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# The reconstituted isolated uncoupling protein is a membrane potential driven $H^+$ translocator

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**The isolated uncoupling protein (UCP) from brown fat adipose tissue mitochondria has been reconstituted into artificial phospholipid vesicles. Because of the high lability of $H^+$ transport, several new steps have been introduced in the reconstitution; the detergent octyl-POE, the addition of phospholipids to mitochondria prior to solubilization and purification, the vesicle formation by rapid removal of detergent with polystyrene beads and of external salts by a mixed ion exchange. In the $K^+$-loaded proteoliposomes, $H^+$ influx can be induced by a diffusion potential on addition of valinomycin. $H^+$ influx is inhibited to more than 90% by GTP addition, in the assay for UCP activity. By reversing $\Delta\psi$ with external $K^+$, $H^+$ efflux is measured, however, at a four times lower rate. In vesicles loaded with internal GTP, $H^+$ influx is fully inhibited but can be activated by Dowex-OH treatment to an even higher rate than that found in the GTP-free vesicles. Binding studies with GTP show that most of the active UCP are oriented with the binding site outside as in mitochondria, and that in GTP-loaded vesicles GTP is also bound at the outside. The rate of $H^+$ transport is linearly dependent on the membrane potential. Despite the ordered orientation, there is no 'valve' mechanism, since there is $H^+$ efflux with a reversed potential. pH dependency is only small between pH 6.5 and 7.5, indicating that the $H^+$-translocating site differs from the highly pH-dependent nucleotide-binding site. The turnover number of reconstituted UCP is commensurate with mitochondrial function and indicates a carrier instead of a channel-type $H^+$ transport. The UCP is thus the simplest $H^+$ transporter yet known.**

*Key words:* reconstitution/uncoupling protein/$H^+$ translocator

## Introduction

$H^+$ transport through membranes is either associated with substrates in the form of $H^+$ co-transport, or driven by a variety of $H^+$ pumps, or coupled to exchange against $Na^+$ and $Ca^{2+}$. In this diversity of $H^+$-translocation modes, the case of a $H^+$ carrier has so far been missing, except for the suggestion that the uncoupling protein (UCP) of brown fat adipose tissue mitochondria may represent this basic case (Nicholls, 1977). Here we report how by incorporating the purified protein into phospholipid vesicles we proved that the UCP is indeed a $H^+$ carrier, the translocation of $H^+$ being driven by a membrane potential. With this function the UCP represents the simplest $H^+$ translocator known.

The inner membrane of mitochondria from brown adipose tissue has large amounts of a $M_r$ 32 kd protein distinguished by its specific binding of purine riboside di- and triphosphates, i.e., ADP, ATP, GDP and GTP (Heaton *et al.*, 1978; Lin and Klingenberg, 1982). This protein has been suggested to be responsible for the thermogenic function of these mitochondria by returning $H^+$ or $OH^-$ in such a manner that the electrochemical $H^+$-potential generated by substrate-oxidation-driven electron transport collapses (see Nicholls and Locke, 1984).

The 'uncoupling protein' has been isolated and purified in our laboratory by adapting the isolation procedure developed for the ADP/ATP carrier from mitochondria (Lin and Klingenberg, 1980, 1982). A structural similarity between the two proteins has recently been substantiated by the amino acid sequences of the proteins (Aquila *et al.*, 1985).

To explore the function of UCP in transport, the isolated protein has to be reincorporated into artificial phospholipid vesicles. A satisfactory reconstitution encountered difficulties because of the sensitivity of the protein towards those detergents which also permit the formation of $H^+$- and $K^+$-tight vesicles. Several new steps had to be developed to achieve a high $H^+$ transport activity which can be fully attributed to the incorporated UCP and which is amenable to quantitative studies.

An early brief communication (Bouillaud *et al.*, 1983) claiming reconstitution showed only spurious $H^+$-flux activities, which were partially sensitive to GTP, and which was based on the system that we used originally in collaboration with Lin (unpublished data). In a recent short report a significant UCP catalyz-

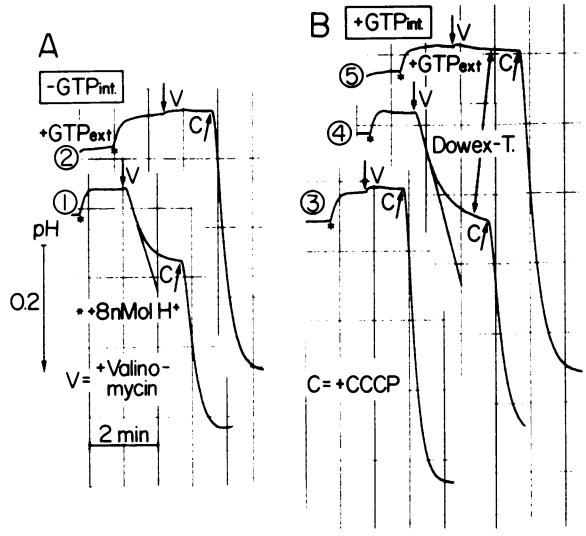


**Fig. 1.** $H^+$ influx catalyzed by UCP in reconstituted phospholipid vesicles. $K^+$-diffusion potential is generated by addition of valinomycin to the $K^+$-loaded vesicles. **A.** Vesicles not loaded with GTP ($-GTP_{int}$). **B.** Vesicles loaded with GTP ($+GTP_{int}$), i.e., prepared in the presence of 50 $\mu$M GTP. For reactivation (traces 4 and 5) they are treated with Dowex $OH^-$, as described in Materials and methods. 50 $\mu$M external GTP ($GTP_{ext}$) was added to inhibit UCP. Vesicles were loaded with 100 mM KCl, 100 mM MOPS, pH 7.2 (see Materials and methods) and incubated with 8.4 mg phospholipid/mg and 62 $\mu$g protein/ml, plus 5 mM choline Cl, 11°C, pH 6.9. The initial $H^+$-flux rates were (**A**) 1690 and (**B**) 2050 $\mu$mol/min/g protein. Valinomcyin (1 nmol) and CCCP (1 nmol) were added.

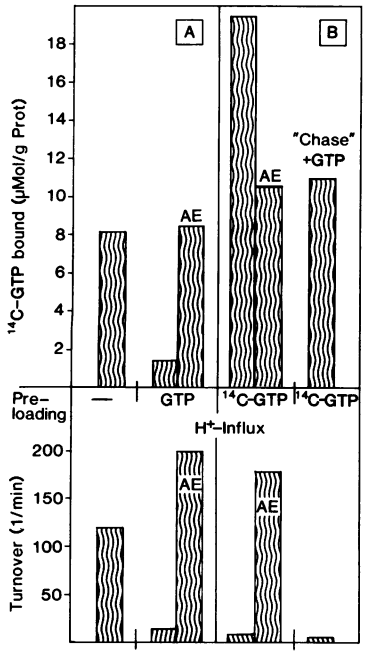


**Fig. 2.** The sided orientation of UCP molecules incorporated into vesicles: probing sidedness of UCP with [$^{14}$C]GTP binding and comparison with $H^+$-flux activity in response to internal and external GTP. **A.** [$^{14}$C]GTP was added to the completed vesicles. **B.** [$^{14}$C]GTP was added prior to completion of the vesicles. In both cases binding was determined before and after removal of the externally bound GTP portion from GTP-loaded vesicles by anion exchange (AE) Dowex $OH^+$. Binding was assayed with [$^{14}$C]GTP as described in Materials and methods. Vesicles were loaded with 100 mM KCl and 100 mM MOPS, pH 7.2. For 'preloading' 30 μM GTP or 30 μM [$^{14}$C]GTP were added. For 'chase', 100 μM GTP was added to [$^{14}$C]GTP-loaded vesicles before anion-exchange treatment.

ed $H^+$-flux activity was described (Strieleman *et al.*, 1985). However, only the functionally less important $H^+$ export was reconstituted with a partially purified UCP and with partial GTP sensitivity.

## Results

### *$H^+$ influx*

The basic experiment demonstrating $H^+$ flux catalyzed by UCP using a recent version of our reconstitution methods is shown in Figure 1. Here the vesicles were loaded with $K^+$ and high buffer concentration and thus prepared to take up $H^+$. Net $H^+$ influx was induced by generating a membrane potential by a $K^+$ diffusion gradient upon addition of valinomycin, as shown by the pH recording of trace 1 in Figure 1A. An initially quasi-linear slope served to evaluate the initial rate of $H^+$ influx. Further addition of the uncoupler carbonyl-cyanide *m*-chlorophenyl-hydrazone (CCCP) should collapse the membrane potential and permitted us to test the capacity of the $H^+$ translocation across the vesicles. The additional large $H^+$ influx can in part be attributed to vesicles not equipped with active UCP. Of great importance is the sensitivity of the $H^+$ flux to purine nucleotide as a criterion for UCP-catalyzed $H^+$ flux. Prior addition of 50 μM GTP nearly completely blocked the valinomycin-inducible $H^+$ influx; when CCCP was added the $H^+$ uptake reached the same value (trace 2). This shows that prior to CCCP addition, nearly all the $H^+$ uptake was due to UCP activity. Control experiments, not shown here, demonstrated that these vesicles are impermeable to $K^+$ and $H^+$. For example, virtually no pH change was observed on addition of valinomycin to these vesicles

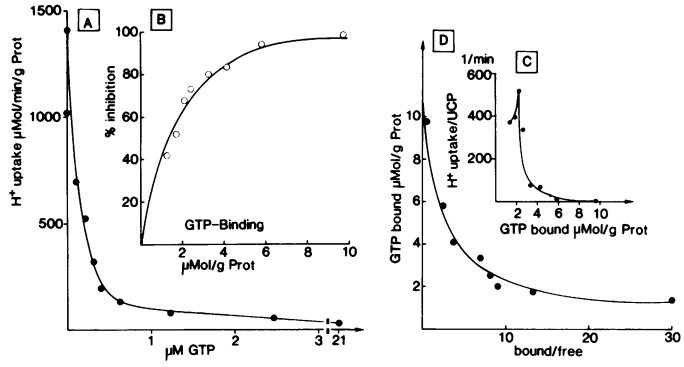


**Fig. 3.** The titration of $H^+$-flux activity by GTP and correlation to [$^{14}$C]-GTP binding. Measurements of $H^+$ flux and [$^{14}$C]GTP binding in identical samples. **A.** The inhibition of $H^+$ flux by increasing concentrations of GTP. **B.** Correlation of $H^+$-flux inhibition with binding of [$^{14}$C]GTP to UCP-containing vesicles. **C.** Relation between incremental flux activity and incremental binding of GTP. Evaluation of the data given in **A** and **B**. $H^+$ uptake/UCP = $\Delta H^+$ uptake/$\Delta$GTP bound. $\Delta$ is the difference between two consecutive values during the titration by GTP of $H^+$ flux and binding. **D.** Mass action plot of GTP-binding for evaluation of binding affinity. Incubation of vesicles (4.6 mg phospholipid, 0.04 mg protein/ml) in 2 mM PIPES, 200 mM sucrose, 10 mM choline $Cl^-$, pH 6.9, 11°C. The vesicles were loaded with 100 mM KCl and 100 mM MOPS. Measurement of binding with [$^{14}$C]GTP, as described in Materials and methods.

without incorporated UCP thus indicating that the vesicles are $H^+$ tight.

### *Orientation of uncoupled UCP*

Anticipating a statistically orientated insertion of UCP molecules in the vesicles, GTP should only partially inhibit the $H^+$ flux, since it can bind only to the right-side-out UCP molecules (i.e., those oriented in the same way as in the mitochondria). One would expect a complete inhibition of $H^+$ flux only when GTP is present both inside and outside the vesicles. The observed nearly complete inhibition by externally added GTP, therefore, indicates that all the active UCP molecules seem to be oriented right-side-out. For this reason we also prepared vesicles in the presence of GTP in order to include GTP into the vesicles (Figure 1B). Surprisingly, $H^+$ flux was nearly fully inhibited despite the usual passage of the vesicles over an ion exchanger (see Materials and methods) for removal of salts and external GTP (Figure 1, trace 3). Only after further treatment with $OH^-$-loaded 'Dowex' resin, was the $H^+$ flux fully activated to an even higher rate and extent, and this flux could be blocked by addition of GTP (trace 4 and 5). These results indicate that by incorporating GTP into the vesicles, GTP also binds to the external sites so tightly that it resists the mixed-bed treatment for removal of external salts. Only by raising the pH to decrease the binding affinity of GTP (see Lin and Klingenberg, 1982) and by simultaneously providing a strong anion trap by Dowex-$OH^-$, was it possible to displace the GTP. The large $H^+$ influx capacity of the GTP-loaded vesicles reflects a larger inner volume of these vesicles (data not shown). The reason for and implications of a strong vesicle size dependency on UCP incorporation will be discussed elsewhere.

To verify directly whether the GTP-loaded vesicles still retain external GTP, binding experiments with [$^{14}$C]GTP were performed parallel to the $H^+$ uptake (Figure 2). The binding capacity for [$^{14}$C]GTP added to the completed vesicles was measured and, for further verification, the binding of [$^{14}$C]GTP which originates from a pre-loading of the vesicles with [$^{14}$C]GTP. In the unloaded vesicles [$^{14}$C]GTP binding is ~8.5 μmol/g protein, whereas in the GTP-loaded vesicles additional binding of [$^{14}$C]GTP is largely

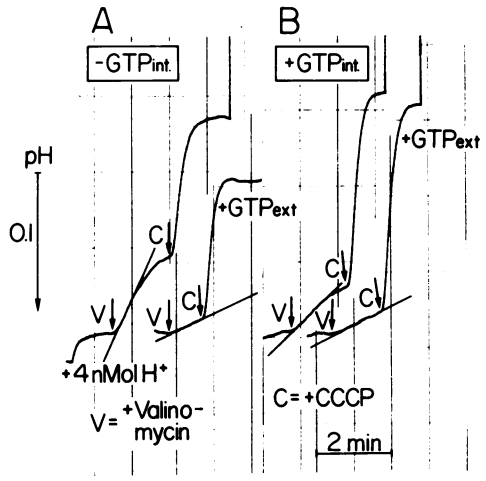


**Fig. 4.** $H^+$ efflux catalyzed by reconstituted UCP in vesicles (2.5 mg phospholipid and 25 μg protein/ml) incubated in 2 mM PIPES, 200 mM sucrose, pH 7.2 11°C. The vesicles were loaded with 100 mM Na phosphate, pH 6.5 and in **B** with 50 μm GTP.

suppressed (Figure 2A). After treatment with Dowex-$OH^-$, [$^{14}$C]GTP binding is increased, parallel to the activation of $H^+$ flux, to about the same level as in unloaded vesicles.

The contribution of GTP loading to external binding was also shown with [$^{14}$C]GTP-loaded vesicles (Figure 2B). The high binding of ~19 μmol/g protein should include internally bound and free GTP in addition to externally-bound GTP. After treatment with Dowex-$OH^-$ the GTP binding was decreased and the $H^+$ flux fully activated. Obviously the externally bound [$^{14}$C]GTP is removed whereas the internally located [$^{14}$C]GTP, 11 μmol/g protein, is retained. The removed [$^{14}$C]GTP is of about the same amount as that bound externally by adding [$^{14}$C]GTP to GTP-untreated vesicles (Figure 1A). Furthermore, the same amount of [$^{14}$C]GTP is displaced by 'chase' with an excess of cold external GTP. All these results confirm that when pre-loading vesicles with GTP, the incorporated UCP molecules also retain externally bound GTP, even after removal of all other external anions. The activation and inhibition of the $H^+$-flux activity goes parallel with the binding of GTP at these external sites, irrespective of whether GTP is added externally or retained from the loading of the vesicles. The internally retained GTP has little influence on the $H^+$-flux activity.

*Heterogeneity of incorporated UCP molecules*

The dependence of the $H^+$ flux inhibition on the GTP concentration shows that as little as 0.5 μM GTP are sufficient to inhibit the activity to 85% (Figure 3A). As a result the $H^+$-flux activity is segregated into portions which are highly or less sensitive to GTP. In the same samples [$^{14}$C]GTP was determined and correlated to the extent of inhibition (Figure 3B). Only 25% of the GTP binding sites are responsible for 90% of the $H^+$-flux activity. By correlating the incremental inhibition with the incremental binding along the titration, the activity distribution of the incorporated UCP molecules was obtained (Figure 3C). Moreover, the maximum performance per UCP molecule can be calculated, undiluted by the activity of the less active molecules. The maximum turnover is thus determined as 500 $H^+$/UCP/min.

The mass action plot of GTP binding also illustrates the heterogeneity (Figure 3D). A small fraction of ~2 μmol GTP/g protein binds with a $K_D$ ~$10^{-6}-10^{-7}$ M, whereas the larger fraction has a $K_D$ ~$10^{6}-10^{-5}$ M. This unusually high affinity has not been observed with the soluble carriers. Two factors seem to be involved: the insertion of UCP in the phospholipid and the low anion concentration of the medium. Anions are not only competitive inhibitors of GTP binding (Lin and Klingenberg, 1982), but also of the $H^+$-flux activity (data not shown). The fact that highest activity correlates with high affinity seems to indicate that these molecules are the most intact ones, because they are more optimally inserted into the membrane or kept more intact during incorporation. This would also agree with the finding that although on average our vesicles contain 3–4 UCP molecules, only about half of the vesicles seem to be equipped with active UCP, as indicated in Figure 1.

**Table I.** Rates of $H^+$ flux

| Conditions | | Rate μmol/min/g prot. | Turnover l/min |
|---|---|---|---|
| Influx aver. | 11° | 500–1400 | 120–220 |
| max. | 11° | – | 500[a] |
| | 20° | 3000 | 450 |
| Reactivated influx | 11° | 2000–4000 | 300–600 |
| efflux | 11° | 300–700 | 50–100 |

[a]Maximum in the activity distribution profile of incorporated UCP molecules (cf. Figure 3C).

*$H^+$ efflux*

In view of the oriented insertion of the active UCP molecules, it is of interest to invert the diffusion potential in order to study $H^+$ efflux. Moreover, a possible 'valve effect' in UCP is to be tested, i.e., whether right-sided oriented UCP allow only $H^+$ influx with $\Delta\psi$ negative inside but no $H^+$ efflux when the $\Delta\psi$ is reversed. The vesicles were loaded with a $K^+$-free buffer kept at low pH, e.g., Na phosphate, pH 6.2. In an external medium with KCl, addition of valinomycin leads to a diffusion potential which is negative outside. An $H^+$ efflux is recorded which can be largely inhibited by added GTP (Figure 4). The rate of $H^+$ efflux is considerably slower than that of the influx in the reverse system. The rate cannot be increased by higher $K^+$ concentrations. The $H^+$ efflux is only about half inhibited in GTP-loaded vesicles. It can be reactivated by treatment with Dowex-$OH^-$ to withdraw externally attached GTP, but this rate is still lower than in the GTP-free vesicles (data not shown). The GTP-inhibition characteristics indicate that most of the $H^+$-efflux activity is due to the UCP molecules directed right-side-out, but residual inhibition by only internal GTP indicates a contribution from inverted UCP molecules.

Since the reversed $\Delta\psi$ drives $H^+$ efflux largely by the right-side-out inserted UCP molecules, the 'rectifier effect' seems not to exist in UCP. The fact that the $H^+$ activity is lower with the reversed $\Delta\psi$ can be explained by the less favorable conditions for the $H^+$ efflux, such as a smaller $K^+$ gradient and therefore a lower $\Delta\psi$ and a smaller $\Delta$pH. Also the higher concentration of external anions, i.e., of $Cl^-$ impairs the UCP activity. The $H^+$-flux data measured under various conditions are summarized in Table I.

*Quantitative relation to membrane potential*

The dependence on the $K^+$ gradient represents a critical assay for the electrical, i.e., electrophoretic nature of the UCP-catalyzed

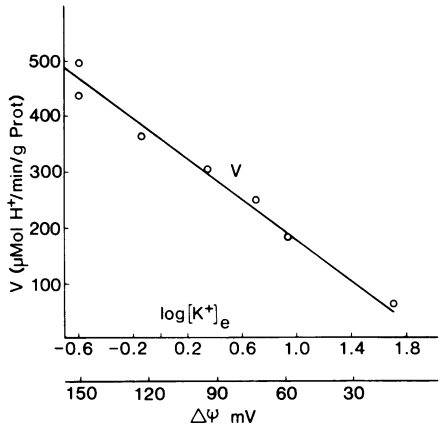


**Fig. 5.** The dependence of $H^+$ influx catalyzed by UCP on the $K^+$-diffusion (membrane) potential. Vesicles incubated in a medium with concentrations of $K^+$ increasing from 0–80 mM, as given on the abscissa by log [$K^+$ mM]. Initial rate of $H^+$ influx is given as measured in Figure 1. Vesicles were loaded with 150 mM $K^+$ phosphate, pH 7.0, and incubated in 1 mM PIPES, 1 mM $MgSO_4$, 5 mM choline $Cl^-$, 200 mM sucrose.

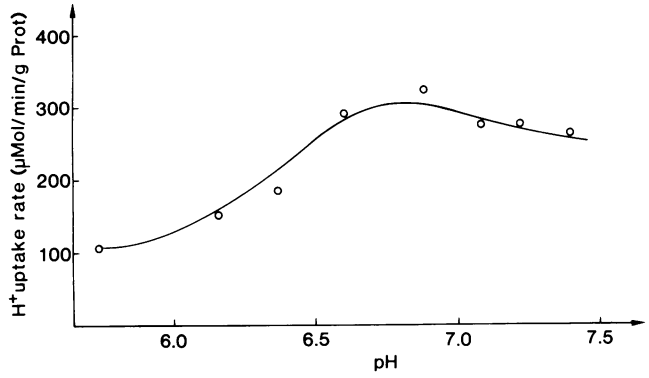


**Fig. 6.** pH dependence of $H^+$-influx rate catalyzed by UCP. Initial rates measured as shown in Figure 1. The rates represent the difference between the uninhibited and GTP-inhibited values. Vesicles were loaded with 100 mM $K^+$ phosphate, pH 7.5, 1 mM PIPES, 100 mM sucrose, 1 mM $MgSO_4$ and 5 mM choline $Cl^-$. The pH was adjusted prior to valinomycin addition in each sample.

$H^+$ flux. In the $H^+$-influx system, where the vesicles are loaded inside with 100–200 mM $K^+$, this dependence can be measured by varying the external $K^+$ concentration over a wide range (Figure 5). As is to be expected, external KCl decreases the initial $H^+$ influx rate drastically. By plotting the rates against log [$K_e^+$], a linear relation of the $H^+$ influx rate to the diffusion potential was obtained. As expected, the rate reaches nearly zero when the external and internal $K^+$ concentrations are equal. The diffusion potential $\Delta\psi$ is calculated from the initial $K^+$-gradient and given on the abscissa. The resulting linear relation of $H^+$ flux *versus* $\Delta\psi$ is approximately described by $\Delta_v = 0.33\ \Delta\psi$[$\mu$mol $H^+$/min/g protein/mV].

*Dependence on pH*

The $H^+$ concentration is expected to be another critical parameter. For the $H^+$-influx system with a given internal pH set at 7.2, the external pH was varied over a wide range from pH 5.8 to 7.9 (Figure 6). There is relatively little change of the uptake rate between pH 6.6 and 7.9 and, surprisingly, it even decreases at lower pH. Obviously the $H^+$ concentration does not limit the influx. This also argues against the notion that the $H^+$ binding group, which has been linked to the GTP-binding site, is catalytically involved in $H^+$ transport. The $H^+$ dependence of GTP binding is a most striking phenomenon (Klingenberg, 1984) and it may seem obvious that this protonization is part of the $H^+$ translocation site. From GTP-binding studies it was concluded that protonization of this site is proportional to $H^+$ concentration down to pH 5. Therefore, it should increase 60-fold from pH 7.9 to 5.7, whereas the $H^+$-influx rate actually decreases. This suggests that the rate-determining $H^+$-translocation site differs from the protonized GTP-binding site. The latter then seems to have a purely regulatory function.

**Table II.** Attempts to demonstrate chloride transport[a]

A. $Cl^-$ influx

| $GTP_i$ | $GTP_e$ | Internal $Cl^-$ $\mu$mol | | $\mu$mol/min | | $H^+$efflux $\mu$mol/min |
|---|---|---|---|---|---|---|
| | | −Val | +Val | −Val | +Val | |
| − | − | 212 | 279 | 80 | 70 | |
| − | + | 248 | 203 | 45 | 31 | 700 |
| + | − | 155 | 271 | 134 | 108 | |
| + | + | 337 | 216 | 81 | 56 | |

B. $Cl^-$ efflux

| $GTP_i$ | $GTP_e$ | mmol | | $\Delta(\pm GTP_e)$ mmol | | $H^+$ uptake mmol |
|---|---|---|---|---|---|---|
| | | −Val | +Val | −Val | +Val | |
| − | − | 1.30 | 1.20 | 0.01 | 0.09 | 2.0 |
| − | + | 1.29 | 1.01 | | | |
| + | − | 0.84 | 0.78 | − | 0.03 | 3.0 |
| + | + | 0.87 | 0.75 | | | |

[a]All values per g incorporated UCP protein. Conditions: A. Vesicles loaded with $NaP_i$, pH 6.2 ± 50 $\mu$M GTP. B. Vesicles loaded with 20 mM KCl, 100 mM MOPS, pH 7.2, ± 50 $\mu$M GTP.

*Is there a $Cl^-$ flux due to the reconstituted UCP?*

$Cl^-$ transport has been associated with the UCP action ever since a GTP-sensitive swelling of mitochondria in $Cl^-$-containing medium but not in $SO_4^{2-}$-containing media was observed (Nicholls, 1974). However, as for the postulated $H^+$ conductance, this evidence relied only on swelling studies of mitochondria. During the various stages of our reconstitution experiments, we carefully monitored the postulated $Cl^-$-flux function of UCP, not only by using indirect evidence but also by directly determining $Cl^-$ fluxes. We have little evidence for a UCP-catalyzed $Cl^-$ transport under various conditions. First, the inhibiting influence of $Cl^-$ on $H^+$ transport could be mimicked by $SO_4^{2-}$ at a concentration about one third of the $Cl^-$ concentration. Therefore, the $Cl^-$ effect was not due to competition of a specific $Cl^-$ flux for the $H^+$- or $HO^-$-flux but rather due to the non-specific anion inhibition of the active site. A second piece of evidence comes from a direct study of the $Cl^-$ flux with reconstituted UCP vesicles using $^{36}Cl$. Both $Cl^-$ influx and efflux were determined and tested for inhibition by external or internal GTP, as shown in Table II. As in the $H^+$-flux system, a membrane potential was generated by a $K^+$-valinomycin gradient, either positive inside for $Cl^-$ influx or positive outside for $Cl^-$ efflux. In the influx system, a minor uptake of $Cl^-$ was observed which was barely influenced by valinomcyin and only slightly inhibited by external GTP; it was not inhibited at all by internal GTP. In the $Cl^-$-efflux system, vesicles were loaded with 20 mM $^{36}Cl$ and the amount of $Cl^-$ retained in the vesicles was measured. Efflux of $Cl^-$ was lower in the vesicles with in-

ternal GTP; but there was no effect of external GTP. Valinomycin exerted no major influence beyond error limits.

## Discussion

It has been suggested, based initially on swelling studies of brown fat mitochondria, that the nucleotide binding factor catalyzes $H^+$ or $OH^-$ flux through the membrane (see Nicholls and Locke, 1984). However, it was by no means clear whether the nucleotide binding protein is the actual $H^+$ translocation or whether it is a regulatory unit associated with another $H^+$-conducting protein, similar to the small lipoprotein bound in the ATP synthase. The present work proves that the UCP is a $H^+$ transporter, in particular for importing $H^+$.

The postulate of an $H^+$ conducting activity had been based on the high oxidative capacity of brown fat mitochondria and on acetate-induced swelling of mitochondria which, however, employs $H^+$ export (Nicholls, 1974, 1976, 1977; Rial *et al.*, 1983; Rial and Nicholls, 1983) instead of the functionally relevant $H^+$ import. The $Cl^-$-transport assay in mitochondria was also based on swelling (Nicholls and Lindberg, 1973). The complications of these indirect assays, together with the complexity of the mitochondria, may be the reasons why direct measurements of transport with purified reconstituted UCP cannot confirm $Cl^-$ transport activity of UCP.

For some time our unsuccessful efforts to demonstrate $H^+$ transport in the re-incorporated isolated UCP seemed to indicate that the $H^+$-conducting unit was missing after the purification and that the isolated nucleotide-binding protein is only a regulatory unit associated with a $H^+$ channel, lost during purification. However, it seems now that preservation of nucleotide-binding activity during isolation or reconstitution does not mean retention of $H^+$-transport capacity. The latter is more sensitive to detergents and therefore several novel steps had to be devised (Klingenberg and Winkler, 1985), which may also become useful for the reconstitution of other transport systems. Although the purity of the reconstituted protein is at least 60% as judged from the GTP-binding capacity (see also Materials and methods), there can be little doubt that all the GTP-sensitive $H^+$-transport activity is mediated by the UCP. All the typical characteristics of the inhibition, i.e., dependence on pH and GTP concentration and competition with anions are also found in the highly purified solubilized UCP.

The procedure for reconstitution of UCP overcame two difficulties encountered with the other methods; i.e., the $H^+$- and $K^+$-leakage of the liposomes and the inactivation of the UCP. A major problem was posed by detergents with a high critical micelle concentration (cmc) which by their easy removal permitted the generation of tight and large vesicles, but tended to inactivate the membrane protein. Octyl polyoxyethylene which has a sufficiently high cmc, proved to be much less offensive than cholate or octylglucose. But even with this detergent, only the extra protection by prior addition of phospholipids to the mitochondria gave good results. The rapid removal of the detergent by polystyrene beads instead of by dialysis was important for the UCP stabilization. The removal of external salts by mixed ion exchange greatly accelerated the proteoliposome preparation.

Surpringly, most of the UCP molecules appeared to be oriented right-side-out in the reconstituted vesicles. The first evidence comes from the nearly complete inhibition by external GTP. More importantly, the molecular accounting based on the measurements of GTP binding agrees with this conclusion. How this orientation is generated during the protein insertion and vesicle formation remains a problem we are studying. The right-side-out orientation of the UCP is different from the orientation of the reconstituted ADP/ATP carrier, where the molecules are to a larger extent directed right-side-in (Krämer and Klingenberg, 1979). However, in the latter system the vesicles were generated by sonication of phospholipids with protein in Triton X-100.

The linear relation between the $H^+$-flux activity of UCP and the imposed membrane potential agrees with the conclusions drawn by Nicholls (1977) from studies on the relation between respiratory activity and $K^+$ gradient in mitochondria. This dependence is similar to the phenomenon observed with the reconstituted ADP/ATP carrier (Krämer and Klingenberg, 1982). Moreover, the influence of $\Delta\psi$ appears to be independent of the orientation of the UCP since $H^+$ import or export is catalyzed by the same right-side-out directed UCP molecules. A valve mechanism, preventing the activation of the oppositely oriented UCP by the membrane potential, might have been inferred from the observed inhibition by external but not by internal GTP. However, this can be ruled out because of the distinctly sided orientation of most UCP molecules shown in the binding studies with external and internal GTP. As a result the UCP activity appears to be controlled by an influence of $\Delta\psi$ on the $V_{max}$ rather than on the $K_m$. We might call it an 'electrophoretic' control of $H^+$-flux activity. The activity relation can also be described by a shift of the activation energy profile under the influence of the membrane potential, in which the barrier decreases proportionally to $\exp(\Delta\psi F/RT)$ (Klingenberg, 1980).

The values of $H^+$-flux rates (Table I) are commensurate with the activity of UCP required in the mitochondria. However, even the maximum molecular activity of UCP (turnover) is still low considering that ions as small as $H^+$ are transported. A channel type of transport seems therefore highly improbable. The UCP activity rather corresponds to a carrier-mediated transport as also inferred by Nicholls (1977). The activity is higher than that of the ADP/ATP carrier but lower, for example, than that of the glucose carrier. It may be possible to increase this activity still more. However, its magnitude agrees with the fact that the mitochondria are equipped with an abundance of UCP molecules.

A most intriguing structural similarity between the UCP and the ADP/ATP carrier, based on significant homologies in the primary structures (Aquila *et al.*, 1985), suggests that the UCP is actually derived from a $H^+$-substrate co-transporter rather than being an *a priori* $H^+$ carrier (Klingenberg, 1985). The low turnover of UCP is reminiscent of a substrate carrier. Another conclusion from this analogy is that UCP translocates only one $H^+$ per catalytic cycle.

The pH dependency of $H^+$ transport is quite different from the strong pH dependence of nucleotide binding, thus suggesting that a different $H^+$-accepting site is involved in the rate-limiting step of $H^+$ translocation. The UCP has been proposed to be an anion transporter for $OH^-$ rather than for $H^+$ (Nicholls, 1974). Since we find only a poor $Cl^-$-transport activity, if any, and taking into account the proposal that UCP is a degenerated $H^+$ co-transporter, the suggestion of an $OH^-$ transport seems unjustified. Also, $P_i$ transport in mitochondria is a $H^+$ co-transport (Palmieri *et al.*, 1970; Guerin *et al.*, 1970; Klingenberg *et al.*, 1974) rather than an $OH^-$-$P_i$ exchange, as originally suggested (Chappell, 1968).

The fact that $Cl^-$ is not transported in the present reconstituted system may either indicate that this activity has been inactivated, or that $Cl^-$ transport is due to another protein as suggested by Kopecký *et al.* (1984). This may explain why Strieleman *et al.* (1985) found a definite but low $Cl^-$-transport activity, since they

used a less pure UCP preparation. Moreover, in the reconstituted UCP no activating influence of fatty acids such as oleate or palmitate was found, although they have been postulated to play an important role in the regulation of $H^+$-transport activity in mitochondria (Rial *et al.*, 1983; Rial and Nicholls, 1983).

In conclusion, UCP seems to represent the most simple $H^+$-translocating membrane system known so far. With the primary structure known, and a good assay in the reconstitution system now available, UCP is a very promising object for elucidating fundamental problems in the mechanism of $H^+$ translocation across biomembranes.

## Materials and methods

Amberlite XAD-4, 20–50 mesh was obtained from Sigma. Before application the material was washed with methanol. 100 g dry amberlite XAD-4, 20–50 mesh were suspended in 600 ml methanol and stirred for 15 min, then washed with 1.5 l methanol on a filter funnel and then three times with 1.5 l water.

Egg yolk phospholipids (EYPL) were prepared from fresh eggs according to Wells and Hanahan (1969). For further purification a second precipitation step was introduced.

Octyl-polyoxyethylene (octyl-POE), a mixture of $C_8E_2$ to $C_8E_{12}$, was kindly donated by Dr. Rosenbusch, Biozentrum, Basel, Switzerland.

Brown fat mitochondria from cold-adapted hamster were prepared as previously described (Lin and Klingenberg, 1982).

*Reconstitution procedure (see also Klingenberg and Winkler, 1985)*

A typical preparation of 4 ml vesicles containing 10 mg phospholipids and 0.07 mg UCP per ml was obtained as follows. 85 mg of twice-purified (see below) egg yolk phospholipids are dissolved in 1 ml $H_2O$ with 130 mg 'octyl-POE', 75 mM MOPS, 1.5 mM EDTA by heating to 50°C. Then 15 mg protein of isolated brown fat mitochondria were added and incubated for 10 min at 0°C. The solubilized preparation was thoroughly mixed with 1.2 g wet hydroxylapatite in a small homogenizer and left for 10 min at room temperature and then for 25 min at 0°C. The paste was centrifuged for 5 min at 5000 *g*. To 1 ml supernatant were added another 0.5 ml of the EYPL-detergent solution and 0.5 ml $H_2O$.

To adjust the composition of the vesicle interior the following additions were made: for measuring $H^+$ influx, 0.2 ml each of 1 M KCl and 1 M MOPS pH 7.2, or 0.4 ml of a 0.5 M K-phosphate solution pH 7.2, which results in a final concentration of 100 mM KCl plus 100 mM MOPS, or 100 mM $KP_i$. For measuring $H^+$ efflux, the additions were 0.4 ml 0.5 M $NaP_i$ solution pH 6.5, or 0.2 ml of 1 M NaCl solution and 0.4 ml of 0.5 MES solution, pH 6.2.

For detergent removal and concomitant vesicle formation 4 g wet amberlite XAD-4 were added, 20–50 mesh, and the mixture shaken at 4°C for 2.5 h. The amberlite was removed by sucking the vesicle suspension through a small sintered polypropylene filter. The amberlite slurry was washed with ~1 ml of isotonic 0.2–0.35 M sucrose solution. The two filtrates were combined.

For removal of the externals salts from the total volume of ~3.5 ml, the pH was adjusted to 7.8, and 4 g wet mixed-bed resin added. The resin was supplemented in advance with 1 ml 1 M sucrose to maintain the external osmolarity at a level about equal to that of the internal salt concentration after external salt removal. After 10 min of gentle shaking, the suspension was filtered as described above, yielding ~4 ml vesicle preparation.

*Characteristics of proteoliposomes*

The characteristic data of the proteoliposomes for an average preparation were as follows. The weight ratio of protein/phospholipid was ~0.01. The molar ratio of UCP to phospholipid was $10^{-4}$ mol/ml. The internal volume, as determined by $P_i$ content, was 1.5–2 μl/mg phospholipid. This corresponds to a vesicle diameter of 600–850 Å. The number of phospholipid molecules per vesicle was $4 \times 10^4$ and the number of UCP molecules per vesicle was 3–4. The incorporated probe had a binding capacity of 8–10 μmol GTP/g protein corresponding to >60% purity. The purity was >80%, as judged from the content of the '32-kd band' on SDS gels.

*Determination of $^{14}$C-GTP binding*

The binding measurements were described in Klingenberg *et al.* (1985). The anion exchange method was used throughout. A 200 μl suspension of vesicles, which had been loaded internally or externally with [$^{14}$C]GTP was added to a small capillary column 2.0 x 60 mm containing 60 mg of Dowex-$Cl^-$ 1 x 8, 100–400 mesh. Immediately after application the sample was chased by squeezing 2 x 220 μl $H_2O$ through the column. The whole procedure should take only 1 min to avoid withdrawal of bound GTP. The eluate of ~600 μl contained the bound [$^{14}$C]-GTP and was used for scintillation counting.

*Measurements of $H^+$ conductance*

$H^+$ conductance was recorded with a combination pH glass electrode (Ingold type M403) in a precisely temperature-controlled water-jacketed vessel. The volume was 0.6 ml, containing 5–10 mg PL, 40–80 μg protein, 5 mM choline $Cl^-$, and 1 mM MOPS adjusted to pH 6.9.

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