Modulation of the Reconstituted Adenine Nucleotide Exchange by Membrane Potential†

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ABSTRACT: Regulation of adenine nucleotide transport is analyzed in a reconstituted system consisting of egg yolk phospholipid liposomes and purified ADP–ATP carrier from beef heart mitochondria. In this relatively simple system the influence of membrane potential and of H^+ gradient on the transport function is elucidated and the exchange is resolved into the single rates for ADP and ATP and for both transport directions. The exchange of ATP and ADP proves to be clearly regulated by the membrane potential, which was generated by diffusion gradients of K^+ in the presence of valinomycin. With $\Delta\psi$ positive outside, exchange of external ADP against internal ATP is strongly favored as compared to ATP uptake against ADP efflux. Membrane potential in the opposite direction stimulates the reverse direction of exchange. Thus, the ADP–ATP carrier is regulated symmetrically in both directions of membrane potential though being oriented asymmetrically in the liposomal membrane. In competition experiments with both ADP and ATP on both sides of the membrane, it can be shown that $\Delta\psi$ also regulates the efflux of ADP vs. ATP. The counterexchange of ADP and ATP is further resolved into the four possible modes of hetero- and homoexchange. With $\Delta\psi$ of $\sim$180 mV, externally positive, the exchange of internal ATP against external ADP is favored more than 30 times over the corresponding heteroexchange of internal ADP against external ATP. ΔpH influences the direction of ADP–ATP counterexchange only slightly. These results also indicate that the adenine nucleotide exchange in the reconstituted system is mostly if not completely electrophoretic. It is concluded that the isolated and reconstituted ADP–ATP carrier is fully competent not only in transport but also in the membrane potential mediated regulation of ADP vs. ATP exchange.

The exchange of ADP and ATP across the inner mitochondrial membrane is influenced by the energy state of the membrane. In "deenergized" mitochondria both ADP and ATP are translocated with similar rates, whereas in the "energized" state exchange becomes asymmetrical with preference for an extrusion of ATP and uptake of ADP (Pfaff & Klingenberg, 1968; Klingenberg, 1975). There is now extensive evidence that the exchange is largely electrophoretic, i.e., driven by the membrane potential (Klingenberg et al., 1969; LaNoue et al., 1978; Klingenberg & Rottenberg, 1977), although also an electroneutral, i.e., ΔpH[1] driven, component of the transport has been measured (Wulf et al., 1978). As a consequence, about one negative charge has to be transported in an electrophoretic type of exchange. Thus, the energy cost of the adenine nucleotide exchange can be estimated to be 20–33% of the total oxidative energy, depending on the stoichiometry of protons generated per coupling site. These considerations emphasize the importance of the ADP–ATP exchange mechanism in the energy balance of the cell.

In intact mitochondria accurate differentiation between ADP and ATP translocation is complicated by highly active phosphate transferases, which can rapidly transform ADP and ATP. Also, the net charge transfer might be influenced by other transport systems, e.g., for phosphate, and by leakage for cations. Internal Mg^{2+}, which decreases the internal free concentrations of ADP and ATP, gives an additional complication. Moreover, in mitochondria the influences of the two regulation factors—membrane potential and pH gradient—can hardly be investigated separately.

Several of these problems can be overcome by the use of an artificial transport system, consisting of only the adenine nucleotide carrier incorporated in a defined phospholipid membrane. As previously described, the isolated and purified ADP–ATP carrier from beef heart mitochondria (Krämer et al., 1977) was functionally reconstituted with respect to its binding (Krämer & Klingenberg, 1977a) and transport activity (Krämer & Klingenberg, 1977b). The reconstituted carrier protein molecules have nearly the same specific transport activity as in mitochondria, though the extent of reconstitution amounts only to $\sim$5% (Krämer & Klingenberg, 1979).

In the present paper the regulation of an anionic transport system by membrane potential is demonstrated for the first time with a purified carrier protein in a reconstituted liposomal system. It permits quantitative elucidation of the regulation phenomena that cause the asymmetry of counterexchange in the energized state of the membrane. Due to the relative simplicity of the artificial system, the influences of membrane potential and H^+ gradient can be separately and quantitatively investigated. The results indicate that the ADP–ATP exchange works in a prevailing electrophoretic manner.

Materials and Methods

Materials. The sources of chemicals were the following: Triton X-100 (Sigma), egg yolk phospholipids (Merck), carboxyatractylate, valinomycin, and nucleotides (Boehringer-Mannheim), radioactive nucleotides (NEN), Dowex 1-X8 (Bio-Rad), Sephadex (Pharmacia). Bongkrekate was a gift from Professor W. Berends (Delft). All other chemicals were of analytical grade. Hydroxylapatite was prepared as previously described (Krämer & Klingenberg, 1979).

Determinations. Protein concentration was determined by the method of Lowry in the presence of 1% sodium lauryl sulfate (Helenius & Simons, 1972), and phosphorus was estimated by the method of Chen et al. (1956).

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[1] Abbreviations used: Tricine, *N*-tris(hydroxymethyl)methylglycine; Pipes, 1,4-piperazinediethanesulfonic acid; $\Delta\psi$, membrane potential; ΔpH, H^+ gradient; $v^{\leftarrow}$, uptake rate; $v^{\rightarrow}$, efflux rate; ADP_i, ADP in the internal volume of liposomes; ADP_e, external ADP.

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Isolation of the Adenine Nucleotide Carrier. The protein was isolated by hydroxylapatite chromatography in a batch procedure with Triton X-100 as described by Krämer & Klingenberg (1979). In the isolated protein assays on residual enzymes were performed in order to exclude possible interconversion of ADP and ATP in the reconstituted system by the presence of ATPase or adenylate kinase. The interconversion activities were so small that less than 0.1% of the nucleotides might be affected in the course of an exchange experiment.

Reconstitution of Adenine Nucleotide Exchange. Liposomes were prepared by sonication of phospholipids in a Branson sonifier (Krämer et al., 1977). The carrier protein was incorporated (Krämer & Klingenberg, 1977a), and the ATP–ADP translocation activity was reconstituted by a second sonication (Krämer & Klingenberg, 1977b, 1979). The sonication buffer was 100 mM NaCl or KCl, depending on the intended direction of membrane potential, and 20 mM Tricine–NaOH, pH 8.0. The concentration of nucleotides is indicated in the corresponding experiments.

Measurement of Adenine Nucleotide Exchange. The adenine nucleotide exchange in the forward direction was measured according to Krämer & Klingenberg (1977b, 1979). After reconstitution the external medium was changed by chromatography on Sephadex G-75 (100 mM NaCl) instead of Dowex-Cl being used. For the reversed case, i.e., back exchange experiments, the reconstituted vesicles were incubated with labeled nucleotides (1 μM) for 15 min at room temperature. Residual external nucleotides were removed on an additional Dowex-Cl column. Back exchange was started by adding unlabeled nucleotides (1 mM).

Adjustment of $\Delta\psi$ and ΔpH. The internal K^+ concentration was adjusted by the K^+ in the buffer used during preparation of liposomes, whereas external K^+ was added directly to the exchange assay. After addition of valinomycin (10 μM), a K^+ diffusion potential is generated. The membrane potential was calculated according to the Nernst equation. We are aware of the fact that the calculated values are somewhat overestimated because internal K^+ concentration may be decreased during chromatography of the liposomes and due to K^+ movements in the exchange experiments. However, since liposomes cannot be separated from the surrounding medium in reasonable times, a determination of membrane potential by distribution of labeled cations is not possible. For further reasons these measurements—including flow dialysis—would not be meaningful. The population of liposomes with incorporated carrier protein is small as compared to the large amount of protein-free vesicles, necessary only for absorption of the excess Triton X-100.

In the experiments with an H^+ gradient, for sonication a buffer with high capacity, Tricine–NaOH or Pipes–NaOH (50 mM), was used. After removal of the external medium, the H^+ gradient was adjusted by adding external buffer solution. The actual pH gradient was determined by the use of the impermeable dye phenol red (Wikström & Saari, 1977), which was added during the preparation of liposomes in these experiments.

Results

Influence of Membrane Potential on the Uptake Rates. For elucidating the regulation of nucleotide exchange, at first a simple system was chosen where only one single nucleotide species is present in the internal and external volume. Membrane potential was generated by diffusion gradients of K^+ in the presence of valinomycin. In Figure 1 it is shown that the membrane potential influences the exchange of nucleotides

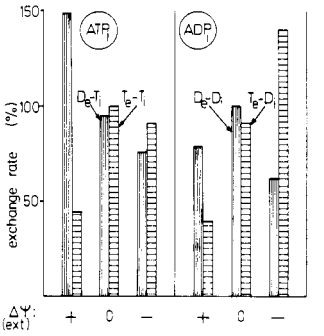

FIGURE 1: Influence of membrane potential on the uptake rates of external ADP or ATP. Only one nucleotide species was present in the external (100 μM) or internal (10 mM) volume. The applied membrane potential was 180 mV, external positive, or 120 mV, external negative. For comparison of the different rates in different experiments, all exchange rates are normalized to homoexchange (TeTi or DeDi) = 100%.

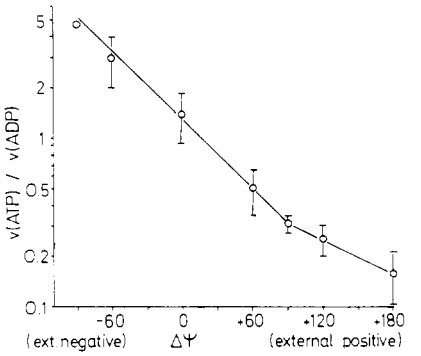

FIGURE 2: Competition of ADP and ATP uptake under the influence of membrane potential. ADP and ATP were present both in the external (50 μM each) and the internal (5 mM each) volume. The individual uptake rates [v(ATP) and v(ATP)] for both nucleotides were measured separately, and the ratio v(ATP)/v(ADP) was thereby calculated. The sum of both uptake rates remains nearly constant over the whole potential range.

in the reconstituted system. When the membrane becomes positively charged outside, the exchange of ADP_e against ATP_i is favored ~3 times as compared to the exchange of ATP_e against ADP_i. The opposite effect is observed when the potential is applied in the other direction. It is remarkable that an influence of the membrane potential is seen on the homoexchange rates. This cannot be understood by a primitive electrophoretic model of exchange regulation.

In further experiments the influence of membrane potential on the reconstituted adenine nucleotide exchange is demonstrated in the presence of both ADP and ATP on each side of the membrane. Thereby, a competition—similar to the physiological situation—takes place between these nucleotides in binding and exchange. With $\Delta\psi$ in the same direction as in energized mitochondria, positive outside, transport of ADP into the vesicles is strongly favored as compared to ATP uptake (Figure 2). Application of a K^+ diffusion gradient in the opposite direction involves stimulation of the reverse direction of exchange. The logarithmic dependence of the ratio of uptake rates ($k_D^{\rightarrow}/k_T^{\rightarrow}$) on the membrane potential is in agreement with the relation $k_D^{\rightarrow}/k_T^{\rightarrow} = e^{\beta F \Delta E/(RT)}$ (Klingenberg et al., 1969), which can be derived on thermodynamical considerations.

One can object to these experiments, where only the uptake rates of both nucleotides are measured, that in the efflux an identical change in the individual rates for ATP and ADP could take place. Thereby, no change of the ATP/ADP ratios in the internal and external pools would occur and no asym-

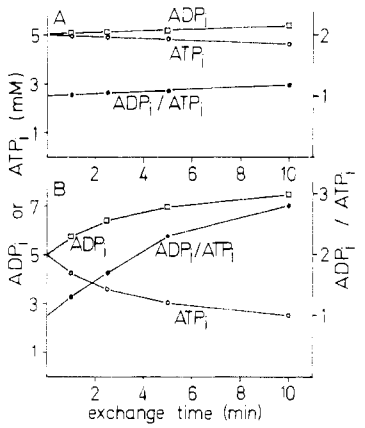

FIGURE 3: Shift in the internal ADP/ATP ratio by the $\Delta\psi$-influenced exchange. The experimental conditions were similar to those in Figure 2, without membrane potential (A) and with an external positive potential of 180 mV (B). The internal nucleotide concentrations were calculated as follows. The concentrations at the beginning of the experiment are known. The corresponding radioactivity of ATP and ADP when starting the back exchange can be measured. In one-half of the experiment the uptake rates for ADP and ATP are determined. Since adenine nucleotide transport is a strict counterexchange, the total uptake rate is equal to the total efflux rate as measured in the other half of the samples. Thereby, the efflux rate is obtained as micromoles per gram of protein per minute and the specific activity of the internal nucleotides is also obtained. For each time in the corresponding samples the change in the internal ADP and ATP concentration is calculated from the known amount of nucleotides taken up and transported out. Thus, the corrected specific activities and the corresponding concentrations of the internal nucleotides after every time interval are obtained.

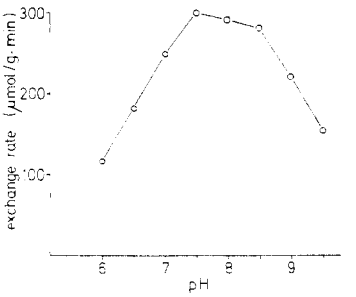

FIGURE 4: pH dependence of the reconstituted adenine nucleotide exchange. Exchange of external ATP (50 μM) against internal ATP (10 mM). The internal pH was 7.5, and the external pH given on the abscissa was adjusted before starting the exchange reaction.

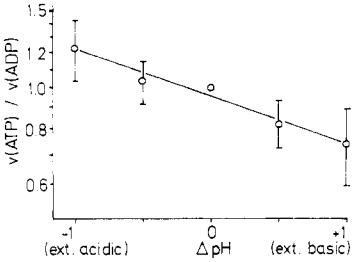

FIGURE 5: Competition of ADP and ATP uptake under the influence of ΔpH. Conditions are as in Figure 2. Due to the pH dependence shown in Figure 4, the sum of both uptake rates does not remain constant over the applied pH range, different from the experiment described in Figure 2. For differentiation of the ΔpH effect from a pH influence, the internal pH, as a basis for ΔpH = 0, was varied between 7.0 and 8.5.

metry in the exchange—expressed as the ratio $(v_T^{\rightarrow}/v_D^{\rightarrow})/(v_T^{\leftarrow}/v_D^{\leftarrow})$—would arise. For proof that a distinct enrichment of one nucleotide species by an asymmetric transport takes place, forward and back exchanges under the influence of membrane potential were measured simultaneously. Thereby, the internal concentration of both ADP and ATP can be calculated (Figure 3), as explained in the legend of this figure. In the deenergized state ($\Delta\psi = 0$), no significant enrichment of one single nucleotide can be detected. On the other hand, application of a membrane potential, positive outside, leads to both a definite accumulation of ADP inside the vesicles and a diminished internal ATP concentration. Since the applied potential is only available at the beginning of this experiment to its full extent and decreases due to the continuing exchange and to unspecific leakage, the asymmetry of nucleotide distribution reaches only a limited extent.

Influence of H^+ Gradient. Investigation of the influence of ΔpH on the reconstituted adenine nucleotide exchange is complicated by several factors. Every phospholipid membrane is proton permeable to some extent, presumably due to variable amounts of free fatty acids, which act as proton carriers (Scarpa & deGier, 1971). It can be shown, however, that the vesicles used in this study sustain H^+ gradients of about one unit for at least some minutes. This was tested by monitoring the internal pH of liposomes which contain the impermeable dye phenol red (Wikström & Saari, 1977) by the use of a dual-wavelength spectrophotometer. The reconstituted transport exhibits a distinct pH dependence with an optimum range between 7.5 and 8.5 (Figure 4). The profile does not change much whether the different pH values are adjusted from the beginning of the experiment (not shown) or immediately before the exchange reaction. Also, reconstituted vesicles, exposed to low (6.0) or high (9.5) pH for 30 min, have afterwards nearly the same pH dependence as the original reconstituted liposomes with only slightly diminished exchange rates (data not shown). These findings confirm that the activity profile in Figure 4 reflects the true pH dependence of the reconstituted exchange reaction and not an inactivation of the carrier protein.

Due to these complications the measured absolute exchange rates and ratios in Figure 5 vary considerably. Therefore, they are normalized to a zero H^+ gradient. Nevertheless, artificially imposed H^+ gradients influence only slightly the direction of ADP–ATP counterexchange. A gradient with a higher external H^+ concentration, resembling the situation in energized mitochondria, enhances slightly the ATP uptake as compared to ADP. It was tried furthermore by continuous pH registration whether during an asymmetric, membrane potential driven exchange of ADP_e against ATP_i a proton efflux can be measured. This was correlated to the extrusion of ATP. Because of the unfavorable, extremely high ratio of external to internal volumes, the accuracy of this method was not higher than 0.1–0.2 H^+/ATP transported. The detectable proton movements did not exceed this value (experiments not shown).

Regulation of the Efflux Rates and Differentiation of the Four Modes of Exchange. It has been shown in mitochondria (Klingenberg, 1975, 1976) that not only the uptake of adenine nucleotides is regulated but also the efflux. These regulation phenomena can be elucidated in the reconstituted system.

The ADP–ATP exchange can be subdivided into four "exchange modes": two homotypes (ATP_e against ATP_i = v_{TeTi} and ADP_e against ADP_i = v_{DeDi}) and two heterotypes (ATP_e against ADP_i = v_{TeDi} and ADP_e against ATP_i = v_{DeTi}).

The relations between these parameters are described by the following equations:

$$v^{\rightarrow} = v^{\leftarrow} \tag{1}$$

$$v_T^{\rightarrow} + v_D^{\rightarrow} = v_T^{\leftarrow} + v_D^{\leftarrow} \tag{2}$$

$$= v_{TeTi} + v_{DeDi} + v_{TeDi} + v_{DeTi} \tag{3}$$

$$v_T^{\rightarrow} = v_{TeTi} + v_{TeDi} \quad (4)$$

$$v_D^{\rightarrow} = v_{DeDi} + v_{DeTi} \quad (5)$$

$$v_T^{\leftarrow} = v_{TeTi} + v_{DeTi} \quad (6)$$

$$v_D^{\leftarrow} = v_{DeDi} + v_{TeDi} \quad (7)$$

For the determination of all these rates, the following set of experiments is necessary. With ADP and ATP on both sides of the membrane, the rates $v_T^{\rightarrow}$ and $v_D^{\rightarrow}$ can simultaneously be obtained by double labeling of the external nucleotides. According to eq 1, the amount of nucleotides taken up is equal to that released. Thereby, the specific activity of internal nucleotides can now be quantitatively evaluated and the ADP and ATP components of both uptake and efflux rates of eq 2 are thus available. For determination of the rates of all four exchange modes (eq 4–7), at least one of these individual rates must be independently measured. This was achieved by carrying out back exchange with either ADP or ATP alone in the external volume. Thus, the ratios v_{TeTi}/v_{TeDi} with respect to v_{DeDi}/v_{DeTi} can be determined and all rates of eq 4–7 can be calculated. In Figure 6 these individual rates are summed up to 100% for total uptake or efflux according to eq 2 and for the four modes according to eq 3. The membrane potential influences not only the inward transport direction as it has already been shown in Figure 2. With outside positive potential also the efflux of ATP is favored ~3 times over that of ADP. It is further demonstrated that the membrane potential in the same direction as in energized mitochondria (positive outside) drastically influences the heterotype modes. The exchange of external ATP against internal ADP (v_{TeDi}) is favored nearly exclusively over the corresponding heteroexchange (v_{DeTi}). A potential in the reverse direction promotes the exchange of external ATP against internal ADP. The homotype modes are less affected by the applied membrane potential.

Discussion

In the present work, for the first time, an electrically driven transport can be achieved with a purified carrier protein in a reconstituted system. This demonstrates that the reconstituted adenine nucleotide carrier not only recovers its exchange activity but also its important regulation by the membrane potential. In this system the dependence of ADP–ATP exchange on the membrane potential and the H^+ gradient can be quantitatively studied.

The reconstituted system also permits the use of a single nucleotide species (either ADP or ATP) or a well established ADP/ATP ratio on either side of the membrane (Figure 1) whereas in mitochondria this is barely possible for the matrix ATP/ADP. The influence of the membrane potential on the nucleotide exchange is less apparent if only ADP or ATP is offered as compared to the competitive situation when both ADP and ATP are present on either side of the membrane (Figure 2). A similar result had been observed in mitochondria (Klingenberg, 1972). The total exchange rate does not change significantly with the membrane potential when both ADP and ATP are present whereas in the exchange the portion of a single nucleotide species is highly sensitive to potential changes.

Moreover, in the reconstituted vesicles the potential can be varied in both directions, either positive inside or outside. Consequently, the uptake ratio for ATP and ADP can even be reversed as compared to the normal case. This is most remarkable if one considers our previously reported observation that the orientation of transport-active carrier molecules in

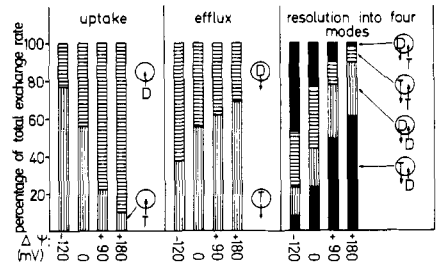

FIGURE 6: Influence of membrane potential on the uptake and efflux rates and on the four exchange modes. Resolution into the single rates is described in the text. The rates are given in percent of total exchange rate. As in Figure 2, the sum of the single rates remains nearly constant over the whole potential range.

the liposomes is in the same direction as in mitochondria (Krämer & Klingenberg, 1979) and that the carrier is regarded as an asymmetric "gated pore" with a fixed orientation in the membrane (Klingenberg, 1976). Thus, a membrane potential positive outside applied to the liposomes can be considered as "physiological". There are no transport-active carrier molecules to which an opposite membrane potential might be also physiological. While the response of incorporated ADP–ATP carrier to the membrane potential is similar to that in mitochondria, the symmetrical response to the reversed membrane potential up to 90 mV, where measurements can still be done with reasonable accuracy, might not have been anticipated. This means that the carrier does not behave like an electrical valve but rather as a "passive" conductor following the electromotive forces.

There is a small, but definite, influence of the ΔpH on the ATP vs. ADP uptake rates which cannot be explained by the difference of the pK values between ADP and ATP, since the influence persists when the internal and external pH is raised above 8, where all nucleotides are completely deprotonated. The influence of the ΔpH should counteract the effects of $\Delta\psi$, as shown in the present experiments, although it is very small as compared to the effects of $\Delta\psi$. Therefore, in the reconstituted system, the ADP vs. ATP exchange seems to have only a very minor electroneutral component.

In the previous studies on mitochondria, after the exchange was recognized to be electrically driven (Pfaff & Klingenberg, 1968; Klingenberg et al., 1969), an electroneutral contribution to the ADP vs. ATP exchange was assumed for reasons of energy balance in oxidative phosphorylation and to account for a small H^+ efflux accompanying the release of ATP (Klingenberg et al., 1972). This release, however, was then associated with interfering H^+ leaks in the mitochondrial membrane (LaNoue et al., 1978; Villiers et al., 1979). In the reconstituted system this complication is eliminated. Here the ADP–ATP exchange can be unequivocally regarded as electrically driven.

It remained to be shown whether the membrane potential also influences the efflux of ADP and ATP in the liposomal system. For this purpose changes of the internal pools of ADP and ATP were analyzed. These data then permitted the resolution of the ADP and ATP transport into the four possible single modes of hetero- (ADP_e–ATP_i and ATP_e–ADP_i) and homoexchange (ADP_e–ADP_i and ATP_e–ATP_i) (Figure 6). The influence of the membrane potential is most strongly expressed on the two heteroexchanges, resulting in a high preference for the exchange of ADP_e against ATP_i (v_{DeTi}) over the exchange of ATP_e against ADP_i (v_{TeDi}) in the externally positive polarized state. This corresponds to the energized state of mitochondria, in which similarly the selection of the ADP_e–ATP_i exchange modes can be measured (M. Klingen-

berg, unpublished experiments). Furthermore, in the reconstituted system the opposite mode can be selected by applying the opposite membrane potential.

In our opinion the necessity of a reconstituted system needs to be critically discussed, in particular the differences of the protein function in the original and in the reconstituted system. It has been demonstrated previously that the relatively small portion of reconstituted adenine nucleotide transport shows nearly the same specific activity as compared to intact mitochondria (Krämer & Klingenberg, 1979). Yet, there are some properties of the reconstituted protein which differ from those of the carrier in intact mitochondria. The response of the exchange to the membrane potential is not as high as observed in intact mitochondria. It has to be taken into account, however, that in the liposomes the membrane potential may be somewhat overestimated as discussed already under Materials and Methods. Another difference of the reconstituted carrier is a pronounced pH dependence, whereas this effect is very weak in intact mitochondria (Pfaff & Klingenberg, 1968). The "sensitivity" to the pH of the surrounding medium may be explained by a higher portion of protein surface exposed to the water phase since the artificial bilayer has a smaller thickness as compared to the protein-rich mitochondrial membrane.

In spite of these restrictions, several advantages in working with the reconstituted system have been shown in this paper. No other interfering transport system or phosphate transferases can cause complications; usually these complications are partially circumvented by the use of inhibitors such as *N*-ethylmaleimide, which might also affect the ADP–ATP carrier (Leblanc & Clauser, 1972). The two components of the mitochondrial $\Delta\mu H^+$, i.e., $\Delta\psi$ and ΔpH, can be applied separately, and the membrane potential can be varied arbitrarily in the nonphysiological direction. Furthermore, all of these measurements are possible without the interference of other nucleotide binding sites and without the complexation of nucleotides by an unknown concentration of internal magnesium.

In conclusion, the experiments reported here prove that the adenine nucleotide carrier itself comprises all the properties not only for transport but also for the regulation of exchange. In the reconstituted system, the carrier protein is separated from both ATPase and the phosphate translocator, to which it is functionally related in the mitochondrial membrane. Thus, it is excluded that the ATPase regulates the activity of the ADP–ATP carrier by a direct interaction between both proteins as has been repeatedly suggested (Out et al., 1976; Vignais et al., 1975). These considerations do not necessarily mean that the carrier–substrate complex follows the membrane potential by simple electrophoretic diffusion. Finally, a regulation mechanism by changing K_m values for ATP and ADP (Souverijn et al., 1973) is not excluded by the data presented here. These questions will be studied in further experiments.

Acknowledgments

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