Biochimica et Biophysica Acta, 735 (1983) 145–159
Elsevier

BBA 71869

INTERACTION OF MEMBRANE SURFACE CHARGES WITH THE RECONSTITUTED ADP/ATP-CARRIER FROM MITOCHONDRIA

REINHARD KRÄMER

Institut für Physikalische Biochemie der Universität München, Goethestrasse 33, 8000 München 2 (F.R.G.)

(Received July 6th, 1983)

Key words: ADP-ATP exchange; Mitochondria; Liposome; Surface potential

Various modulating influences of negative and positive membrane charges on binding and transport properties of the reconstituted ADP/ATP carrier from mitochondria were investigated. The results are interpreted in terms of functional and structural asymmetries of the adenine nucleotide carrier embedded in the liposomal membrane. The surface potential of liposomes was measured directly either by potential-dependent adsorption of the fluorescent dye 2-*p*-toluidinylnaphthalene 6-sulfonate (TNS) or by the p*K* shift of the lipophilic pH indicator pentadecylumbelliferone. These results were correlated with the following observations. (1) Negative surface potentials increase the apparent dissociation constant, K_d, for binding of the negatively charged inhbitor carboxyatractylate to the reconstituted carrier protein. (2) Surface potentials modulate the apparent transport affinity, K_m, of the reconstituted adenine nucleotide carrier for ADP and ATP. The interaction of surface charges with the transport function was investigated with carrier proteins oriented both right-side-out and inside-out. Thus the influence of the surface potential on the function of the ADP/ATP carrier could be determined for the internal and external active sites of the translocator on the outer side of the membrane. Large discrepancies were observed not only between the potentials measured directly (fluorescent dyes) and those measured indirectly (binding and transport affinities), but also between the different surface potentials determined from the influence on the alternatively oriented carrier proteins. The effect of surface charges was rather weak on the cytosolic side of the translocator, whereas there was a strong influence of surface charges on the active site at the matrix side. The most obvious explanation, i.e., screening of negative membrane charges by positively charged amino acid residues at the protein surface, could be ruled out. Besides the modulation of binding affinities for substrates and inhibitors, an additional side-specific effect of surface charges on the transport velocity was observed. Again, the influence on the internal active site of the ADP/ATP carrier was found to be much higher than that on the cytosolic site. The observed effects can be explained by a definite structural asymmetry of the carrier embedded in the liposomal membrane. That site which is physiologically exposed to the cytosol is located at a considerable distance from the plane of the membrane, whereas the opposite site seems to be in close proximity to the membrane surface. Moreover, a spatial equivalence of carboxyatractylate binding site and nucleotide binding site at the external side of the carrier protein was concluded.

Introduction

The adenine nucleotide carrier from the inner mitochondrial membrane is influenced and regulated by a wide variety of factors. Many of these,

Abbreviations: CTAB, cetyltrimethylammonium bromide; C15U, pentadecylumbelliferone; PC, phosphatidylcholine; PE, phosphatidylethanolamine; TNS, 2-*p*-toluidinylnaphthalene 6-sulfonate; Tricine, *N*-tris(hydroxymethyl)methylglycine; ψ_0, surface potential; σ, surface charge density.

such as membrane potential [1,2], divalent cations [3] and composition of the phospholipid membrane with respect to headgroups [4,5] and charge [5] have been investigated in the reconstituted system. The most important regulatory factor of the nucleotide exchange activity, the membrane potential [6–8], has been shown to influence predominantly one single kinetic parameter in the reaction cycle of nucleotide transport, namely the rate constant of ATP import and export [2]. An influence of membrane potential on the binding affinity (K_d) of the carrier protein could be more-or-less excluded. However, there are also factors directly modulating the apparent carrier affinity for nucleotides, the most significant of which is presumably the surface potential.

The inner membrane of mitochondria, like most biological membranes, bears a high net negative surface charge which gives rise to a negative surface potential. This will affect the concentration of cations and anions in the region adjacent to the membrane and may thus influence kinetic parameters of membrane-bound enzymes [9–11] and carriers [12,13]. This should be of importance in particular for the adenine nucleotide carrier, since its substrates, ATP and ADP, are highly negatively charged. The effects of surface potential on the transport of various anions in mitochondria from heart and liver have been previously studied indirectly by the dependence of transport velocities on the ionic strength of the surrounding medium [14,15].

The influence of negative surface potential on the ADP/ATP transport is of such interest not only because of the presence of negatively charged phospholipids in the inner mitochondrial membrane, but also because they have been shown to stimulate the reconstituted adenine nucleotide exchange [4,5,16,17]. Functional dependence on negatively charged phospholipids has been reported for several membrane proteins (e.g. Refs. 18,19). However, it is not yet clear whether the stimulation observed is always due to direct interaction of the protein with the individual phospholipid species or whether this dependence is caused predominantly by the anionic charge.

Besides the quantitative correlation of actual surface potentials with their effect on the ADP/ATP transport, the present study will focus especially on the fact that both the phospholipid distribution between the outer and inner side of the membrane [20] and, of course, the location of proteins within the inner mitochondrial membrane [21,22] are asymmetric. This explains the importance of investigating the influence of surface charges separately on each side of a transmembrane carrier protein. Since the translocator molecules turn out to be oriented in the two possible directions after insertion into the liposomal membrane [23] and since these two populations of carrier molecules can be functionally separated, the reconstituted system is particularly suitable for determining any asymmetric interaction of the ADP/ATP carrier with the two different membrane surfaces.

Materials and Methods

Chemicals

The chemicals and their sources were as follows: Triton X-100 (Sigma); carboxyatractylate, valinomycin and nucleotides (Boehringer-Mannheim); radioactive nucleotides (Amersham-Buchler); Dowex 1-X8 (Bio-Rad); Sephadex (Pharmacia); dicetyl phosphate, cetyltrimethylammonium bromide, tetraphenylborate and 2-*p*-toluidinylnaphthalene 6-sulfonate (Sigma). Pentadecylumbelliferone was a gift from Professor Fromherz (Ulm) and bongkrekate was a gift from Professor Berends (Delft). Hydroxyapatite was prepared as described previously [23]. All other chemicals were of analytical grade. [^{3}H]carboxyatractylate was prepared according to Ref. 24.

Determinations

Protein concentration was determined by the method of Lowry et al. in the presence of 1% sodium dodecyl sulfate [25] and phosphorus was estimated by the method of Chen et al. [26].

Lipids and liposomes

Egg yolk phospholipids were prepared according to Ref. 27. Soybean phospholipids were partially purified [28]. Purification and separation of single phospholipid species were performed as described previously [29]. For the preparation of liposomes with added negatively or positively charged lipids, the individual lipids were mixed in

chloroform, evaporated to dryness and sonicated under nitrogen atmosphere in a Branson sonifier.

Isolation, reconstitution and assay of ADP/ATP carrier protein

The adenine nucleotide carrier was isolated from beef-heart mitochondria by hydroxyapatite chromatography in a batch procedure using Triton X-100 as described previously [23]. The carrier protein was incorporated into preformed liposomes and the ADP/ATP-translocation activity was reconstituted by a freeze-thaw procedure [30] and a second sonication [4]. The sonication buffer included all ions and nucleotides which had to be present afterwards in the internal liposomal volume. For most experiments, the internal space contained 10 mM ATP/30 mM Na_2SO_4/10 mM Tricine-NaOH (pH 8.1). The external medium was exchanged by chromatography on Sephadex G-75 columns in order to obtain the desired external ionic conditions, as indicated in the corresponding experiments. Reconstituted adenine nucleotide exchange in the forward [4,2] and backward direction [23] has been described previously. Extrapolation of true exchange velocities and of individual kinetic parameters was carried out according to Ref. 2. In exchange experiments with simultaneous measurement of surface potential, an aliquot of the liposome pool from the Sephadex G-75 columns was titrated with TNS or C15U and analyzed for its phospholipid content by phosphate determination.

In some experiments, the two populations of reconstituted nucleotide carriers (right-side-out and inside-out) were analyzed further. This was achieved by alternate titration of the exchange with carboxyatractylate and bongkrekate, respectively. The procedure has been described in detail previously [23]. After stopping the exchange reactions with carboxyatractylate and/or bongkrekate, as indicated in the corresponding experiments, radioactive external nucleotides were removed by ion-exchange chromatography on Dowex 1-X8 columns (Cl^- form). 100–200 μl of liposomal suspension was subjected to columns 5 mm × 30 mm. In order to minimize loss of lipid and protein, the columns were preequilibrated with egg-yolk phospholipid liposomes (3–5 mg phospholipid/column) and bovine serum albumin (2 mg/column). The liposomes of the exchange assay were eluted with a defined volume of 50 mM NaCl. Aliquots of the eluate were analyzed by liquid scintillation counting of the amount of internal radioactively labeled nucleotides.

Carboxyatractylate binding assay

In order to determine carboxyatractylate binding in a rapid procedure, suitable for large experimental series, advantage was taken of the very high affinity of carboxyatractylate for the reconstituted ADP/ATP translocator. After incubation of the reconstituted nucleotide carrier with ^3H-labeled carboxyatractylate, 300–500 μl of this incubate were added to 50 mg of Dowex-Cl^- which had been dried on filter paper. After 30 s, the Dowex material was quickly centrifuged (10 s, 15 000 × g). The supernatant was immediately separated from the ion exchange resin and aliquots of the supernatant were analyzed in a scintillation counter.

Measurement of surface potential

Two different methods were used for the determination of surface potential:

(i) The potential-dependent adsorption of the fluorescent dye 2-*p*-toluidinylnaphthalene 6-sulfonate (TNS) and calculation of the corresponding surface potentials are described by Eisenberg et al. [31]. The fluorescence of TNS (0.25 μM) was measured with a Perkin-Elmer MPF 44a fluorescence spectrometer in the presence of 0.05–0.15 mg phospholipid/ml. The excitation wavelength was 321 nm (2 nm bandwidth) and emission was measured at 439 nm (5 nm bandwidth). The appropriate corrections, described in Ref. 31, were carried out. The surface potential was determined in dependence on added NaCl and the corresponding surface charge densities were calculated (see theoretical section).

In order to confirm these measurements and to allow their extension also to positive surface potentials, a technique was applied whereby negative surface charges in the liposomal membrane were titrated back with externally added CTAB and positive surface charges with sodium tetraphenylborate, respectively. An example of this kind of measurement is shown in Fig. 1. The surface potential of liposomes consisting of egg-

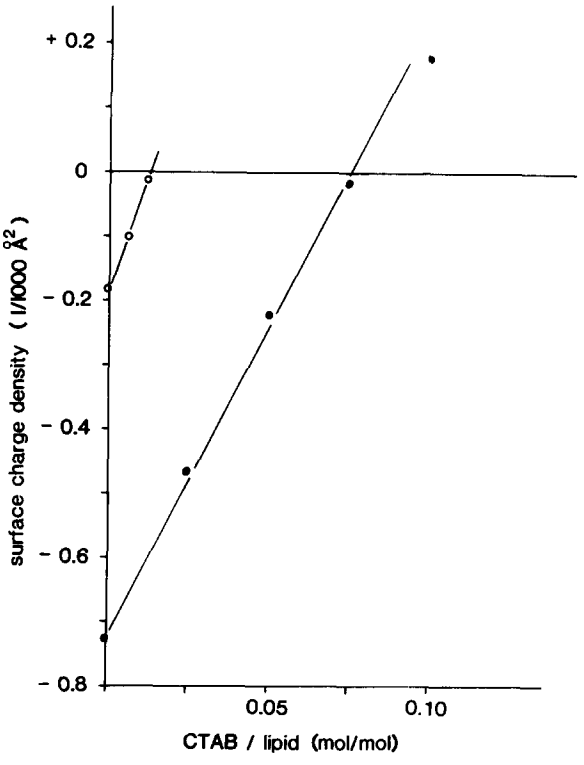

Fig. 1. Titration of negatively charged liposomes with CTAB. The indicated amounts of CTAB were added to liposomes consisting of purified egg-yolk phospholipids (○–○) or egg-yolk phospholipids with 10 mol% dicetyl phosphate (●–●). The surface potential was measured at Na^+ concentrations of 10–100 mM by TNS fluorescence and the corresponding surface charge densities were calculated and averaged over this range of ionic strength.

yolk phospholipids and negatively charged dicetyl phosphate was determined in dependence on the amount of added CTAB. The point of equilibration between dicetyl phosphate in the membrane and added CTAB can be extrapolated. This value clearly depends on the amount of originally incorporated dicetyl phosphate. The complication arising due to the two faces of the liposomal bilayer membrane will be discussed later (see Fig. 3).

(ii) Alternatively, the fluorescence of the lipophilic pH indicator C15U was used to determine surface potential [32,33]. The interfacial pK of the membrane-incorporated pH indicator can be determined by acid/base titration, since only the basic form of the indicator in fluorescent. The pK can thus be obtained by measuring pH and fluorescence intensity [32]. The pH indicator was dissolved in a methanolic stock solution and added in a concentration of 0.2–0.5 μM to the liposomal suspension (0.15–0.3 mg phospholipid/ml). Excitation was at 366 nm (2 nm bandwidth), emission at 448 nm (5 nm bandwidth). The pK was determined from simultaneous measurement of steady-state fluorescence and pH with a microglass electrode and calculcated by graphic extrapolation.

There remains, however, a severe methodological problem. Using the dye C15U, a definite negative surface potential is measured also in pure PC liposomes, probably due to the geometry of the phosphatidylcholine headgroup [34]. This potential does not contribute to the apparent surface potential as calculated, for example, from ζ-potential determinations or from fluorescence of TNS, but it can be detected with the lipophilic pH indicator C15U, because of the location of its potential-sensitive OH group. Since PC is always the predominant lipid in the phospholipid mixtures used for these experiments the pK value for pure PC liposomes, measured at equivalent ionic strength, was taken as zero surface potential.

Theoretical considerations

Ligand binding

The concentrations of ions with z negative or positive charges in the aqueous phase at the membrane/solution interphase (c_m) is given by the Boltzmann relation

$$c_m = c_b \cdot \exp\left(\frac{z \cdot q \cdot \psi_0}{kT}\right) \quad (1)$$

where c_b in the bulk phase concentration of the ions, q the negative electron charge and ψ_0 the surface potential. For the present experiments, this relation is important in several respects.

(a) The concentration of H^+ is influenced according to the equation

$$pH_m = pH_b + \psi_0/60 \quad (2)$$

where pH_b is the bulk phase pH and pH_m the pH at the membrane/water interphase. Eqn 2 also explains the apparent pK shift of C15U caused by the surface potential.

(b) The concentration of TNS anions is modulated likewise by the surface potential.

(c) The concentration of carboxyatractylate^{4-} is strongly influenced by surface charges. This results in an apparent K_d shift according to

$$K_{d\,app} = K_d \cdot \exp\left(\frac{4 \cdot q \cdot \psi_0}{kT}\right) \quad (3)$$

(d) In the same way, the binding affinity of membrane-bound enzymes [11] or the transport affinity of carrier proteins is modulated by the surface potential, leading to a change in the apparent K_m values:

$$K_{m\,app} = K_m \cdot \exp\left(-\frac{z \cdot q \cdot \psi_0}{kT}\right) \quad (4)$$

At pH 8.2, used in these experiments, z for ATP and ADP is -4 and -3, respectively. The following investigations were carried out without application of membrane potentials; therefore the complication due to modulation of other kinetic constants that are also contained in the K_m of ADP/ATP exchange [2] can be neglected.

Gouy-Chapman potential

The values for surface potential derived from the different methods described here are used to calculate surface charge density (σ) according to the equation

$$\frac{A\sigma}{c} = \sinh(z \cdot q \cdot \psi_0 / 2kT) \quad (5)$$

where A is a temperature-dependent constant [35] and c is the bulk aqueous electrolyte concentration. To estimate the distance between membrane surface and the plane at which different potential monitors sense the actual potential, the extended Gouy equation [35] was used.

Results

(1) Influence of surface potential on the adenine nucleotide exchange

The anionic nature of ADP and ATP, the substrates of the adenine nucleotide carrier, results in a repulsion of these ligands from negatively charged membrane surfaces. As already shown for membrane-bound enzymes [9–11] and transport proteins [12,13], this repulsion leads to an increase in the apparent K_m of ADP and ATP transport (see also Theoretical section). Thus, the fundamental question arises: to what extent does the surface potential influence the nucleotide exchange at the two sides of the membrane and which steps in the reaction cycle of the carrier protein [36] are modulated?

Since the adenine nucleotide carrier is a transmembrane protein, exposing its nucleotide binding sites to both sides of the membrane, it is of basic interest to find out whether the surface potential influences the carrier protein symmetrically at the outside and the inside. The inside of liposomal vesicles is, however, not easily accessible to direct measurement. Therefore advantage was taken of the fact that during reconstitution the carrier protien is incorporated into the membrane in both possible directions [23]. In the 'physiological' orientation, i.e., right-side-out (R), the nucleotide carrier exposes its carboxyatractylate-binding site to the outside, whereas in the reverse orientation, i.e., inside-out (I), the bongkrekate-binding site faces the outside. The two contrarily oriented populations of carrier molecules can be differentiated by titration with the site-specific inhibitors carboxyatractylate and bongkrekate [23]. In this way, the influence of surface charges on the two binding sites of the nucleotide carrier can be investigated at the outer side of the liposomal membrane. Complications due to asymmetric phospholipid distribution, which has been reported not only for mitochondrial membranes [20], but also for liposomes [37,38], can thus be avoided.

The apparent K_m values for adenine nucleotide exchange measured with right-side-out (R) and inside-out (I) carrier molecules are summarized in Table I. In both types of orientation, the influence of negative surface charges on the transport affinity in liposomes with different phospholipid composition is detected. As expected, the increase in the apparent K_m is enhanced when a higher density of negative charges is applied. As predicted by the theory of the diffuse layer [35], the surface potential and its effect on the apparent transport affinity, K_m, can be screened by high ionic strength. It should be emphasized that the data of Table I clearly demonstrate an asymmetric influence of the surface potential on the two binding sites of

TABLE I

TRANSPORT AFFINITY K_m (μM) FOR ATP OF THE RECONSTITUTED ADP/ATP CARRIER

The liposomes used for reconstitution consisted of PC/PE/cholesterol = 65 : 20 : 15 mol% with addition of the indicated amounts of dicetyl phosphate. The transport affinity K_m was measured for the right-side-out- (R) and inside-out- (I) oriented carrier protein (see text). The external Na^+ concentration was adjusted with NaCl, osmolarity was balanced with sucrose.

Dicetyl phosphate added (mol%)	Carrier orientation	External Na^+ concentration (mM)						
		4	7	10	20	40	60	100
0	R		7	8	8	11	14	16
	I		18	17	20	26	28	37
3	R				15			
	I				105			
6	R	118	67	36	31	19	17	19
	I		1800	1400	950	570	210	
10	R	187	115	72	43		20	
	I				2050		390	

the carrier. Apparently, the transport affinity of the inside-out-oriented carrier protein (I) is modulated very strongly, whereas the effect on the right-side-out-oriented carriers (R) is relatively weak. The increase in K_m values at high ionic strength can be explained fully by competition effects of anions, in this case Cl^-, at the nucleotide binding site (unpublished results).

The interpretation of the observed K_m modulation as an electrostatic interaction between substrates and the membrane surface is corroborated not only by variation of the surface charge density and the ionic strength, as shown in Table I, but also by variation of the substrate charge from four (ATP) to three (ADP) negative charges. As seen in Table II, the influence of negative surface charges on the transport affinity is in fact diminished when ATP is replaced as substrate by ADP. The data from Table I and Table II are used in the following sections for the calculation of the actual surface potential (Table V).

TABLE II

TRANSPORT AFFINITY K_m (μM) FOR ADP AND ATP

The liposomal membranes consisted of egg-yolk phospholipid/cholesterol = 85 : 15 mol% with addition of dicetyl phosphate where indicated. The reconstituted exchange was measured only for right-side-out-oriented carrier molecules.

Dicetyl phosphate added (mol%)	Substrates	External Na^+ concentration (mM)			
		6	10	20	100
0	ATP	6	8	8	16
	ADP		7	9	
10	ATP	154	80	45	20
	ADP	65	36	21	15

(2) Influence of surface potential on inhibitor binding

The surface potential modulates not only the binding of the charged transport substrates ADP and ATP, but influences also the binding of inhibitor ligands like carboxyatractylate and bongkrekate, which carry about four and three negative charges at pH 8.2, respectively. The binding step of adenine nucleotides cannot be easily separated from the overall transport reaction due to the fact that conformation change and translocation step are obligatorily coupled in the reaction cycle of the nucleotide carrier [36]. Using specific inhibitors, however, the influence of surface charges on the dissociation constant can be detected directly.

Binding of the hydrophobic inhibitor bongkrekate, as assayed in the reconstituted system with its high phospholipid : protein ratio, leads to rather unsatisfactory results in the binding analysis, due to unspecific binding to liposomal membranes. In

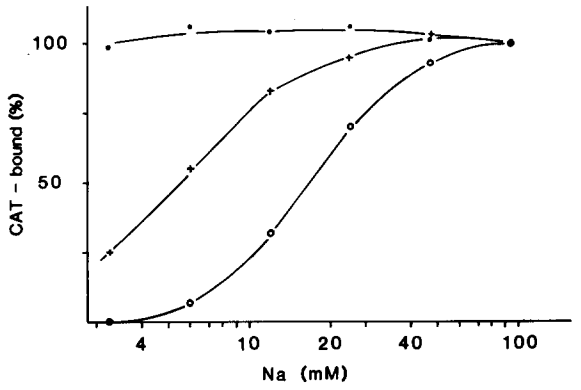

Fig. 2. Binding of carboxyatractylate (CAT) to the reconstituted ADP/ATP carrier in liposomes with different surface charges. ^{3}H-labeled carboxyatractylate (100 nM) was bound to the reconstituted adenine nucleotide carrier in liposomes consisting of egg-yolk phospholipids (●–●, neutral), soybean phospholipids (○–○, negatively charged) and a 1/1 mixture of both (+–+). The amount of bound inhibitor at 100 mM Na^+ was taken as 100%.

contrast, binding of the more hydrophilic inhibitor carboxyatractylate can be well analyzed with regard to the modulation caused by negative surface potentials.

In Fig. 2, binding of radioactively labeled carboxyatractylate to carrier protein reconstituted in egg-yolk lipid (1–2% negatively charged phospholipids) and soybean phospholipid (more than 20% negatively charged lipids) is analyzed. Obviously, variation in the lipid composition of the liposomal membrane may, in fact, have a dramatic influence on the binding properties of the nucleotide carrier. Although the effects of the potential are strongly enhanced because of the low ionic strength used in this experiment, this observation explains why asolectin is such a poor material for the reconstitution of functionally active ADP/ATP exchange. It has to be taken into consideration that the affinity of ADP and ATP for the carrier protein is about three orders of magnitude lower than that of carboxyatractylate. Substrate binding to the active site would therefore be virtually impossible in soybean phospholipid membranes, even at higher ionic strength.

The influence of surface potential on the binding affinity of carboxyatractylate for the nucleotide carrier reconstituted into membranes with defined negative surface charge is analyzed in Table III. The results are similar to those obtained for the transport affinity K_m of ADP and ATP (Tables I and II): both charge density and ionic strength determine the apparent affinity changes, in accordance with the theory of the diffuse layer. These data, too, are used in the following sections to calculate actual surface potentials at the carboxyatractylate-binding site (Table V).

(3) Direct determination of the surface potential

In order to correlate these functional measurements with the true surface potential, the latter was determined by direct methods. Several methods were used to obtain reliable values for the surface potential: (a) salt titration with the fluorescent dye TNS [31]; (b) salt titration with the lipophilic fluorescent pH indicator C15U [32,33]; and (c) titration of negatively charged liposomes with $CTAB^+$ or positively charged vesicles with

TABLE III

DISSOCIATION CONSTANTS, K_d (nM), FOR THE CARBOXYATRACTYLATE-CARRIER COMPLEX

^{3}H-labeled carboxyatractylate was bound to the adenine nucleotide carrier reconstituted in liposomal membranes with the indicated phospholipid composition. The external Na^+ concentration was adjusted by addition of NaCl, osmolarity was balanced with sucrose. Binding to the carrier protein in PC membranes was carried out in multiple determinations ($n > 5$), wherease the other values each represent the average of 2–4 binding assays. EYPL, egg-yolk phospholipid; DCP, dicetyl phosphate.

Liposome composition (mol%)	External Na^+ concentration (mM)					
	1	3	7	10	20	75
PC			5±2[a]			
EYPL		14		8	10	
EYPL+6% DCP	210		34	25	18	11
EYPL+10% DCP		190	78	31	23	14

[a] For all Na^+ concentrations.

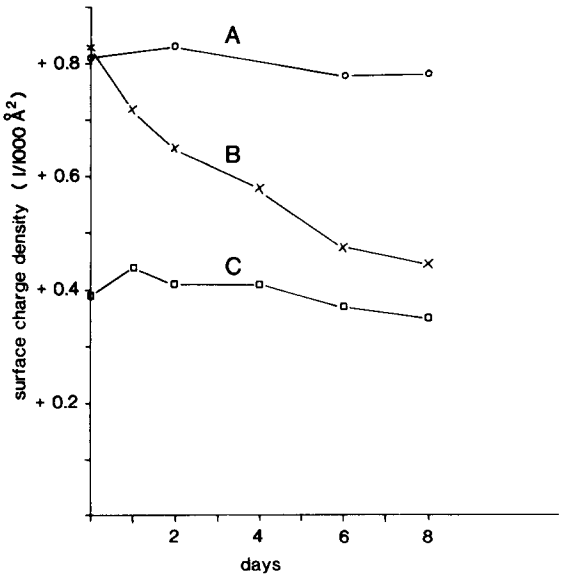

Fig. 3. Titration of CTAB in pure PC liposomes. CTAB was added to pure PC liposomes (PL/CTAB = 94:6 mol/mol) and the surface potential was determined either immediately after the addition of positively charge lipid (A) or after the time intervals indicated on the *X*-axis (B+C). Furthermore, at time zero, a liposome/CTAB mixture equivalent to (B) was frozen five times in liquid nitrogen, thawed and finally resonicated (C). Measurement of surface potential was carried out as described in Fig. 1. Thus line (A) represents a series of determinations after repeated new addition of CTAB to stored PC liposomes, whereas lines (B) and (C) reflect repeated measurements at the indicated times with the same liposome/CTAB mixtures, which were prepared separately for (B) and (C) at time zero.

the tetraphenylborate anion and determination of the charge equilibration by TNS or C15U as monitors of the potential.

In all these experiments, it must be remembered that only the outer layer of the liposomal membrane is measured. This is clearly demonstrated in Fig. 3. Basically, the neutral PC liposomes are made positive by addition of CTAB from the outside. The positive surface charge, titratable with tetraphenylborate from the outside, gradually diminishes, with a half-life of several days. The interpretation of these results as disappearance of positive charges from the outside is further corroborated by an experiment in which the positively charged lipid is equilibrated from the beginning (Fig. 3, line C). Addition of CTAB, with subsequent repeated freezing, thawing and sonication, results in liposomes with a diminished amount of externally accessible CTAB.

The calculated surface charge densities derived from measurements of surface potential are listed in Table IV for several types of liposome with different phospholipid composition. The negative surface potential created by addition of dicetyl phosphate to the liposomal membrane is measured both by C15U and TNS. Several results, which are of special interest both for the following experiments as well as for the interpretation of sections 1 and 2, are pointed out here. (a) The charge densities determined directly by C15U and TNS are relatively close to the calculated values. (b) The data obtained by titration with CTAB (last column) are in good agreement with the calculated data when they are multiplied by a factor of about 1.7, which resembles the ratio of total lipid/lipid in the outer layer of the liposomes. (c) Addition of CTAB and DCP in equimolar amounts during the preparation of liposomes results in virtually uncharged or chargecompensated liposomes. (d) Isolated and purified egg-yolk phospholipids have only few negative charges, whereas soybean phospholipids are highly negatively charged.

(4) Comparison of directly and indirectly measured surface potentials

In Table V, values of surface potentials measured in two kinds of negatively charge liposome are summarized. The data are either calculated on the basis of lipid composition or measured directly by TNS and C15U fluorescence, or they are derived from the apparent K_m and K_d values for adenine nucleotide transport and carboxyatractylate binding, respectively. On the one hand, there is an unequivocal correlation of the increase in the negative surface charge caused by increasing amounts of dicetyl phosphate with the shift in the binding affinity of carboxyatractylate and the transport affinity of ATP for the carrier protein. On the other hand, striking discrepancies are observed between the results obtained by the different methods for calculating the surface potentials.

The experimental data of Table V, together with the results achieved with various other types of liposome (not shown) can be arranged in the following sequence, using decreasing values of

TABLE IV

DIRECT MEASUREMENT OF SURFACE POTENTIALS USING FLUORESCENT DYES

The surface charge density of liposomes with different lipid compositions was calculated from values of surface potential measured by the following methods. (1) σ was calculated from the lipid composition assuming an area of 70 Å^2 for phospholipids, 50 Å^2 for dicetyl phosphate, and 25 Å^2 for CTAB. (2) The surface potential was measured directly by pK titration of externally added C15U in the presence of various Na^+ concentrations. The charge densities calculated from the obtained potentials at 10, 20 and 40 mM Na^+ were averaged. The surface potential for pure PC liposomes was taken as zero (see Materials and Methods). (3) The surface potential was measured by salt titration of TNS fluorescence. The charge densities calculated from the potentials at 10, 20, 40 and 80 mM Na^+ were averaged. (4) The surface potential was titrated by addition of CTAB to negatively charged liposomes and monitored by TNS fluorescence. The values of this column have to be multiplied by a factor of approx. 1.7 in order to obtain the correct charge density, since only the outer layer of the membrane is titrated (see Materials and Methods and Fig. 1). DCP, dicetyl phosphate; EYPL, egg-yolk phospholipids; AL, asolectin (soybean phospholipids).

Liposome composition (mol%)	Surface charge density, σ (1/1000 Å^2)			
	Calculated (1)	Measured		
		C15U (2) (direct)	TNS (3) (direct)	TNS (4) (CTAB titration)
PC	0	0 (2)	0±0.03	
PC+5% DCP	−0.72	−0.6	−0.40	−0.39
PC+10% DCP	−1.47	−1.15	−0.79	−0.74
PC+5% DCP+5% CTAB	0		−0.06	
EYPL		−0.27	−0.16	−0.14
EYPL/AL = 1:1		−1.7	−1.05	
AL		−2.5	−1.7	

TABLE V

SURFACE POTENTIAL (mV) OF LIPOSOMES AS DETERMINED BY DIRECT AND INDIRECT MEASUREMENTS

The potentials derived from K_m measurements are based on the data of Tables I–IV and on further experiments. For the calculation of surface potentials from their influence on the transport affinity of ATP towards the right-side-out-oriented carrier, K_m^{ATP} (R), a value of 7 μM was used for neutral membranes. The corresponding value for inside-out-oriented carrier molecules, K_m^{ATP} (I), was 15 μM. EYPL, egg-yolk phospholipids; DCP, dicetyl phosphate; CAT, carboxyatractylate.

Liposome composition (mol%)	Basis for potential determination	Na^+ concentration (mM)					
		7	10	20	40	60	75
EYPL+6% DCP	calculated	−65	−58	−46	−33	−28	−25
	C15U		−49	−45	−36	−30	
	TNS	−49	−42	−31	−22	−19	−17
	K_m^{ATP} (I)	−32	−29	−27	−22	−16	
	K_m^{ATP} (R)	−14	−10	−9	−6	−5	
	K_m^{CAT}	−13	−11	−8			−5
EYPL+10% DCP	calculated	−86	−77	−61	−47	−40	−36
	C15U	−78	−69	−60	−51	−45	−37
	TNS	−68	−60	−44	−34	−29	−26
	K_m^{ATP} (I)			−32		−21	
	K_m^{ATP} (R)	−18	−15	−12		−6	
	K_m^{CAT}	−18	−12	−10			−6

measured surface potentials:

$$\psi_0 \text{ (calculated)} \simeq \psi_0 \text{ (C15U)} > \psi_0 \text{ (TNS)} > \psi_0 \left(K_m^{ATP} \text{ (I)}\right)$$

$$\gg \psi_0 \left(K_m^{ATP} \text{ (R)}\right) \simeq \psi_0 \left(K_d^{CAT}\right)$$

where CAT is carboxyatractylate. Evidently, the actual surface potential at the carrier-binding site for inhibitor and transport ligands is not the same as the potential at the membrane surface and – what is even more surprising – it makes a considerable difference whether the cytosolic binding site or the matrix binding site of the carrier is investigated.

The difference between the calculated value of the surface potential and the potentials measured by fluorescent dyes is relatively small and may be explained on methodological grounds (see Discussion). The influence of negative surface charges on the apparent K_m of the transport by right-side-out-oriented carrier protein (K_m^{ATP} (R)) leads to a significant underestimation of the true surface potential. An equivalent deviation from the potential measured by TNS or C15U is seen when the surface potential is calculated on the basis of the apparent dissociation constant of CAT-binding to the carrier protein (K_d^{CAT}). In contrast to these observations, much higher values are obtained when the surface potential is derived from its influence on the inside-out-oriented carrier protein (K_m^{ATP} (I)). These values are comparable to surface potentials as determined by the fluorescent dyes.

(5) Correlation of the influence of surface potential with the structure of the carrier protein

When searching for possible explanations for these discrepancies in the measurement of surface potential, one has to take into account that in the reconstituted system – in contrast to the situation in mitochondria – the surface charges of the phospholipid membrane cannot be screened by surrounding proteins in the direct neighborhood of the adenine nucleotide carrier. This possibility is ruled out because of the very low amount of carrier proteins in the liposomal membrane; therefore, the simple view of the carrier protein symmetrically embedded in the membrane (Fig. 4A) does not apply in the reconstituted system. Two alternative explanations remain, which are illustrated in Fig. 4. Either the negative potential at the cytosolic side is specifically compensated by positive charges at the protein surface in the micro-environment of the active site, which is exposed to the cytosol (Fig. 4C) or the binding area for nucleotides and inhibitors at the cytosolic carrier site protrudes very far from the plane of the membrane (Fig. 4B).

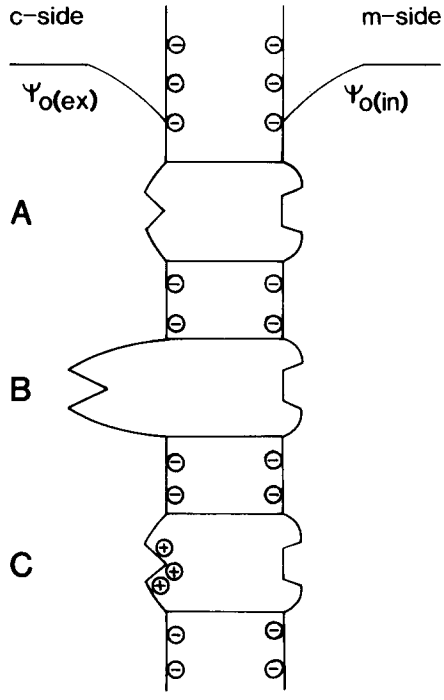

Fig. 4. Correlation of carrier structure with effect of surface potential. The figure illustrates the influence of external ($\psi_{o(ex)}$) and internal ($\psi_{o(in)}$) surface potentials on the binding sites of the reconstituted ADP/ATP carrier at the external and internal side of the membrane (c-side and m-side). For interpretation see text.

In the reconstituted system, it is possible to decide between these alternatives. If a completely neutral or a charge-compensated membrane is used for reconstitution, the presence of positive charges at the active site, influencing the binding step of nucleotides, should be clearly detectable on the basis of electrostatic interaction between negatively charged substrate and positively charged binding site. In this case, in contrast to the results of Tables I and II, an increase of ionic strength in the surrounding phase should result in an increase in the apparent transport affinity K_m. Thereby the

TABLE VI

TRANSPORT AFFINITY K_m (μM) OF ATP EXCHANGE IN NEUTRAL LIPOSOMES

The transport affinity for ATP was determined in the presence of different NaCl concentrations. The surface charge density was calculated on the basis of surface potential measured by salt titration of TNS fluorescence. EYPL, egg-yolk phospholipid.

Liposome composition (mol%)	Surface charge (1/1000 Å^2)	Na^+ concentration (mM)			
		3	10	30	60
EYPL + 1.5% CTAB	0 ± 0.02	8	10	10	12
PC/PE/chol = 7:2:1	−0.03	16	12	14	17

local surface potential near the active site would be calibrated.

Two different types of electrically neutral liposome were used to decide between the alternatives mentioned above (Table VI). Neutral liposomes were obtained either using phospholipid mixutres of purified PC and PE, or by titration of slightly negatively charged membranes (egg-yolk phospholipids) with CTAB, monitored by fluorescent dyes. In reconstitution experiments with both liposomes, increasing ionic strength does not lead to a significant change in the transport affinity K_m, which would be expected for a high local concentration of positive surface charges at the active site. As discussed already in Table I, the small increase in the K_m upon higher ionic strength can be explained by competition effects of anions.

(6) Influence of positive surface charges

The reconstituted system offers an additional possibility for testing the results and interpretations obtained so far. Incorporation of the cationic lipid $CTAB^+$ into the membrane results in a net positive surface charge of the liposomes and should consequently increase the apparent affinity for nucleotides due to electrostatic attraction of negatively charged substrates.

In the following experiments, two possible effects of incorporated CTAB have to be considered, namely firstly the influence of its positive charge and secondly the inhibitory effect due to its detergent nature. When investigating nucleotide transport by the right-side-out-oriented carrier (R), both influences can in fact be observed. The apparent transport affinity is slightly increased by CTAB (Table VIIA), whereas the exchange velocity becomes increasingly inhibited (Table VIIB). Although positive surface charges obviously modulate the transport affinity at the cytosolic surface of the carrier, again the effect is very small as compared to the calculated and measured positive surface potential. The same was found for negative charges (Table V).

The situation turns out to be completely different when the nucleotide carrier is oriented in the opposite direction. Even very low amounts of added CTAB severely inhibit the adenine nucleotide exchange in this direction (Table VIIB), leading to a very strong depression of the exchange velocity. However, it remained to be elucidated whether the transport by the carrier protein with reversed polarity is inhibited by the positive surface charges or by the mere presence of CTAB molecules. This question was solved in a set of experiments applying different modes of introducing charges into the liposomal membrane.

It has been demonstrated in the Materials and Methods section and in Fig. 3 that negative and/or positive charges can be introduced at the outer surface of the lipid bilayer by in situ titration of the liposomes with positively charged CTAB and negatively charged tetraphenylborate, respectively. As shown in Table VIII, the inhibition by CTAB of nucleotide transport in inside-out-oriented carrier proteins is completely reversed when the membrane surface is titrated back with tetraphenylborate, leading again to a net negative surface charge. This obviously holds true whether tetraphenylborate is titrated to membranes with 2% or 4% added CTAB. Furthermore, when tetraphenylborate is titrated to slightly negatively charged egg-yolk lipids, a stimulation by the increased

TABLE VII

TRANSPORT AFFINITY K_m AND MAXIMUM EXCHANGE RATE V_{max} OF THE RECONSTITUTED ADP/ATP CARRIER IN POSITIVELY CHARGED LIPOSOMES

The liposomes consisted of egg-yolk phospholipids with increasing amounts of CTAB, which was added prior to the vesicle preparation. The different Na^+ concentrations were adjusted by Na_2SO_4 and NaCl. (A) Transport affinities for ATP-exchange were measured for the right-side-out-oriented carrier. (B) Maximum exchange rates were measured for right-side-out- (R) and inside-out- (I) oriented carrier. The exchange rates are given in percent of the transport velocity catalyzed by the right-side-out-oriented translocator without addition of CTAB. Thereby, the possibility of variation due to varying reconstitution efficiency is eliminated [2]. The maximum velocity (= 100%) corresponds to 1.5–3.0 $mmol \cdot g^{-1} \cdot min^{-1}$ in the different experiments.

(A) $[Na^+]$ (mM)	CTAB added (mol%)	K_m (μM)					
		0	1	2	3	6	9
4		11				3	
10		9	7	5	6	6	
25		9				7	6
65		13				9	7
100		16	12	8	8	9	9

(B) $[Na^+]$ (mM)	Carrier orientation	CTAB added (mol%)	V_{max} (%)				
			0	1	2	3	6
16	R		100	79	72	55	37
	I		36	9	7	5	
100	R		100	87	86	67	63
	I		19	7	4	4	

TABLE VIII

KINETIC PARAMETERS OF THE RECONSTITUTED ADP/ATP EXCHANGE IN LIPOSOMES WITH DIFFERENT SURFACE CHARGES

The liposomes consisted of egg-yolk phospholipids/cholesterol = 85 : 15 (mol%). CTAB and sodium tetraphenylborate (TPB) were added to the liposomes after reconstitution prior to the measurement of the exchange. In experiments with addition of both CTAB and tetraphenylborate, the two substances were titrated to the liposomes in the two possible sequences with virtually the same results. Surface potential was measured by TNS fluorescence in the presence of 20 mM Na^+. The positive potentials could be measured by titration with tetraphenylborate (see Materials and Methods). The maximum exchange rate is normalized to the activity in untreated liposomes (= 100%, see explanation in Table VII). Both V_{max} and K_m were determined with right-side-out- (R) and inside-out- (I) oriented carrier proteins.

Addition of (mol%)		Surface potential (mV)	V_{max} (%)		K_m (μM)	
CTAB	TPB		R	I	R	I
–	–	−10	100	20	9	26
–	2	−22	121	41	10	125
–	4	−32	120	50	14	440
2	–	−3	98	7	9	20
4	–	+6	92	8	7	12
2	2	−16		22		55
2	4	−21	110	27	12	140
4	2	−6		11		42

negative charge is observed. In addition to this influence on the transport velocity, a strong modulation of the transport affinity of inside-out-oriented carrier proteins can be detected (Table VIII) which is caused by the increase of negative surface charges. This is in good agreement with the results shown in Table I.

Discussion

Various aspects of interaction between charged lipids and the adenine nucleotide carrier were investigated in the reconstituted system. It may be either a direct interaction between certain phospholipid molecules and the carrier protein, or it may also be an indirect interaction mediated by the surface potential which originates from the charged lipids. The present study focuses mainly on the latter possibility.

The surface potential was measured directly with the fluorescent dyes TNS and C15U. Although the lipophilic pH indicator C15U gives quite accurate values for the surface potential (Tables IV and V) due to its position near the potential-generating plane of the membrane [34], its application is limited by the high pH values which are required for pK titration. The fluorescent dye TNS leads to an underestimation of the true surface potential; however, this drawback can be largely eliminated by titration of surface charges with lipids or lipophilic ions carrying opposite charges (Fig. 1 and Table IV). Because of its simple application, predominantly TNS was used to monitor the surface potential of liposomes in the reconstitution experiments reported here.

The calculation of surface potentials on the basis of their influence on binding parameters and kinetic constants of the adenine nucleotide carrier, as described in the present paper, may be considered as a very indirect procedure. However, it has turned out to be a reliable and a well-reproducible method to elucidate the interaction between charged lipids and the reconstituted carrier protein. It should be mentioned here that the same indirect method has also been used successfully to determine the dissociation constants of adenine nucleotides for divalent cations [3].

Although the different methods for measuring surface potential have, in fact, led to different results, a definite sequence in the measured apparent potentials was obtained in each case, independent of the type of liposome used for reconstitution (see Results, section 4). The most interesting feature of this sequence is the distinct behavior of the external and internal ligand binding sites of the carrier protein.

The binding affinity for carboxyatractylate and the transport affinity for ATP and ADP at the cytosolic side of the carrier are only weakly influenced by negative surface charges (Tables I and V). This may be explained either by a considerable distance between the nucleotide binding site and the potential-generating plane of the membrane or, alternatively, by the possible presence of a certain number of positive charges at the binding site of the carrier – an explanation which would be all the more obvious when considering the observed participation of positive charges in binding and transport processes [39,40]. However, these positive charges cannot be the reason for the lack of response to negative surface potentials, as has been demonstrated here on the basis of two experimental results: (a) salt titration of ATP binding affinity in neutral or charge-compensated phospholipid membranes does not reveal any influence of positive charges at the active site (Table VI); and (b) positive membrane surface charges, too, which should not be screened by cationic groups at the active site, have a very weak effect on the nucleotide binding (Table VIIA). Assuming that the lack of response to the true surface potential is due to the distance between binding site and membrane surface, one can calculate from the data of Table V that this distance must be larger than 25 Å. As discussed previously (Tables I and VI), the competitive effects of anions on the apparent K_m at high ionic strength must be taken into consideration when interpreting the data of Table V.

Independent of the accuracy of this determination, there is another fact in Table V that should be emphasized. The surface potential modulates the binding of carboxyatractylate and of adenine nucleotides at the external site of the carrier to the same extent. This suggests a spatial equivalence of nucleotide and inhibitor binding sites at the cytosolic side of the ADP/ATP translocator.

In contrast to these results, the transport affinity of ATP for the internal binding site is strongly

influenced by both negative (Table I) and positive (Table VII) surface charges. The steep increase in the apparent transport affinity in negatively charged membranes leads to high values for the corresponding surface potentials (Table V). Although considerably lower than the values calculated from the phospholipid composition, they are nearly as high as the potential measured directly with TNS. Compared to the situation at the cytosolic site of the carrier, this would mean that the internal nucleotide binding site (matrix side) is in close proximity to the potential-generating plane of the membrane.

In order to analyze these interactions between charged phospholipids and the carrier protein more extensively, the effect of the added lipid molecule itself and that of the charge had to be separated (Table VII). There is yet another problem to be considered. When testing the exchange velocity in liposomes with different lipid compositions, one has to take into account that during the complete reaction cycle of nucleotide transport both the 'forward' (transport to the inside) and the 'backward' (transport to the outside) half-reactions may be influenced, even though the internal space always disposes of saturating concentrations of nucleotides and other ions. These difficulties were overcome by the experiments shown in Table VIII. Here, negative and positive charges were added to intact liposomes in situ from the external phase. Therefore they create a surface potential only at the exterior half of the phospholipid bilayer. In this way, the influence of surface charges on one type of nucleotide binding site can be investigated separately, without a concomitant effect on the other site.

When assuming a considerable distance between binding site and plane of the membrane, the influence of both negative and positive charges on substrate and inhibitor binding to the external site in principle reflects classical effects of surface potential. Apart from this modulation of the substrate affinity the exchange velocity is only slightly affected by negative and/or positive surface charges (Table VIII). Quite a different situation is revealed when the inside-out-oriented carrier protein is concerned. Indeed, at the internal site, the transport affinity is strongly influenced by negative surface charges, in accordance with a small distance between binding site and membrane plane. But in this case not only modulation of the transport affinity is observed, but also a definite stimulation of the exchange velocity be negative charges and a severe inhibition by positive charges. These effects are not based on interaction of the translocator with the lipid molecule itself, since both stimulation and inhibition are reversible by back-titration (Table VIII). It has to be pointed out once more here that the results of Table VIII cannot be compared directly with those of Tables I–VII, since addition of charged lipids before (Tables I–VII) or after (Table VIII) preparation of liposomes leads to formation of quite different phospholipid vesicles.

Thus, besides the difference in influence of surface potential on ligand binding to the two alternative carrier sites, an additional side-specific effect on the transport velocity is observed. From the results of Table VIII, it can be concluded that the carrier protein is inhibited by positive and stimulated by negative surface charges, present at that side of the membrane where the internal binding site is located. On the other hand, the cytosolic site does not seem to be strongly influenced by negative or positive charges. Whether these effects are due to direct lipid-protein interaction, which may be different for the two sides of the membrane, or whether they are caused by influence of the surface potential on the corresponding binding site, is being investigated at the moment. These questions are of special interest in view of the well-established dependence of the reconstituted ADP/ATP transport on negatively charged phospholipids and/or PE [4,5,16,17].

The influence of surface potential on the catalytic function of carriers can of course also be observed in several mitochondrial anion transport processes in general [14] and relating to the ADP/ATP carrier in particular [15]. The exact correlation of these results in the reconstituted system with the situation in mitochondria, in mitoplasts and also in inverted sonic particles, however, is beyond the scope of this paper. This will be discussed in detail in a further publication.

Acknowledgements

I appreciate the continuous interest and support of Professor Dr. M. Klingenberg. I wish to thank

Gabriele Kürzinger and Styliani Tsompanidou for their expert assistance in the experiments. I am also indebted to Professor Dr. P. Fromherz for providing the dye C15U and for giving me helpful instructions. This work was supported by a grant from the Deutsche Forschungsgemeinschaft.

References

1 Krämer, R. and Klingenberg, M. (1980) Biochemistry 19, 556–560
2 Krämer, R. and Klingenberg, M. (1982) Biochemistry 21, 1082–1089
3 Krämer, R. (1980) Biochim. Biophys. Acta 592, 615–620
4 Krämer, R. and Klingenberg, M. (1977) FEBS Lett. 82, 363–367
5 Krämer, R. and Klingenberg, M. (1980) FEBS Lett. 119, 257–260
6 Klingenberg, M., Wulf, R., Heldt, H.W. and Pfaff, E. (1969) in Mitochondria: Structure and Function (Ernster, L. and Drahota, Z., eds.), Vol. 17, pp. 59–77, Academic Press, New York
7 Klingenberg, M. and Rottenberg, H. (1977) Eur. J. Biochem. 73, 125–130
8 LaNoue, K., Mizzani, S.M. and Klingenberg, M. (1978) J. Biol. Chem. 253, 191–198
9 Goldstein, L. (1972) Biochemistry 11, 4072–4084
10 Møller, I.M., Schwitzguebel, J.-P. and Palmer, J.M. (1982) Eur. J. Biochem. 123, 81–88
11 Wojtczak, L. and Nalecz, M.J. (1979) Eur. J. Biochem. 94, 99–107
12 Roomans, G.M. and Borst-Pauwels, G.W.F.H. (1979) Biochem. J. 178, 521–527
13 Roomans, G.M., Theuvenet, A.P.R., Van den Berg, T.P.R. and Borst-Pauwels, G.W.F.H. (1979) Biochim. Biophys. Acta 551, 187–196
14 Meisner, H. (1971) Biochemistry 10, 3485–3491
15 Meisner, H., Palmieri, F. and Quagliariello, E. (1972) Biochemistry 11, 949–955
16 Brandolin, G., Doussiere, I., Gulik, A., Gulik-Krzywicki, T., Lauquin, G.J.M. and Vignais, P.V. (1980) Biochim. Biophys. Acta 592, 592–614
17 Shertzer, H.G. and Racker, E. (1976) J. Biol. Chem. 251, 2446–2452
18 Fry, M. and Green, D.E. (1980) Biochem. Biophys. Res. Commun. 93, 1238–1246
19 Kadenbach, B., Mende, P., Kolbe, H.V.J., Stipani, I. and Palmieri, F. (1982) FEBS Lett. 139, 109–112
20 Op den Kamp, J.A.F. (1979) Annu. Rev. Biochem. 48, 47–72
21 Klingenberg, M. (1971) in Energy Transduction in Respiration and Photosynthesis (Quagliariello, E., Papa, S. and Rossi, C.S., eds.) pp. 23–34, Adriatica Editrice, Bari
22 Capaldi, R.A. (1982) Biochim. Biophys. Acta 694, 291–306
23 Krämer, R. and Klingenberg, M. (1979) Biochemistry 18, 4209–4215
24 Babel, W. (1975) Diploma Thesis, Ludwig-Maximilians-Universität München
25 Helenius, A. and Simons, K. (1972) J. Biol. Chem. 247, 3656–3661
26 Chen, D.S., Jr., Toribara, T.Y. and Warner, H. (1956) Anal. Chem. 28, 1756
27 Wells, M.A. and Hanahan, D.J. (1969) Methods Enzymol. 14, 178–184
28 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5488
29 Krämer, R. (1982) Biochim. Biophys. Acta 693, 296–304
30 Kasahara, M. and Hinkle, P.C. (1977) J. Biol. Chem. 252, 7384–7390
31 Eisenberg, M., Gresalfi, T., Riccio, T. and McLaughlin, S. (1979) Biochemistry 18, 5213–5223
32 Fromherz, P. and Masters, B. (1974) Biochim. Biophys. Acta 356, 270–275
33 Fernández, M.S. (1981) Biochim. Biophys. Acta 646, 23–26
34 Haase, A. (1980) Thesis, Justus-Liebig-Universität, Gießen
35 McLaughlin, S. (1977) in Current Topics in Membranes and Transport (Bronner, F. and Kleinzeller, H., eds.), Vol. 9, pp. 71–144, Academic Press, New York
36 Klingenberg, M. (1976) in The Enzymes of Biological Membranes (Martonosi, A.N., ed.), Vol. 3, pp. 383–438, Plenum Press, New York
37 Berden, J.A., Barker, R.W. and Radda, G. (1975) Biochim. Biophys. Acta 375, 186–208
38 Litman, B.J. (1975) Biochim. Biophys. Acta 413, 157–162
39 Klingenberg, M. and Appel, M. (1980) FEBS Lett. 119, 195–199
40 Block, M.R., Lauquin, G.I.M. and Vignais, P.V. (1981) Biochemistry 20, 2692–2699