Biochimica et Biophysica Acta 933 (1988) 200–211
Elsevier

BBA 42718

ATP hydrolysis induces variable porosity to mannitol in the mitochondrial inner membrane

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(Received 24 June 1987)
(Revised manuscript received 17 November 1987)

Key words: ATPase; ADP/ATP carrier; Osmometry; Proteoliposome; Mitochondrion; (Rat liver)

Osmotic titration of ATPase activity in rat liver mitochondria was consistent with enhanced porosity of the mitochondrial inner membrane to mannitol due to ATP hydrolysis even when endogenous respiration was inhibited by rotenone. The occluded ATPase activity, which exhibits osmotic activation with an optimum near isotonicity, depends both on the ATPase activity per se and on the activity of the ADP/ATP carrier. Purified ADP/ATP carrier incorporated into small, unilamellar liposomes was critically shown to exhibit dependence of its activity on the osmotic pressure differences across the membrane, with maximal activity corresponding to isotonicity, regardless of the actual internal tonicity.

Introduction

Recent studies from our laboratory, based on detailed measurements of various activities associated with mitochondria and oxidative phosphorylation as well as erythrocytes as a function of the osmolality of the external polyol media, led to certain unique observations: (i) the mitochondrial membrane exhibits variable porosity to external polyols as a function of respiration [1,2]; (ii) the activity of a variety of membrane proteins, including enzyme complexes, transporters and even channels varies with the external osmotic pressure, or the volume of the particle [1–8]; (iii) the coupled processes exhibit continuous variation in the activities, degree of coupling (respiratory control ratio) and even the stoichiometry (ADP/O ratio), depending on the osmotic stretch of the membrane, though the inner membrane is physically intact (i.e., in the non-lytic domain) [1–7]. Osmotic modulation of the activity of membrane-bound enzyme systems was considered forbidden on a priori thermodynamic grounds that pressure compression of chemical reactions would not be possible by osmotic means in biological systems [9]. Nor was it possible to assign any specific class of proteins the property of osmotic modulation, since the activities of a variety of hydrophobic proteins were shown to exhibit osmotic modulation (cf. also Refs. 10–13). The possibility of one activity (e.g., respiration and phosphate transport) conferring osmotic modulation on another, otherwise inert, activity (e.g., the dicarboxylate transporter activity) was also encountered in studies on mitochondrial transport (Rao, N.M. and Sitaramam, V., unpublished observations).

Two major lines of evidence were yet unavailable for a comprehensive description of reciprocal

Abbreviation: DNP, 2,4-dinitrophenol.

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modulation of enzymatic activities associated with the mitochondria on one hand and osmotic properties of mitochondria on the other. Firstly, if biological membranes, on energization, exhibit variable porosity to polyols, would ATP hydrolysis also induce variable porosity in the mitochondrial inner membrane? Secondly, the assumption that the osmotic modulation of the activity resides in the protein itself, manifest when embedded into its lipid milieu, remains to be proven in a system in which a purified protein is incorporated into liposomes. We report here direct experimental evidence that ATP hydrolysis induces variable porosity in the mitochondrial inner membrane to mannitol. We also report the first ever demonstration in membrane biology that a purified transporter, the ADP/ATP carrier, incorporated into liposomes, exhibits osmotic modulation of its activity, which accounts, in part, for the osmotic modulation of the ATPase activity.

Materials and Methods

Materials

Sucrose, mannitol, ATP, rotenone, 2,4-dinitrophenol, atractyloside, carboxyatractyloside and 6-carboxyfluorescein were obtained from Sigma, U.S.A. All other reagents were of analytical grade.

Methods

Enzyme osmometry. The theory, methodology, precautions and reproducibility of the technique of enzyme osmometry have been discussed earlier [1–3,5,7,14]. Briefly, membraneous vesicles do not expand or contract indefinitely in the face of varied external osmolality. The linear limits, manifest in both the hypo- and hypertonic domains, can be evaluated as points of intersection of independent regression lines (i.e., breakpoints), defining the true osmotic response. The advantages are: (i) the breakpoint, expressed as a critical external solute concentration/osmotic pressure, permits comparison of different activities/phenomena as well as different osmolytes on a common osmotic pressure scale; (ii) these measurements obviate the need to assess the volume of the particles, a notoriously error-prone measurement [15]; (iii) several membrane-bound enzyme systems, transporters and even ion channels were shown to exhibit hypoosmotic activation [1–13]. One can, therefore, utilize such activities to assess breakpoints; and (iv) it was repeatedly confirmed that a change in a breakpoint (i.e., the corresponding constant critical volume, V_c), in time, t, relates to fluxes (J) such that,

$$\Delta \text{ breakpoint} = \frac{RT}{V_c}\left\{\int_0^t J_p - \int_0^t J_q\right\}_{1\to 2 \quad 2\to 1} \tag{1}$$

The break-points increase or decrease on the osmolality scale, depending on the net influx ($1 \rightarrow 2$) or efflux ($2 \rightarrow 1$) of the external (p) or internal (q) osmolytes. Thus, one can measure reflection coefficients to osmolytes even in dynamic functional states (cf. Ref. 1).

Enzyme osmometry of respiration and ATPase activity. ATPase activity in rat liver mitochondria was measured as described earlier [7]. Respiration was measured polarigraphically with a Clark oxygen electrode [1,7].

Studies on ADP/ATP carrier. The well-characterized bovine heart ADP/ATP carrier was purified to homogeneity and incorporated into liposomes (with egg yolk phospholipids) of defined internal osmolality, as described earlier [16,17]. The small unilamellar vesicles were prepared by sonication, the preferred method in view of the marked lability of the activity of the carrier to detergents such as cholate. Integrity of liposomes was assessed by measuring the loss of 6-carboxyfluorescein incorporated into the liposomes at various osmolalities (cf. Ref. 18). These liposomal preparations were not leaky in an osmotic gradient range ($0.5 \leqslant \pi_{\text{external}}/\pi_{\text{internal}} \leqslant 4$) as measured by leakage of 6-carboxyfluorescein. The fluorescence assays could detect the leakage of the fluorescent marker for at least 1%.

Osmometric studies on ADP/ATP carrier were carried out in different preparations of the carrier reconstituted into liposomes with variable internal tonicity, which was adjusted primarily by varying sucrose, at nearly constant (approx. 0.13 M) NaCl. Initial velocities of the external, labelled nucleotide uptake were measured at constant internal nucleotide, by subtraction of control values obtained by prior addition of the specific inhibitor, carboxyatractyloside. Since not all liposomes may contain the carrier, the functional population was

monitored by another control: the end point value (after 30 min) of the exchange reaction in the reconstituted system (which resembles the isotopic equilibrium between the external and the internal substrate, i.e., in active proteoliposomes) was directly dependent on the amount of internal substrate. This value was found to be nearly constant over the entire range of osmotic gradients employed, suggesting that functionally active liposomes must have remained intact with regard to the topography of the localization of nucleotides. Osmometric studies were carried out primarily by varying external sucrose. Osmolality of media was routinely monitored by a Wescor vapour pressure osmometer 5100C. Osmometric analyses of turbidity, ATPase (latent and uncoupler (2,4-dinitrophenol)-stimulated) and related methodology in rat liver mitochondria were carried out as described earlier [1,7]. Isolated mitochondria were routinely evaluated for their functional integrity by assessing ADP/O ratio and respiratory control and were comparable to the results published earlier from our laboratory [1,7]. *

Results and Discussion

Respiration as well as ATP hydrolysis increases the free energy available to the system. The term, energization of the membrane, has been used interchangeably in the past to account for a myriad of energy-dependent activities or energy-associated changes in the mitochondrial inner membrane. These range from reversal of electron flow [19] to the binding of the ANS [20]. The term, energization, should not be used within the framework of the chemiosmotic hypothesis .(since the definition of a chemical potential of protons requires that for changes in internal energy, $dU = 0$) [21,22]. We use the term 'energization' of the membrane to depict any event that implies both the presence of a free energy generator activity and the evidence for the work done, be it permeation of thermodynamically forbidden polyols, ATP synthesis, transport or even an instability such as lysis. An important distinction should be among the various free-energy generators. Since respiration itself induces variable porosity to polyols, the effects of ATP hydrolysis needed to be carefully distinguished from the effects of respiration by specific inhibitors such as rotenone.

Effect of rotenone on the osmometric behaviour of turbidimetric profiles in sucrose and mannitol media

Fig. 1 illustrates the osmometry of turbidimetry in rotenone-treated mitochondria from rat liver. The breakpoints in hypo- and hypertonic domains (as expected of the linear limits of volume changes as a function of osmolality, bounded by lysis in the hypotonic domain and the linear limit of contraction in the hypertonic domain) were similar in sucrose and mannitol media. It may be recalled that the onset of lysis of mitochondria also yielded identical breakpoints in sucrose and mannitol media, corresponding to approx. 0.1 M solutions of either polyol [1]. This was in contrast with the turbidimetric profiles of respiring mitochondria reported earlier, wherein sucrose media yielded a similar breakpoint (0.31 M or 0.36 os/kg), indicating a lowered reflection coefficient to mannitol induced by respiration [1]. Thus, rotenone-treated mitochondria, in which endogenous respiration was inhibited for at least 95%, behaved similar to non-respiring mitochondria with reflection coefficients to sucrose and mannitol indistinguishably small (i.e., approx. 1.0, or nearly impermeable).

Osmometry of ATPase activity in rotenone-treated mitochondria

Fig. 2 illustrates osmometric profiles of ATPase activity in mitochondria pretreated with rotenone to inhibit the influence of endogenous respiration: (i) the ATPase activity exhibited biphasic profiles in sucrose media, and was osmotically compressible in the non-lytic domain; (ii) the breakpoints shifted to the left on addition of the uncoupler, 2,4-dinitrophenol; (iii) the osmotic profiles of activity in mannitol media exhibited only a monotonic profile of inhibition, indicating that the break-point for the inhibition of ATPase activity

* It should be noted that the measurement of initial velocities at all osmolalities is of critical importance in such osmometric studies, be it transport or catalysis (cf. Ref. 5 for a more comprehensive discussion of the kinetic basis of enzyme osmometry). In all experiments on transport as well as enzyme catalysis reported in this paper, initial velocities were measured without exception.

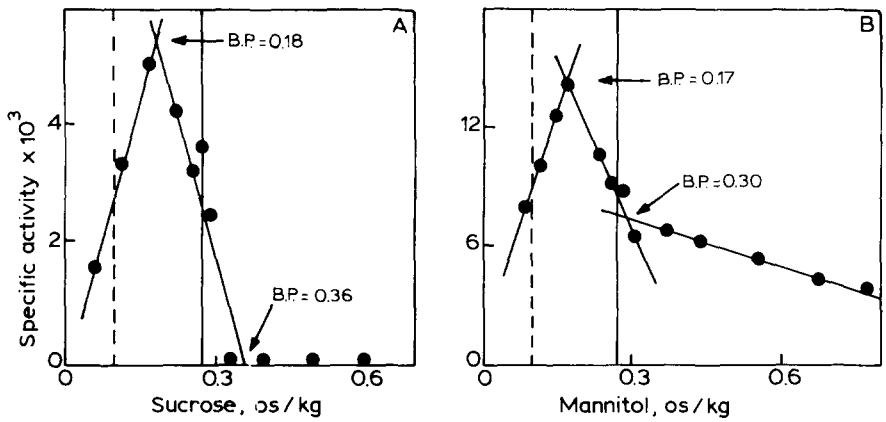

Fig. 1. Osmometry of turbidimetry in rotenone (1.0 μg/mg protein)-treated rat liver mitochondria. Specific activity, i.e., maximal rate of change in absorbance (at 520 nm) per min per mg protein plotted as a function of osmolality. (A) Sucrose media. (B) Mannitol media. The breakpoint (B.P.) (os/kg) is indicated by arrows, the onset of lysis (release of matrix enzymes) by a dashed line, apparent isotonicity (0.27 os/kg) by a continuous line.

shifted significantly to the right, consistent with enhanced permeability to mannitol; (iv) peak activity did not correspond to hypotonicity in sucrose media, but was actually to the right of apparent isotonicity (= 0.25 M sucrose or 0.27 os/kg) in latent ATPase and to the left in DNP-stimulated ATP activities; (v) similar profiles were observed even in control mitochondria which were not treated with rotenone (data not given); (vi) the activity profiles in sucrose media appeared to be

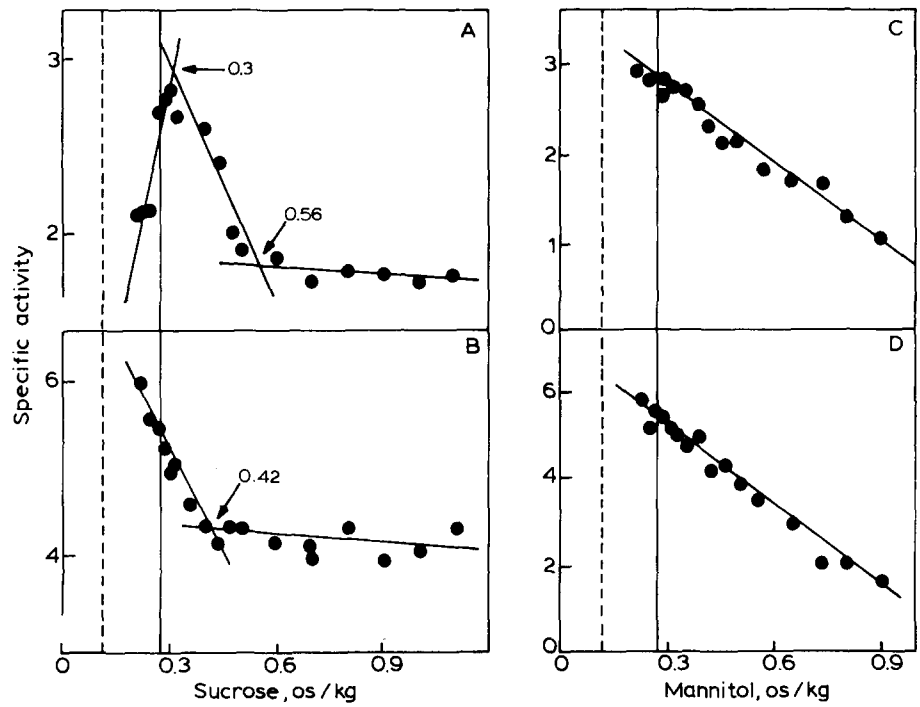

Fig. 2. Osmometry of latent (A, C) and DNP-stimulated ATPase (B, D) activities (μmol P_i/h per mg of rat liver mitochondrial protein) in sucrose (A, B) and mannitol (C, D) media. Osmometric profiles plotted as in Fig. 1.

parabolically related to the external osmolality with peak activity near isotonicity, since the phenomena were observed in an apparently non-lytic domain (more than 0.1 os/kg) of external osmolality (cf. Ref. 1).

The shift to right observed in mannitol media could not be ascertained with DNP-treated mitochondria due to the limited solubility of the polyol. The osmometric data clearly showed that ATP hydrolysis induced enhanced permeability of the mitochondrial inner membrane to mannitol. Several observations remained puzzling, e.g., the leftward shift with DNP, peak activity corresponding to near isotonicity rather than to the onset of lysis (independently determined to be at approx. 0.1 M sucrose; cf. Ref. 1). This could mean that either the onset of lysis shifted to right under these conditions or that a rate-limiting step in ATPase activity (particularly, in the case of the latent ATPase activity) exhibited a parabolic profile with maximal activity at isotonicity. It was critical to make a distinction between these possibilities, since the physical basis of modulation of the ATPase activity would not be known unless the relevant physical parameter responsible for the modulation of the activity is identified.

Influence of KCl in the assay medium

We showed earlier that the conventional assay media for ATPase activity with 75 mM KCl, without external sucrose, were markedly hypotonic resulting in 5–7-fold activation of latent ATPase with uncouplers [1]. This was reduced to merely 2-fold in the presence of 0.25 M sucrose. We investigated the effect of the presence of 75 mM KCl on osmometry of ATPase activity in sucrose and mannitol media, since it could influence the ionic strength of the polyol medium. Data in Fig. 3 show that: (i) absence of KCl induced only a monophasic activation of ATPase in both sucrose and mannitol media wherein the peak at isotonicity disappeared; (ii) presence of KCl led to peak activity of ATPase near isotonicity in sucrose media and at nearly twice the isotonicity in mannitol media; (iii) the activity levels as well as the greater permeability to mannitol were clearly seen regardless of whether KCl was present or not.

ATPase activity in the presence and absence of KCl in the assay media offered intriguing possibilities in the interpretation of the contribution of KCl to either the activity per se or the osmometric profiles. The breakpoints were identical in the presence as well as in the absence of KCl (0.51 and 0.48 os/kg, respectively, in sucrose media and approx. 0.9 os/kg in mannitol media) suggesting that enhanced permeation to KCl was not observed on ATP hydrolysis in the hypertonic domain. However, the activity profiles in the hypotonic domain were of different nature, the most glaring difference was the parabolic profile in the presence of KCl and linear inhibition in the absence of KCl. It is questioned whether this could be an ionic strength effect. However, even mannitol media led to linear inhibition, whereas nearly 50% activity was osmotically incompressible with or without 2,4-dinitrophenol and with or without KCl. It was clear that the coupling of activity to osmotic pressure would be very complex in the case of ATPase activity. The osmotic activation/inhibition could well be due to component activities of ADP/ATP carrier, phosphate carrier, etc. The attendant proton fluxes may not be important, since the osmotic compressibility was also seen in uncoupler-treated mitochondria (Fig. 2).

A simpler question to seek an answer was whether the profiles represented the osmotic behaviour of mitochondria or any peculiar response of the complex ATPase activity per se. The presence of endogenous respiration was found to be without influence on osmometric profiles or activities in Figs. 2 and 3, since assays in the absence of rotenone yielded similar results (data not given). The osmometric profiles of endogenous respiration were directly assessed under the conditions of ATPase activity to compare the profiles of respiration with those of ATPase.

Osmometric profiles of endogenous respiration

Fig. 4A–C shows the effect of external osmolality on endogenous respiration under ATPase conditions (including 5 mM ATP and 75 mM KCl as in Fig. 2, but without rotenone). The profiles were similar regardless of the presence of DNP or atractyloside. The breakpoints were also comparable with those of ATPase in that respiration showed peak activity corresponding to isotonicity rather than lysis. DNP produced a shift to left

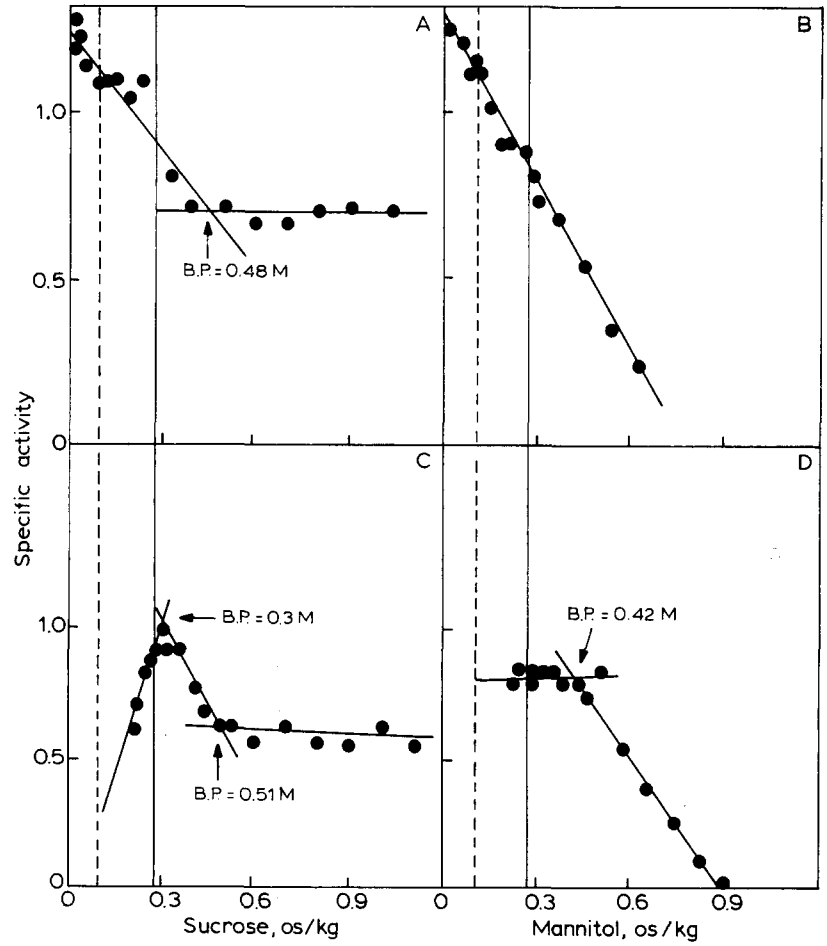

Fig. 3. Osmometric profiles of latent ATPase in the presence and absence of 75 mM KCl in sucrose and mannitol media of varying tonicity. Osmometric profiles plotted as in Figs. 1 and 2: A and B, without KCl, C and D, with 75 mM KCl. Specific activity, μmol P_i/h per mg of mitochondrial protein.

similar to osmometric profiles with ATPase. The presence of atractyloside (therefore, ADP/ATP carrier activity as well as ATPase activity) did not affect the osmometric profiles of respiration.

Osmometric profiles of respiration under conditions of oxidative phosphorylation

Osmometric profiles of respiration under ATPase conditions would be akin to State I respiration (i.e., due to endogenous substrates). Fig. 5 shows a comparison of State I respiration in sucrose and mannitol media. Clearly, mannitol was more permeable than sucrose even in State I respiration, with a clear rightward shift in the break-points. These profiles differed from those under ATPase conditions, as in Fig. 4. State I respiration showed marked daily variation in the level of activity as well as osmometric profiles (i.e., the breakpoints) except for two common observations: (i) State I respiration was inhibited by osmolality well beyond isotonicity (0.5–0.8 M) sucrose; (ii) DNP showed marked inhibition of State I respiration, whereas it stimulated State II respiration; and (iii) DNP also produced a marked leftward shift of State I respiration, as also KCl (75 mM) (data not given).

These studies showed that the osmotic profiles could be a complex interplay of ionic strength,

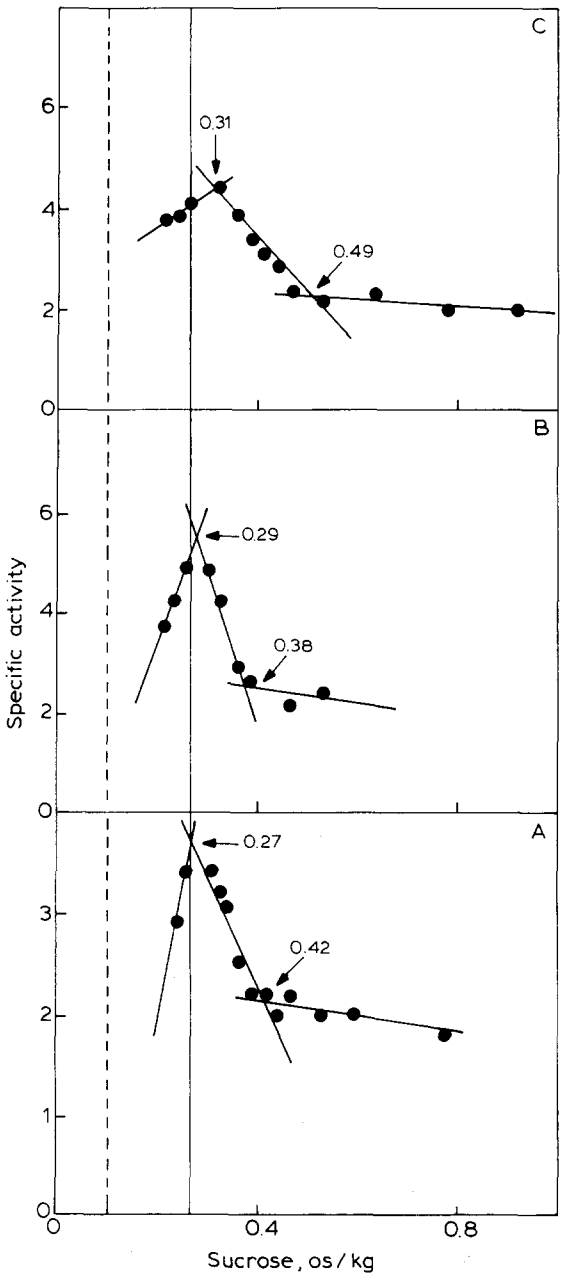

Fig. 4. Osmometry of endogenous respiration in rat liver mitochondria under conditions of ATPase activity (including 5 mM ATP) in control (A), DNP-treated (B) and atractyloside-treated (C) mitochondria. Rat liver mitochondria were pre-treated with 0.2 mM DNP or 0.25 μmol per mg protein of atractyloside prior to assay in the presence of these agents. Osmometric profiles plotted as in Figs. 1–3. Specific activity, expressed as natoms of oxygen per min per mg of protein, was measured polarigraphically.

rates of respiration, permeability to external polyols, etc. Thus, it would not be possible to evaluate the contribution of the component reactions by osmometric profiles alone. However, the osmometric profiles gave consistent evidence with regard to the permeability to external polyols regardless of the assay conditions, in a reliable manner. Our studies also showed that the phosphate transporter could be osmotically compressible, whereas dicarboxylate transporter activity per se may not be (unpublished results). It was necessary to examine the central assumption of osmotic compressibility of the activities of hydrophobic proteins with a purified transporter, which is otherwise well characterized. The ADP/ATP translocase of bovine heart mitochondria was chosen due to its ready availability, as an expedient solution to the problem of osmotic compressibility of the activities of hydrophobic proteins.

Studies on bovine heart ADP/ATP translocase

We considered the osmometric profile of ATPase in intact mitochondria as a composite profile, as in Eqn. 2. When the active site of the enzyme faces the interior of a vesicle, the activity, under initial velocity conditions, is influenced by the permeability to the external substrate such that the rate becomes

$$J_r = \frac{KA_0}{1 + K/P} \tag{2}$$

where P and K are the rate constants of permeability (either of diffusion or of the activity of a relevant transporter) and of the enzyme, respectively [7,14]. Under initial velocity conditions one may observe variations in the transporter activity, provided that the kinetic rate constant of the enzyme is not limiting. However, it was realized at the very outset that the ADP/ATP carrier need not be rate limiting due to its high turnover number in mitochondria [23]. Indeed, the mitochondrial ATPase activity would have so many component reactions and coupled processes [24] that an extrapolation to the carrier activity must be considered very carefully. For this reason, one must also be wary of studies on exchange activity of the carrier using intact mitochondria due to the de-

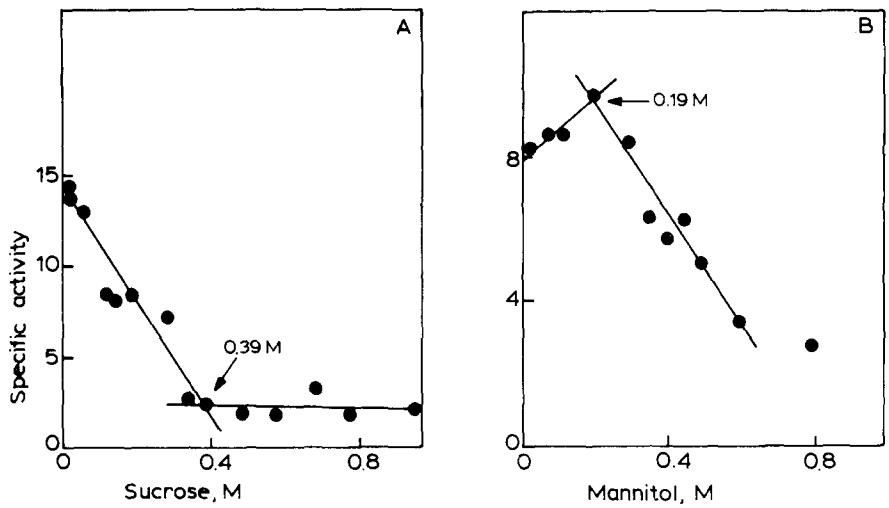

Fig. 5. Osmometry of respiration under conditions of oxidative phosphorylation, i.e., in the presence of 10 mM sodium phosphate buffer (pH 7.4), 0.2 mM EDTA, 3.3 mM $MgCl_2$, 10 mM Tris-HCl (pH 7.4) and varying concentrations of the polyol as specified. Substrates of oxidative phosphorylation were omitted, such that the assay corresponded to State I respiration. Breakpoints indicated by an arrow were obtained as in Fig. 1. The breakpoint for mannitol was in excess of 0.6 M.

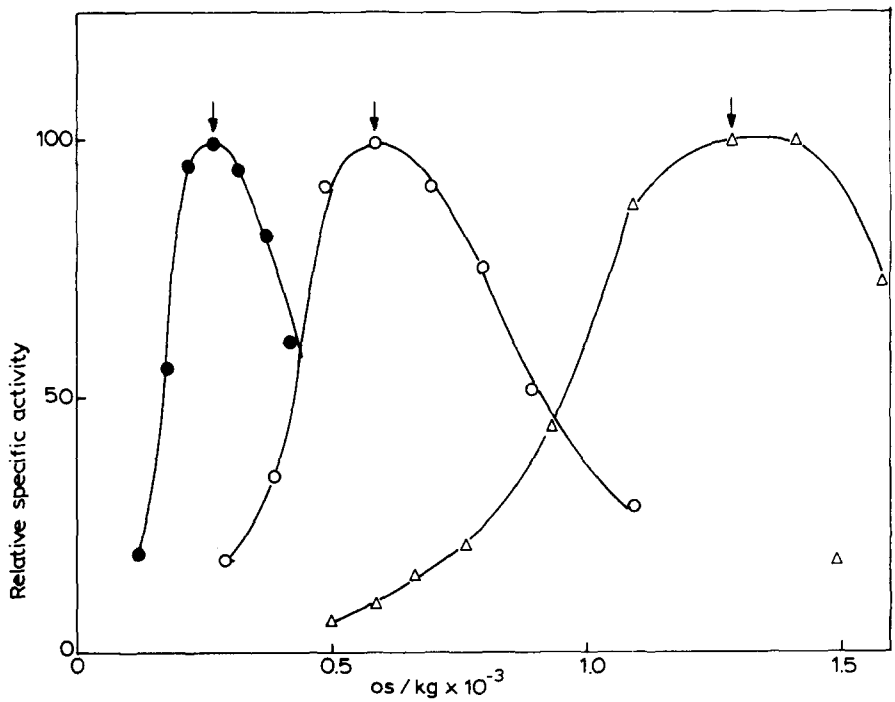

Fig. 6. Osmometry of ADP/ATP carrier activity reconstituted into small unilamellar vesicles. The specific activity of each preparation, obtained at different internal osmolalities (absolute specific activity in the range of 1–3 mmol per min per g protein at isotonicity) were normalized such that the activity at $\pi_{external} = \pi_{internal}$ was taken as 100% (indicated by a vertical arrow). Internal osmolalities, obtained with 0.13 M NaCl and varying amounts of sucrose, were: 280 mos/kg (●———●), 600 mos/kg (○———○); 1300 mos/kg (△———△).

pendence of the activity on the physical state of the membrane. A reconstituted system would offer a special advantage in that one may make observations specific to the carrier per se in a system that may be tested over a wide range of external osmolality than what one can hope to achieve with intact mitochondria (which lyse both in hypo- and hypertonic domains [7]). Another advantage was that the osmotic integrity could be tested over a wide range of external osmolality readily by leakage of the entrapped 6-carboxyfluorescein, which could detect as little as 1% lysis. Thirdly, since the proteoliposomes would always be contaminated with liposomes without the carrier, it was possible to provide a functional control of the intactness of the proteoliposomes per se (vide supra).

Fig. 6 shows the activity of ADP/ATP carrier in small unilamellar liposomes, as a function of external osmolarity. Interestingly, the peak activity coincided with isotonicity at widely different internal osmolalities, such that the activity represented a parabolic profile around isotonicity. The ATPase activity, particularly in sucrose media, in the absence of DNP and in the presence of KCl, also exhibited a parabolic relationship (Figs. 2 and 3). Under these conditions, even respiration exhibited a parabolic relationship around isotonicity (Fig. 4). The parabolic relationship between activity and osmolality, however, remained unexplained. The regulation of the activity of the carrier was known to be related to membrane potential, ionic milieu and the physical state of the membrane (e.g., activation by incorporation of cholesterol [16,17,25]. Membrane potential would exhibit only a monotonic and inverse relationship to volume (or $1/\pi$), if any, and not a biphasic profile [26]. On the other hand, the elastic energy profile of a vesicle indeed exhibits a parabolic relationship

$$E = \frac{1}{2}\frac{K(a-a_0)^2}{a} \tag{3}$$

where a_0 is the interfacial head group area at isotonicity and K, the compressibility modulus [27]. Within the Boyle-Van 't Hoff linear range, the surface area, a, of a sphere would relate to $(1/\pi)^{2/3}$. Therefore, we may obtain an osmotic pressure-dependent, elastic-energy parameter for the vesicle

$$E_\pi = K^* \frac{\left\{\left(\frac{1}{\pi}\right)^{2/3} - \left(\frac{1}{\pi_{iso}}\right)^{2/3}\right\}^2}{\left(\frac{1}{\pi}\right)^{2/3}} \tag{4}$$

where K^* is an appropriate constant. Thus the term E_π/K^* would be expected to exhibit a linear relationship to enzyme/carrier activity within the linear Boyle-Van 't Hoff domain. Fig. 7A–C shows that such a highly significant relationship did exist ($P < 0.01$) for latent ATPase

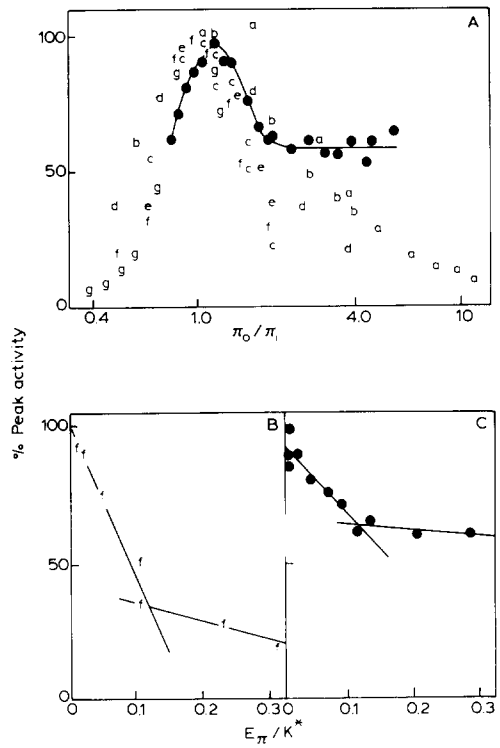

Fig. 7. Osmometric profiles of reconstituted, bovine heart mitochondrial ADP/ATP carrier in comparison with the latent ATPase activity in rat liver mitochondria. (A) ADP/ATP carrier activity, as in Fig. 6. Peak activities (100%) correspond to 1–3 μmol per min per mg protein at various internal osmolalities (os/kg: (a) 0.18; (b) 0.19; (c) 0.28; (d) 0.3; (e) 0.5; (f) 0.6; (g) 1.3). Solid line, latent ATPase activity (peak, 0.5 μmol P_i per min per mg of protein); B and C: E_π/K^* plotted ($(os/kg)^{-2/3}$) against normalized activity of the carrier (f, chosen as an example) and latent ATPase activity from data in Fig. 7A.

activity and the reconstituted carrier activity. The breakpoints, characterizing the linear limits of the Boyle-Van 't Hoff relationship, were also clearly seen in these plots.

Concluding remarks

The experiments reported here established that: (i) the technique of osmometry could yield information on complex systems that can be verified in purified reconstituted systems; (ii) the activity of the purified transporter exhibited strong osmotic dependence; (iii) variations in break-points in polyol media were consistent with variations in reflection coefficients; (iv) a quantitative interpretation of the osmometric profiles of ATPase activity in mitochondria require a clear understanding of the contribution of ionic strength, presence of uncouplers, etc., before one can assign specific meaning to the activity levels, particularly in the hypotonic domain. However, shifts in breakpoints in the hypertonic domain were consistent with variable porosity induced in the mitochondrial membrane to mannitol. The superiority of the osmometric technique in the measurement of reflection coefficients is clear, since other methodology (e.g., tracer fluxes) would not specify the compartment into which the osmolyte actually permeates [14].

Two different quantitative descriptions were suggested for the relationship between the activity of an enzyme/transporter and the external osmotic pressure: a linear inhibition such that the activity becomes

$$A = A_{max} - \tilde{K}\pi \qquad (5)$$

where $\tilde{K}$ is a linear empirical constant coupling activity, A, to the external osmotic pressure, π, as we described in intact systems thus far [1–8], and the present data where in $\tilde{K}$ acquired a parabolic relationship to π such that A_{max} occurred at $\pi_{internal} = \pi_{external}$ (cf. Eqn. 4). A comparison between the intact organelles (which are usually of much larger diameter) and the sonicated proteoliposomes (which exhibit a very small diameter) may be spurious. The latter would correspond in geometry to submitochondrial particles which were shown to be devoid of osmotic activation of any measured activities including respiration or ATPase activity [1,7]. Submitochondrial particles were even shown to be permeable to sucrose [28]. In the absence of methodology that permits an evaluation of ADP/ATP carrier activity in large unilamellar vesicles, the significance of parabolic activation profiles would remain unresolved. *

Does ATP hydrolysis involve enhanced permeation only to mannitol or also to sucrose? ATP hydrolysis may not be competent to induce enhanced permeability to sucrose. Preliminary studies with cytochrome oxidase activity in mitochondria (with the highest possible rate of oxygen consumption) showed a similar situation wherein permeability to mannitol, but not sucrose, was enhanced (unpublished observations). The thermodynamic competence of a given free-energy generator for the induction of the degree of porosity to polyols should also be considered. The change in free energy for respiration with Site III substrates or with ATP hydrolysis would be much less than that for respiration with Site I and Site II substrates.

A number of unanswered questions remain with regard to the osmometric studies: how does the addition of DNP lead to leftward shift in the osmometric profiles? Was it due to diminished permeation of sucrose or due to decreased compressibility of respiration? What would be the equivalent pore radius of the induced pores? What is the biophysical mechanism of modulation of enzyme activity by osmotic pressure? Preliminary studies indicate that cytochrome oxidase activity, in intact mitochondria and on reconstitution of the purified enzyme from a number of species into

* A fundamental distinction between solute effects and osmotic effects needs to be reemphasized: the former would not be related to osmotic gradients across the bilayer nor would these be expected to exhibit marked non-linearities exemplified by the breakpoints. Solute effects tend to be generally monotonic (cf. Refs. 1–3, 5, 7 and 14). Profiles as in Fig. 7 cannot be ascribed to non-osmotic phenomena, though the mechanistic basis of the interaction of osmotic pressure/volume with the activity of the hydrophobic proteins (enzymes and transporters) needs to be established. Thus, the proteoliposomes would be osmotically active particles, consistent with the absence of free-energy generators such as respiration or ATP hydrolysis, unlike in submitochondrial particles whose permeation properties would be essentially variable.

liposomes, was insensitive to osmotic pressure gradients (Sitaramam, V. and Kadenbach, B., unpublished results). Studies are currently under way to delineate the osmotic compressibility of respiration, coupling and phosphorylation at each of the sites to examine whether the osmometric studies could aid in indicating the rate-limiting step(s) in respiration in various respiratory states.

These studies clearly show an induction of variable porosity in the mitochondrial inner membrane by ATP hydrolysis and the novel phenomenon of osmotic compressibility of the transporter activity. The evidence was clear that free-energy generators led to an increase in the free energy of the membrane (i.e., $dU \neq 0$). It requires further investigations to determine whether uncouplers specifically interfere with this process of 'energization', whatever their influence be on transmembrane proton gradients. An important sequel to this variation in internal energy could be the shifts in equilibria, since

$$\Delta G = -RT \ln K_{eq} \quad (6)$$

If so, an increase in internal energy ($dU \neq 0$) could mean a shift in several equilibria, from the planar structure of the bilayer to the kinetic constants of the membrane-bound enzymes. Whether such a shift in equilibria could replace the need for protonmotive force as the energy intermediate requires further evaluation of the relative magnitudes.

Induction of variable porosity to polyols was shown to be a significant exception to the chemiosmotic hypothesis [29], since (i) proton gradients cannot exist across a membrane that readily admits large polyols such as sucrose or mannitol [1]; and (ii) variable porosity to sucrose must imply variations in the state of entropy of the membrane consequent to its energization [1,30]. Thus, the measurement, and even the definition, of protonmotive force need to be reevaluated in terms of the underlying physical and thermodynamic assumptions.

Lastly, the present studies particularly require to be emphasized for their novel empirical content alone, regardless of the implications with regard to the prevailing hypotheses. These studies represent the first ever demonstration of the responsivity of a purified and reconstituted membrane transporter to osmotic gradients across the bilayer. This, in turn, falsifies the a priori conclusion that membrane-bound activities would not directly respond to osmotic gradients on the untested and irrelevant premise that chemical reactions are not compressible (cf. Ref. 9). As we pointed out earlier, these findings bring to fruition the sustained efforts over the last 2–3 decades in the identification of osmosensors in living systems [31,32]. The membrane itself would be the primary osmosensor and membrane-bound proteins would be the secondary sensors as direct links to the relevant metabolic regulations [4,6].

Acknowledgements

This work was supported, in part, by a grant from the Department of Science and Technology, India.

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