kopecky et al., 1984; Rial et al., 1983) where complications might distort the quantitative determinations. H^+ transport by UCP depends on the presence of free fatty acids (FAs). Although this was shown first with isolated mitochondria (Locke and Nicholl, 1981), the physiological role of FA activation has recently been contested (Mar González-Barroso et al., 1998). In the reconstituted system an absolute dependence on FA can be demonstrated, once traces of endogenous FA have been removed during the reconstitution (Winkler and Klingenberg, 1994). In mitochondria there may be a constant supply of small amounts of FAs, which cannot completely be trapped by serum albumin. For activation a minimum FA chain length of C_{10} or more is required. In general, the specificity requirements for fatty acids are low. Unsaturated FAs are also good activators. Substitutions with hydrophilic groups, such as hydroxy or bromo either in ω- or β position, still permit fair to high activities. Even larger substituents such as doxyl (spin label) did not markedly inhibit the activation capability (Winkler and Klingenberg, 1994). A maximum activation of H^+ transport requires large amounts of FAs. Up to 8 molar % of oleate or laurate to phospholipids accumulated in the vesicles for maximum H^+ transport without causing unspecific H^+ leakage, as evidenced by the inhibition of its GDP. Probably fatty acids are recruited by UCP from the lipid phase and, in line with this structural indifference, it is assumed that the hydrophobic moiety largely remains in the lipid phase of the bilayer.

H^+ transport was found in the reconstituted system to be linearly dependent on the $\Delta\Psi$ adjusted by an increasing K^+ gradient in the valinomycin system (Klingenberg and Winkler, 1985; Winkler and Klingenberg, 1992). Also in mitochondria up to $\Delta\psi = 100$ mV, a linear relation between H^+ transport activity and $\Delta\psi$ was observed (Nicholls, 1977). At higher membrane potential, the conductivity appears to increase nonlinearly and becomes GDP insensitive, although it partially depends on fatty acids (Nicholls, 1977). At variance, we assume that H^+ leakage at this high $\Delta\psi$ region is a more unspecific effect and not essential for regulation of H^+ transport in BAT. It can be argued that in BAT a low $\Delta\psi$ prevails during thermogenesis (La Noue et al., 1986) and thus a linear relation of H^+ transport current to $\Delta\psi$ is relevant for regulation.

Anion Transport

UCP1 has a remarkable anion transport capability. It is best characterized by Cl^- transport, but a number of other small anions are being tolerated (Nicholls and Lindberg, 1973). This anion transport is also inhibited by GDP. However, no FAs are needed for activation. On the contrary, inhibition of anion transport by FAs has been claimed (Jezek *et al.*, 1994). At variance, in studies, by Rial and Nicholls (Rial *et al.*, 1983) and by us (unpublished data), no competition has been found both with mitochondria and reconstituted UCP. The discrepancy may be explained by the fact that FA activated H^+ transport and concurrent Cl^- transport compete for the limited capacity of membrane diffusion potential under certain conditions. H^+ transport is much more active and can suppress the about 5- to 20-fold slower Cl^- transport. A strong competition is observed with Cl^- transport by alkyl sulfonates in BAT mitochondria and in the reconstituted vesicles (Jezek and Garlid, 1990). Short-chain (C_3–C_6) alkyl sulfonates are taken up in mitochondria probably by the small anion pathway of UCP1 (Sanchis *et al.*, 1998). Medium-chain (C_{11}) alkyl sulfonate inhibits the fatty acid-stimulated H^+ transport (Garlid *et al.*, 1996) and this effect is argued to be due to the competitive usage of the putative FA anion transport path in UCP. SDS also inhibits H^+ transport, although it is not transported by UCP (unpublished results). This argues that the inhibition by C_{11}-sulfonate occurs by competitive removal of FAs rather than competitive transport and also raises the question whether H^+ and Cl^- transport use different pathways within UCP, as might be suggested by the independence and noninterference of FA with Cl^- transport. Although this cannot be excluded at present, the major consensus argues for a common channel analogous to occurrence of common H^+ and CL^- pathways in the halobacterial retinal proteins. The selectivity for H^+ and Cl^- can here be modulated through elimination or introduction of aspartyl groups.

Mechanisms of H^+ Transport and the Role of Fatty Acids

In several respects, UCP1 is an elementary H^+ transporter. It does not involve H^+ substrate cotransport or requires counterexchanges as in the H^+–Na^+ exchanger. It does not involve ATP-driven machinery. H^+ transport in UCP is driven only by the membrane potential, similar to that in ion channels. Yet, UCP acts more like a carrier than a channel. The mechanism of H^+ translocation is different from a channel-type membrane potential-driven transport of other small ions because of the very low H^+ concentrations and the use of chains of H_2O molecules, along which H^+ are conducted. Higher effective H^+ concentrations for a sufficient H^+ transport rate are created by the installation of H^+ donor/acceptor groups within the translocation channel. These are mostly carboxyl groups of which the pK_a can be modified in a wide range by the environment. Within this general scheme, the role of FAs in UCP is visualized to provide one or more carboxyl groups along the translocation channel, thus facilitating the H^+ transport (Winkler and Klingenberg, 1994). In addition to resident H^+ donor/acceptor groups, FA may fill one or more gaps in the translocation channel. Such an arrangement permits the regulation of uncoupling activity by UCP through changes of the FA concentration. For a mobile oscillation of carboxyl groups within the translocation channel, resident H^+ translocation groups have also been discussed by Rial *et al.* The FA carboxyl groups may oscillate between resident H^+ translocating groups.

In a modified version (Klingenberg and Huang, 1999), the FA headgroup oscillates from the aqueous phase into the protein channel and delivers there H^+ to an acceptor group, which, in turn delivers H^+ into the matrix (Fig. 2). UCP facilitates the return of the undissociated FA to the cytosolic site for another H^+ delivery cycle. The high concentration needed for saturating H^+ transport indicates only loose binding of FA to UCP and a rapid distribution of FA between the lipid and hydrophilic phases of UCP. The cytosolic section of the H^+ path may be a relatively unspecific water channel where the protein provides a favorable environment for the movement of the carboxyl groups toward the H^+ acceptor. The matrix oriented section of the path is suggested to be under control of the nucleotide-induced conformation change by UCP. This mechanism also accommodates the structural model with three loops protruding from the matrix into the membrane area. Here the path may provide "a selection filter" analogous to the structure recently described for the K^+ channel.

There are other variant models of the role of FA in UCP. FA can be visualized to be an effector rather than a cofactor by inducing a conformational change (Rial *et al.*, 1983). A surface membrane potential effect
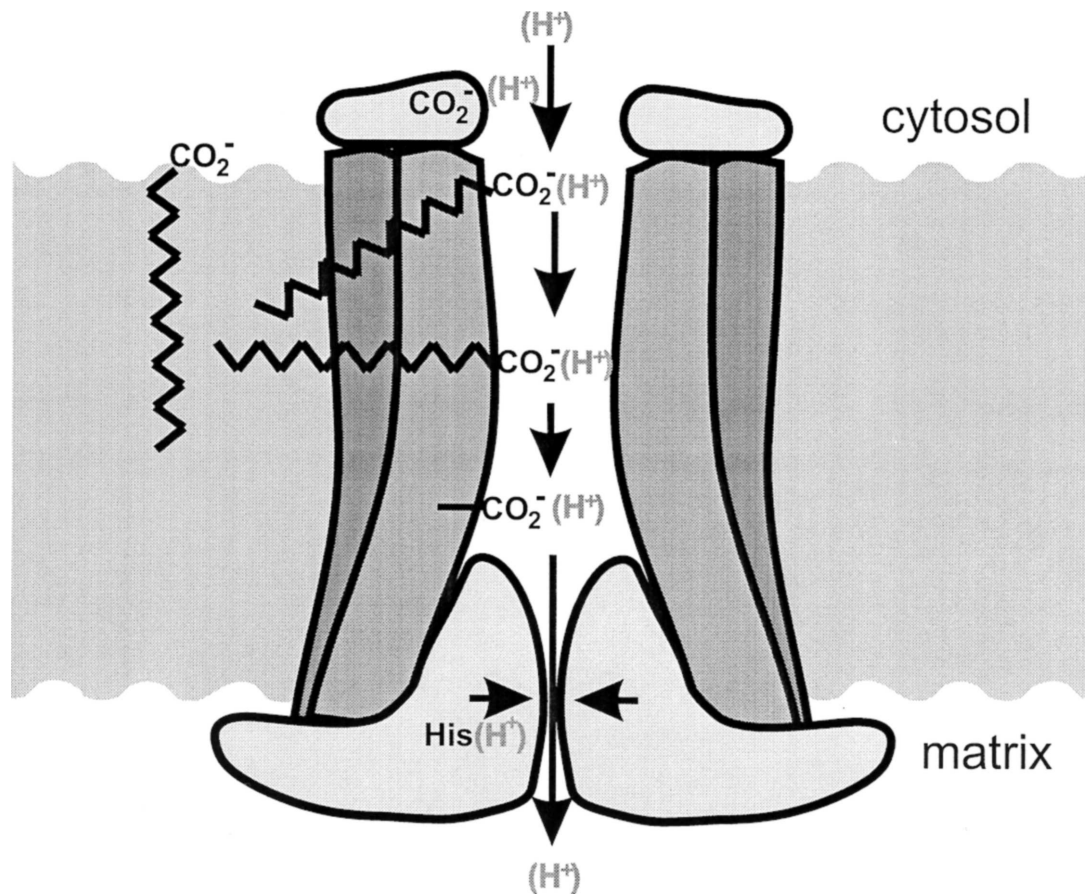
Fig. 2. A model of the mechanism of H^+ transport by UCP1 and the role of fatty acid. The fatty acid is visualized to penetrate from the lipid phase with its carboxy group into the H^+ translocation path. Here it acts as H^+ donor/acceptor between resident carboxyl groups of UCP1. The H^+ transport path 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.

caused by FA was also discussed (Rial *et al.*, 1983). As an alternative, the anion transport model of FA has received wide attention (Jezek *et al.*, 1994). The tenet of this model is the function of UCP as transporter for fatty acid anions rather than for H^+. The H^+ transport occurs via the flip-flop, as undissociated fatty acids through the lipid bilayer and the FA anion return via the UCP. The main support for this model according to Garlid and his co-workers, comes from the translocation of alkyl sulfonates by UCP (Jezek and Garlid, 1990; Jezek *et al.*, 1990), which are considered to be models of fatty acid anions. However, the competitive inhibition by C_{11} sulfonate of H^+ transport with the same chain-length lauric acid is poor (Jesek *et al.*, 1994). In addition the high amounts of GDP needed

for inhibiting the C_{11} sulfonate effects indicate that much of the C_{11} sulfonate may be transported by another path, for example, together with K^+ valinomycin as a ternary complex. A further problem with this model is the inability of short chain-FA to catalyze H^+ transport in UCP1. They should translocate as anions even easier through the channel of UCP1. Garlid *et al.* (1996) proposed, partially in response to this problem, that the FA anion is translocated at the protein± lipid interface.

The pH dependence of H^+ transport is another source for understanding the role of FA. H^+ transport has a clear maximum, which depends on the chain length of the FA used (Winkler and Klingenberg, unpublished results). Typically, H^+ transport is low at

low pH and increases quite strongly to a pH maximum, which depends on the FA employed (Fig. 3). The pH dependence is somewhat different for H^+ uptake than for H^+ release. The pH maximum increases strongly with the chain length of the FA, from $pH_{max} = 6.2$ with C_{10} to $pH_{max} = 8.2$ with C_{18} (oleic). The increase of the pH_{max} parallels the increase of the pK of fatty acids with the chain length when bound to a membrane. This correlation shows that for effective H^+ transport, a balance of dissociated and undissociated FA must exist. Further, the external rather than the internal pH dominantly controls the pH dependence. The fact that most of the UCP are incorporated rightside outside (Winkler and Klingenberg, 1992) indicates that the FA, localized in the outer leaflet of the bilayer, are of primary importance for the H^+ transport activation. Both the dependence of the pH maximum on the fatty acid chain length and the dependence primarily on the external pH are thus in support of the role of FA in the cytosol-oriented section of the H^+ pathway (Fig. 2).

Additional support for this model comes from the finding that azide can replace FA for H^+ transport activation (Winkler, Echtay, and Klingenberg, unpublished). Azide can be transported as other small anions by UCP and, because of its high pK_a, it can recycle
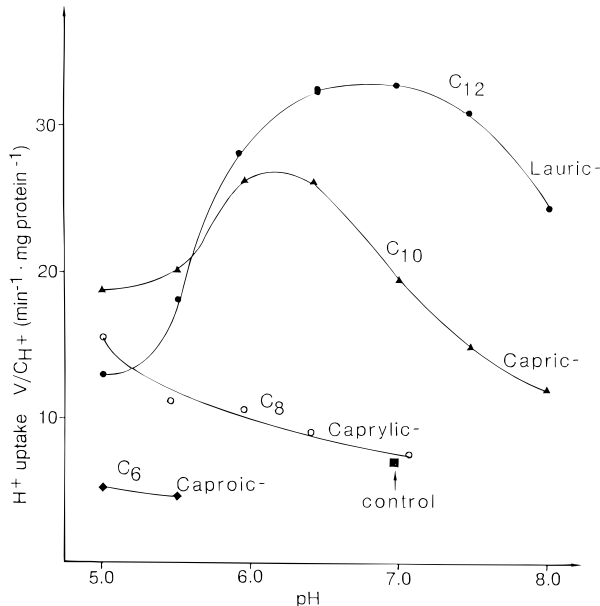

Fig. 3. pH Dependence of H^+ uptake by the reconstituted UCP1. The experiment illustrates the strong dependence of the H^+ transport rate on the fatty acid chain length. It further illustrates the pH dependence of H^+ transport and the strong increase of the pH maximum with the fatty acid chain length. Experimental conditions as in (Winkler and Klingenberg, 1994) (unpublished results).

as undissociated acid through the membrane, thus forming an acid/anion cycle. However, the rate of H^+ transport measured with azide is about 10 times higher than the rate-limiting anion transport through UCP1. This high rate of H^+ transport activated by azide indicates that azide acts similar to FA as a local catalyst within UCP. Stimulation of H^+ transport by azide has been previously observed in bacteriorhodopsin mutants in which an aspartate group has been eliminated (Tittor *et al.*, 1989). This again supports the postulate that azide like fatty acids substitutes for missing H^+ catalyzing carboxyl groups in the H^+ translocation channel. Strong support for the catalytic role of azide comes from the inability to stimulate H^+ transport in those mutants of UCP1 (H145Q, H147N) where the H^+ transport is largely abolished. The same mutants, however, are able to transport small anions like chloride. On the other hand, a mutant of UCP in which chloride, but not H^+ transport, has been decreased also still displays full azide-stimulated H^+ transport.

NUCLEOTIDE BINDING

The inhibition of H^+ transport by purine nucleotides has been a key to track down the source of uncoupling in brown adipose issue. After discovering the recoupling of respiration by GDP and the detection of a GDP binding site on the outer surface of the inner membrane (Rafael and Heldt, 1976; Nicholls, 1976), a protein component could be identified by photoaffinity labeling with azido ATP, which then led to the isolation of UCP1 (Heaton *et al.*, 1978). The binding to UCP1 by nucleotides has been investigated on the isolated protein in great detail (Lin and Klingenberg, 1982; Klingenberg, 1988; Huang and Klingenberg, 1995b, 1996; Huang *et al.*, 1998). The particular characteristics of the nucleotide binding gives important clues of how H^+ transport and thus thermogenesis is regulated. In studies with mitochondria, GDP and GTP were preferred to ADP and ATP because these allow to segregate binding and inhibition from the interference by the oxidative phosphorylation system, including the ADP/ATP carrier. UCP1 accepts a broader range of nucleotides than the AAC, with a strong preference for the purine nucleotides. GDP and GTP bind with somewhat higher affinity than ATP and ADP (Klingenberg, 1988) although within the BAT, because of its abundance ATP, should be the primary inhibitory ligand. There are contrasting features of nucleotide binding between UCP1 and AAC. UCP1 is tolerant to

purine nucleotides, whereas AAC is highly specific only for ADP and ATP. In UCP1, nucleotide binding causes inhibition of transport activity, while in AAC the nucleotides activate their own transport. Binding affinity of the nucleotides is much higher in UCP1 than in AAC. In UCP1, nucleotides bind only from the c-side whereas in AAC binding occurs from both sides. Nucleotide binding transforms UCP into a more rigid state while nucleotides loosen the AAC structure, as evidenced by increased sensitivity to proteases. This is in agreement with the induced transition fit theory for carriers (Klingenberg, 1991) according to which the binding causes a strong conformation change by inducing a substrate fit of the binding center in the transition state. The intrinsic binding energy in the transition state is compensated largely by the energy required for the conformation change resulting in a low binding affinity. In UCP, however, the intrinsic binding energy is largely recovered in the binding affinity because the binding site is *a priori* largely adapted to the nucleotide structure.

Fluorescent nucleotide derivatives such as dimethylaminonaphthoyl (DAN) and dansyl nucleotides bind to UCP1 with a strongly enhanced fluorescence (Huang and Klingenberg, 1995; 1996; Klingenberg, 1984). Both equilibrium and kinetic binding studies are made possible with these derivatives. It is interesting to note that, in contrast to UCP, at the AAC the DAN nucleotides bind only from the matrix side.

Measurements of the binding capacity of UCP, both with radiolabeled nucleotides or fluorescent nucleotides, give a maximum of 16 μmol/g protein, which corresponds to the binding of one molecule nucleotide per UCP dimer (Lui and Klingenberg, 1982). However, there are variant reports of binding stoichiometry of two GDP per UCP1 dimer, based on immunological quantitation (Feil and Rafael, 1994). Intrinsic tryptophan fluorescent quenching by nucleotide binding of UCP using ATP is compatible with one dimer stoichiometry, in contrast to reports of two-dimer stoichiometry which, however, disregards internal quenching by GDP (French *et al.*, 1988). The binding of one nucleotide per dimer indicates anticooperativity between the two subunits induced by the nucleotide binding. Similar anticooperativity was observed in the AAC with the specific inhibitors, carboxyatractylate and bongkrekate.

Two-Stage Nucleotide Binding

Several phenomena can be only explained by assuming that there is a loose binding followed by slow transition to a tight binding state of the nucleotides (Huang and Klingenberg, 1996). The fast kinetics of initial binding can be resolved by using fluorescent nucleotides and the subsequent slow transition to the tight complex by anion exchanger, which separate the free nucleotides from the very slow dissociating tight complexes. The transition rate to the tight complexes is about 10^{-3} slower than the initial binding to a loose complex (Huang *et al.*, 1998). This slow rate indicates a major conformational change of UCP induced by the nucleotide. In fact, a tightening of the structure could be inferred from the sensitivity of UCP toward tryptic digestion (Eckerskorn and Klingenberg, 1987), which could be quantitatively correlated to the extent the tight complex was formed under the influence of various nucleotide derivatives (Huang and Klingenberg, 1996). Of greatest importance is the correlation of tight binding state of UCP1 with the inhibition of H^+ transport. The inhibition by various nucleotides of H^+ transport also correlates well with the ability to protect against the trypsin digestion (Huang and Klingenberg, 1996). Some of the fluorescent nucleotide derivatives such as DAN–ADP and DAN–ATP bind fully to the UCP1, however, do not or only partially inhibit H^+ transport. With these derivatives, binding remains in the loose complex, i.e., the distribution equilibrium between loose and tight complexes is more in favor of the loose complex whereas with the natural nucleotides the equilibrium is strongly in favor of the tight complex. In line with the two-stage model, excess of the loose binding dansyl AMP could even competitively reactivate H^+ transport in reconstituted UCP inhibited by ATP.

pH Dependence of Binding

A most important feature of nucleotide binding to UCP is the strong pH dependence. It is best observed with the isolated protein (Klingenberg, 1988), but less clear in mitochondria (Klingenberg, 1993) because of the masking by endogenous ATP (see below). Both the nucleotide di- and triphosphate exhibit strong pH dependency, but with well-defined differences (Klingenberg, 1988; Huang and Klingenberg, 1996). This can be most clearly analyzed in a pK_d/pH diagram, which shows a downturn at the curve at around pH 6.8. The slope changes to minus 1, but only with the triphosphate does the slope above pH 7.1 become even steeper to minus 2. The pH dependence was interpreted as an interplay of H^+ dissociation of the terminal nucleotide phosphate, and two ionizable residues in UCP with a $pK = 4.0$ and 7.2. The binding niche

for the phosphate was proposed to exist in a binding and nonbinding state, depending on the protonation of a putative Glu or Asp residue (Fig. 4). In the nonbinding state, this group forms an internal ion pair locking the binding pocket. Only on protonization, does the gate open to accept the ligand. Analysis of the binding revealed a 50-fold stronger affinity for the unprotonated nucleotides, such as NTP^{4-} and NDP^{3-} over the protonated form. A second residue within UCP, with a pK of 7.2, contributes to the pH dependency for NTP only. Upon protonation of the putative His residue, the binding niche is further enlarged to create space for accommodating the γ-phosphate of NTP. Thus the protonation of the Glu and of His superimpose to a slope of minus 2 in the pK_d/pH plot. As shown below, this model was confirmed and extended by mutagenesis and identification of the involved residues.

The pH dependence probably plays a major role in relieving the inhibition of H^+ transport by nucleotides. At higher pH, the nucleotide binding becomes increasingly limited by the availability of UCP1 in the binding state. In particular, the binding of the physiologically major nucleotide ATP is drastically diminished at increasing pH. Whether and how the pH is increased in the cytosol is unknown. Support comes from a report that in perfused liver, the cytosolic pH increases on rapid oxidation of medium-chain fatty acids (Soboll *et al.*, 1984).

Masking/Unmasking

Various reports suggested that nucleotide binding sites increased in short-term warm to cold adaptation or
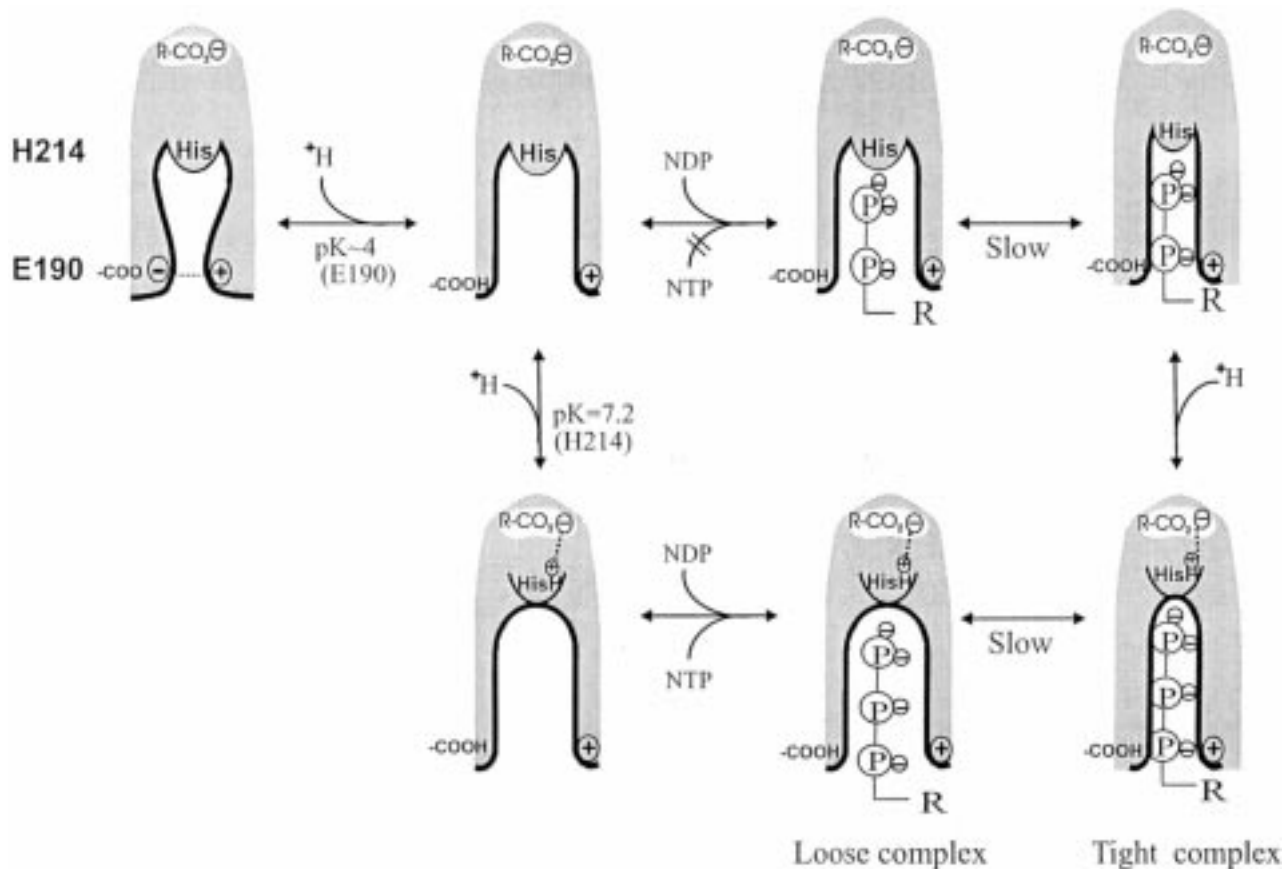

Fig. 4. Model for the pH control and the two-stage nucleotide binding. The phosphate binding niche is under control of two pH sensors. E190 forms a gate at the entrance, which opens on protonation of E190 by releaving an ionic bridge. At the bottom of the niche, H214 regulates the access of the γ-phosphate and thus the binding of the nucleoside triphosphate only. Neutral H214 protrudes into the niche, thus preventing NTP binding. On protonation, H214 is retracted by D209. The nucleotide binding has at least two distinct stages, a loose binding, where H^+ transport is not inhibited and after a slow transition, a tight binding, induced by a major conformation change. From (Klingenberg and Huang; 1999).

with β_3-noradrenoreceptor agonists (Swick and Swick, 1986; Swick *et al.*, 1986; Nedergaard and Cannon, 1987; Milner *et al.*, 1988; Peachy *et al.*, 1988). This was interpreted as an unmasking of binding sites. The reason for the masking, however, remained unknown. More recent work in our laboratory showed that endogenous ATP, tightly bound to UCP1, is occupying a part of the UCP molecules in mitochondria and thus decreases the binding of external nucleotides (Huang and Klingenberg, 1995a). The reason is the extremely slow dissociation of ATP from UCP and, therefore, endogenous ATP remains bound on isolation. Further, in most binding measurements, incubation with the labeled nucleotide have been too short to reach equilibrium because of the slow endogenous ATP dissociation. Pretreatment of mitochondria with anion exchanger and prolonged binding incubation unmasked the binding sites. The masking could be reproduced by addition of low external concentration of ATP. Obviously UCP1 from acutely warm-adapted hamsters retains more endogenous ATP than from the cold control.

Mapping the Nucleotide-Binding Site

Profiting from the relatively high tolerance of UCP1 toward nucleotide derivatives, covalently labeled derivatives were incorporated into UCP for identifying the nucleotide-binding sites. With 8-azido-ATP and still more specifically 2-azido-ATP, radiolabel could be inserted into UCP by photoaffinity labeling (Winkler and Klingenberg, 1992; Mayinger and Klingenberg, 1992). Both types of azido ATP incorporated in the last repeat domain. Surprisingly the covalently labeled residues, T259 and T264, were located in regions that were supposed to be located on the matrix site, although nucleotides bind exclusively from the c-side. With fluorodinitrophenyl-ATP, a cysteine C253 was covalently attacked which is also located on the matrix site near the photoaffinity labeled residues (Mayinger and Klingenberg, 1992). Similarly, previous in the AAC with azido-ATP (Mayinger *et al.*, 1989) or with pyridoxal phosphate (Bogner *et al.*, 1986), residues also in the matrix-localized section (however, in the second repeat domain) were labeled. These data led to the conclusion that part of the matrix section protrudes as a loop into the membrane area lining the translocation path and thus becomes accessible from the c-side.

Further, residues interacting with the nucleotides were identified by amino acid reagents and by mutagenesis. From the pH dependence of binding, a carboxyl group was predicted to be at the gate of the phosphate moiety binding niche (Klingenberg, 1988).

The Woodward reagent K (WRK) at a molar ratio as low as of two to the UCP1 inhibited largely nucleotide binding (Winkler *et al.*, 1996). Apparently the binding niche for nucleotides also preferentially accepts the anionic WRK, where it has time to react. By reducing the adduct with [^{3}H]BH$_3$ it was possible to identify the radiolabel incorporation at E190. In addition, the role of a predicted histidine as a pH sensor for the nucleoside triphosphate binding only, was verified by using the reagent diethylpyrocarbonate (Echtay *et al.*, 1998). It inhibited nucleoside triphosphate binding much more than diphosphate binding. H214 at the cytosolic surface was then identified by mutagenesis as the nucleoside triphosphate pH sensor.

MUTAGENESIS OF UCP1

The heterologous expression of hamster UCP1 in yeast permitted the manipulation of a number of residues by mutagenesis identifying their role in transport and nucleotide binding. Ricquier and co-workers performed primarily mutagenesis on a proposed nucleotide-binding region (Bouilland *et al.*, 1992, 1994) whereas we and also Garlid's group (Moudriansky *et al.*; Murdza-Inglis *et al.*, 1994) concentrated on single residue mutagenesis by neutralizing primarily charged residues in various parts of UCP1. In our work, residues were identified by mutagenic charge neutralization, which affected (1) primarily H$^+$ transport but less Cl$^-$ and nucleotide binding, (2) other residues, which affected primarily Cl$^-$ transport, (3) residues essential for nucleotide binding, (4) those which drastically change the pH dependence of nucleotide binding without inhibiting it.

A striking pair of two His (H145 and H147), on the matrix site near the center of the sequence was found to be essential for H$^+$ transport in UCP1 (Bienengraeber *et al.*, 1998). Mutagenesis of the conspicuous UCP-specific D27 located in the first helix also caused inhibition of H$^+$ transport (unpublished). Although less specific for UCP, helix terminating D195 and D233 are also important for H$^+$ transport. In contrast, inhibition primarily of Cl$^-$ transport, is caused by neutralizing residues E167, D34, and D134 at the end of the first and third helix. Mutagenesis of the three intrahelical arginines, which regularly are positioned in the second helix of each domain, completely inhibits the nucleotide binding. These arginines are typical for the mitochondria carrier family and not specific for UCP1.

Residues involved in the pH dependence of nucleotide binding, the so-called pH sensors, are highly specific

for UCP and have been confirmed and identified by mutagenesis. Thus the neutralizing mutation of E190Q abolished the pH dependence NDP and NTP (Echtay *et al.*, 1997) binding. In H214N, the pH dependence of nucleotide triphosphate was strongly diminished but that of diphosphate was not influenced (Echtay *et al.*, 1998). The nearby positioned D209 and D210 also influenced the pH dependence, but only of triphosphate binding. We, therefore, propose that the protonated H214 forms charge pairs with D210 and D209 and thus is retracted from the binding pocket, making space for the γ-phosphate. As pH increases the deprotonated H214 is released and protrudes to the γ-pocket, preventing triphosphate binding. This model is supported by a strong decease in nucleotide binding affinity, where H214 is replaced by the bulkier residue tryptophane H214W, whereas in H214N the affinity is unchanged.

Variants of UCP: UCP2, UCP3, and UCP4

Whereas UCP1 has been confirmed to be located exclusively in BAT, in more recent years, the occurrence of other proteins of the mitochondrial carrier family with characteristics similar to UCP1 have been identified and found to occur in various other tissues. These are UCP2 (Gimeno *et al.*, 1997; Fleury *et al.*, 1997), UCP3 (Boss *et al.*, 1997; Fleury *et al.*, 1997; Vaidl-Puig *et al.*, 1997; Liu *et al.*, 1998), and UCP4 (Mao *et al.*, 1999; Sachis *et al.*, 1998) with decreasing similarity to UCP1. Because of only 60 to 30% similarity among these UCPs, we prefer to call them UCP variants rather than UCP isoforms.

Each of these UCP variants has a very specific tissue distribution. UCP1 occurs exclusively in BAT, UCP2 is found more ubiquitous UCP3 is found primarily in skeletal muscle, while UCP4 is found exclusively in the brain. All these tissue distribution assays are based on mRNA levels and the true protein contents are not known, beside of UCP1 in BAT. The mRNA level of UCP2 and more of UCP3 is strongly influenced by various stress conditions. Stimulants of UCP2 and UCP3 expression are β_3 agonists, leptin, and thyroids (Liu *et al.*, 1998; Gong *et al.*, 1997; Zhou *et al.*, 1997; Lanni *et al.*, 1997). Stimulants of UCP3 only are food restriction and physical exercise. The latter are linked to enhanced circulating FA levels. The sequence similarity to UCP1 is 58, 55, and only 29% for UCP2, UCP3, and UCP4, respectively. Among UCP2 and UCP3, the similarity is 80%. Significant is the conservation of several critical residues in the UCP variants, which form specific signatures for the UCP family. These are an Asp

in the first helix (numbers refer to UCP1 in Fig. 1) (D27), a second Arg in the second helix (R91), a Glu in the fourth helix (E190), and a His at the fifth helix (H214). As shown above, these residues are associated with specific functions in UCP.

Besides these markers, evidence that these new variants are actually uncoupling is still quite evasive. For example, even on boosting expression with triiodothyronine, the measurement of uncoupling by UCP3 in isolated mitochondria from skeletal muscle has been unsuccessful, since the UCP3 level is very low as compared to BAT mitochondria (unpublished data). The expression in yeast has been the system of choice for all three variants to detect uncoupling in the cells or on the level of mitochondria, using cell counting cytofluorometry with a membrane potential fluorescent indicator. There is a clear downshift of fluorescence with all three variants, which was interpreted as uncoupling. However, the same effect should occur when the yeast cells expressing these UCP isoforms contain less mitochondria. In fact, yeast strains containing UCP2 and UCP3 grew much slower than those expressing UCP1 on a nonfermentative carbon source. Only low yields of mitochondria could be isolated from these cells (unpublished).

Most of the attention was focused on UCP3 because of its possible role in obesity and its abundance in skeletal muscle. Isolated mitochondria for UCP3-expressing yeast cells showed poor response of respiration, $\Delta\psi$, and H^+ uptake to substrates, FA and nucleotides, as compared to the large responses seen with UCP1-containing mitochondria (unpublished, Surwit *et al.*, 1998; Zhang *et al.*, 1999; Hinz *et al.*, 1999). This was surprising since these mitochondrial fractions also contained large amounts of UCP3. This paradox was resolved by the finding that UCP3 was not dissolved in Triton, but only in sarkosyl, leading to the conclusion that UCP3 in yeast is almost quantitatively deposited in inclusion bodies rather than in mitochondria (unpublished).

This poor growth indicates that mitochondria contain some, although only traces, of UCP3, which is sufficient to partially uncouple oxidative phosphorylation. Also from yeast respiration, it was inferred that UCP3 is, in fact, uncoupling. Why then is a high content of UCP1 not harmful for the yeast cells? The growth response of UCP1-containing cells indicates that UCP1 is dormant because of lack of FA. Is UCP3 then active, because it does not require FA acids for activating H^+ transport? The H^+ transport activity of UCP3 still evades experimental scrutiny. Further, H^+

transport cannot yet be reconstituted from UCP1 or UCP3 expressed in inclusion bodies, obtained by expression either in yeast or in *E. coli*. Recently, we succeeded in reconstituting Cl⁻ transport from inclusion bodies both with UCP1 and UCP3 (Echtay *et al.*, 1999) This transport was fully inhibited by nucleotides with the same low K_i as with reconstituted UCP1 from mitochondria. Most interesting, the response to GTP (ATP) versus GDP (ADP) differed drastically between UCP1 and UCP3. In UCP1 the nucleoside triphosphate, in UCP3 the nucleoside diphosphate are the stronger inhibitors. The inhibition constant ratio K_i^T/D_i^D change eight-fold between UCP1 and UCP3. This can be rationalized on the basis of the different physiological competence of the UCP variants. In BAT, UCP1 is a major thermogenetic factor and activator when mitochondria are largely uncoupled, i.e., ATP/ADP ratio is low (Fig. 5). In skeletal muscle, thermogenesis by uncoupling is only required in the resting state when the ATP/ADP ratio is high, i.e., UCP3 is activated when the ADP level is low. So far these results on UCP3 are the only definitive biochemical characterizations known.

OUTLOOK

There are two major issues in the research on uncoupling proteins: (1) their actual role and regulation
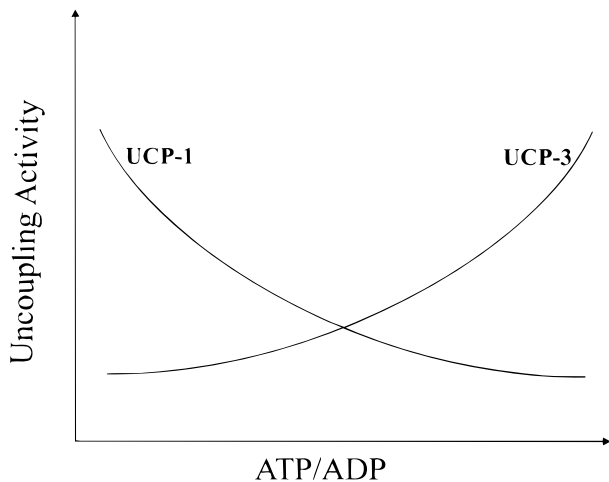

Fig. 5. Different regulation of transport activity by UCP1 and UCP3. Uncoupling activity controlled by the ATP/ADP ratio reflecting the cellular phosporylation potential. UCP1 in brown adipose tissue is active at low potential, whereas UCP3 in skeletal muscle is active at the high potential of the resting state. From (Echtay et al; 1999).

in cells and (2) their molecular mechanisms, i.e., the structure–function relationships. Some inroads have been made on both issues for UCP1. Much less knowledge is available on the other UCP variants UCP2, UCP3, and UCP4. The heterologous expression in yeast was of great benefit for studies on UCP1, but much less fruitful for the UCP variants, of which no native protein has been isolated so far. New approaches in the expression of these variants are mandated. A further difficult issue is the elucidation of the structure even with pure UCP1. The notorious resistance to crystallization of membrane carriers or transporters extends to the mitochondrial carrier family, in particular. Here we are still waiting for a seminal breakthrough.

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