Spermine binding to submitochondrial particles and activation of adenosine triphosphatase

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Studies on the effects of polyamines on oligomycin-sensitive ATPase activity of ox heart submitochondrial particles showed that, of the polyamines tested, only spermine affected the enzyme activity. Spermine within the physiological concentration range increased the $V_{\max}$ of the enzyme, but the K_m for ATP was virtually unaffected. Binding studies of [^{14}C]spermine to submitochondrial particles, under the same conditions as used for the ATPase assay, showed that the spermine binds to submitochondrial particles in a co-operative way; Hill plots of the data gave a Hill coefficient of 2 and a K_d of $8\ \mu\text{M}$. When submitochondrial particles were treated with trypsin, ATPase was not stimulated by spermine and the amount of spermine bound concomitantly was drastically decreased. The ATPase activity of isolated F_1 -ATPase was not affected by spermine. Removal of the natural protein ATPase inhibitor did not suppress either the stimulation of the ATPase activity by spermine or the spermine binding to the particles. The results obtained suggested that the polyamine binds and acts at the level of the liaison between the coupling factor F_1 and the membrane sector F_0 of the ATPase complex.

The polyamines spermidine and spermine are non-protein nitrogenous bases widely distributed in Nature. They are regarded as organic cations which replace inorganic cations in various biological systems (Bachrach, 1973). These molecules, however, are highly flexible and their interaction with cellular macromolecules appears to be more specific. Their actions on cellular metabolism is broad, and they are preferentially involved in processes important for cell life. In fact, they are required in DNA synthesis (Fillingame *et al.*, 1975; Inoue *et al.*, 1975; Kreuze & Cozzarelli, 1980), RNA synthesis (Moruzzi *et al.*, 1975; Galston *et al.*, 1978; Kaur-Sawheny *et al.*, 1978) and protein synthesis (Atkins *et al.*, 1975; Hunter *et al.*, 1977; Igarashi *et al.*, 1977; Seidenfeld & Marton, 1979). Since the first efforts aiming to clarify the physiological roles of polyamines, effects of these biological amines on the stabilization of mitochondrial membrane have been described (Tabor, 1960; Herbst & Witherspoon, 1960). More recently, studies concerning the possibility that polyamines might have a modulat-

ing role in mitochondrial oxidative phosphorylation have been reported (Chaffee *et al.*, 1979; Phillips & Chaffee, 1982; Byczkowski *et al.*, 1982). Those authors suggested that alterations in intracellular polyamine concentrations might play a role in metabolic regulation of mitochondria *in vivo*. However, no information has been reported on the sites of interaction of physiological concentrations of polyamines affecting mitochondrial functions.

In this paper we describe the binding of spermine to submitochondrial particles, the effect on the kinetic parameters of mitochondrial ATPase and the possible binding site of the polyamine on the enzyme complex.

Materials and methods

Submitochondrial particles (ETP_H) derived from bovine heart mitochondria were prepared as described previously (Lenaz *et al.*, 1975).

ATPase activity was measured by the following procedures. (a) The reaction mixture contained $25\ \mu\text{mol}$ of Tris/acetate, pH 8.5, $25\ \mu\text{mol}$ of potassium acetate, $0.3\ \text{mmol}$ of sucrose, $2\ \mu\text{mol}$ of MgCl_2 , $0.2\ \mu\text{mol}$ of NADH, $0.5\ \mu\text{mol}$ of phosphoenolpyruvate, 2.5 units of lactate dehydrogenase, 2.5 units of pyruvate kinase and $1\ \mu\text{g}$ of rotenone in

Abbreviations used: F_1 , coupling factor 1, soluble part of the ATPase complex; F_0 , membrane sector of the ATPase complex; F_6 , coupling factor 6, one of F_0 's subunits.

a final volume of 1 ml. ETP_H suspension (100 µg of protein) was added to the mixture equilibrated at 30°C and the reaction was started with 2 µmol of ATP (pH 7). The decrease in NADH, which is a measure of ADP formation from ATP, was followed at 340 nm on a Zeiss PMQ III spectrophotometer equipped with a Servogor recorder.

(b) The reaction mixture contained 50 µmol of Tris/HCl (pH 8.5) and 7.5 µmol of MgCl₂ in a final volume of 1.5 ml. ETP_H suspension (400 µg of protein) was added to the mixture and the reaction was started by the addition of 5 µmol of ATP (pH 7.5). The reaction was stopped after 5 min by addition of 0.5 ml of 20% (w/v) trichloroacetic acid. P_i released was determined colorimetrically (Fiske & Subbarow, 1925).

ATP formation was determined by disappearance of P_i in a glucose/hexokinase trap system. Submitochondrial particles (2 mg), inhibited with rotenone (2 µg), were incubated aerobically by shaking with 0.25 M-sucrose, 20 mM-glucose, 2 mM-MgCl₂, 0.5 mM-EDTA, 20 mM-succinate, 1 mM-ADP, 6 mM-phosphate and hexokinase (20 units), in 20 mM-Tris/HCl buffer, pH 7.5. The total volume was 1.5 ml and the temperature 30°C. Samples were taken for determination of P_i uptake as described above.

Trypsin treatment of the particles was performed in a reaction mixture containing 2 mg of protein/ml, 0.25 M-sucrose, 10 mM-Tris/HCl, pH 7.5, and 40 µg of trypsin/mg of protein at 20°C for 20 min. The proteolysis was stopped by addition of trypsin inhibitor. Submitochondrial particles were centrifuged down at 100 000 g for 40 min and resuspended in 0.25 M-sucrose/10 mM-Tris/HCl, pH 7.5, before assay of the ATPase activity.

Inhibitor-depleted particles (ammonia-Sephadex particles) were prepared as described by Racker & Horstman (1967).

Protein was determined by the biuret method (Gornall *et al.*, 1949) in the presence of 1% deoxycholate.

The binding of [¹⁴C]spermine to ETP_H was

performed under the conditions of the ATPase assay in method (b). To the incubation mixture various concentrations of [¹⁴C]spermine (122 mCi/mmol) (The Radiochemical Centre, Amersham, Bucks., U.K.) were added. The binding was stopped by pipetting the reaction mixture on to Millipore filters (GS WP 03700). The filters were washed twice with 2 ml of buffer and put in scintillation vials. The radioactivity present was determined by scintillation counting after the addition of 10 ml of Filtercount (Packard). The background radioactivity was independent of the amount of protein in the sample, but was proportional to the amount of radioactivity passing through the filter.

Results

Polyamine interaction with submitochondrial particles

Putrescine, spermidine and spermine were tested to evaluate their effect on oligomycin-sensitive ATPase activity of submitochondrial particles.

Although spermine activated the hydrolytic activity of oligomycin-sensitive ATPase, putrescine and spermidine showed a slight inhibitory effect at the concentrations tested (10–100 µM). As shown in Fig. 1, at pH 8.5 the rate of ATP hydrolysis increased as spermine concentration was increased up to 30 µM. The stimulation was specifically evoked by spermine only, and it could not be ascribed simply to a change in the ionic conditions. A synthetic amine, triethylenetetramine, which bears a number of charges similarly to spermine, showed a slight inhibitory effect on ATPase activity, as did the other polyamines tested (Fig. 1). Moreover, addition of comparable concentrations of Mg²⁺ to the reaction mixture could neither replace the polycation nor modify the stimulatory effect of spermine (Table 1).

The kinetic constants of ATPase were derived from studies in the substrate concentration range 0.03–0.5 mM by using the ATP-regenerating system

Table 1. *Effects of spermine on the specific activity of ATPase in submitochondrial particles after various treatments*
Experimental conditions were as described in the text. The enzyme activity was assayed by determining colorimetrically the P_i released.

Treatment	ATP hydrolase activity (µmol/min per mg of protein)
None	1.50
+ 25 µM-spermine	2.56
+ 1 mM-Mg ²⁺	1.60
+ trypsin	3.72
+ trypsin + 25 µM-spermine	3.60
Ammonia-Sephadex particles	4.06
Ammonia-Sephadex particles + 25 µM-spermine	4.98

Fig. 1. Effect of polyamines on ATPase activity of submitochondrial particles

The hydrolysis rate was measured by determining P_i released. Experiments performed by using the ATP-regenerating system for the enzyme assay gave similar results. The activity is expressed as μmol of P_i liberated/min per mg of protein. $\circ$, Spermine; Δ , spermidine; $\square$, putrescine; $\bullet$, triethylenetetramine.

Fig. 2. Double-reciprocal plot for ATP hydrolysis by submitochondrial particles

Δ , Control; $\circ$, in the presence of $25 \mu\text{M}$ -spermine. The hydrolysis rate, measured by determining NADH oxidized, is expressed as μmol of ATP hydrolysed/min per mg of protein. The concentration of ATP ranged from 0.03 to 0.5 mM. For experimental details see the text.

for the enzyme assay. The reaction obeyed Michaelis-Menten kinetics, and double-reciprocal plots of the data (Fig. 2) showed apparent K_m values not significantly different when determined in the absence or in the presence of spermine (0.38 and 0.36 mM respectively). The maximum velocity, V , was 80% greater when $25 \mu\text{M}$ -spermine was present in the reaction mixture; its value increased from 1.50 to 2.70 units.

Spermine (0.01–0.5 mM) did not affect the oligomycin-sensitivity of the ATPase activity of submitochondrial particles, nor did it have any effect on the ATPase activity of isolated F_1 -ATPase, nor did it have any appreciable effect on the rate of ADP phosphorylation under the experimental conditions described above (results not shown).

Experiments were performed to evaluate the binding of $[^{14}\text{C}]$ spermine to submitochondrial particles. Fig. 3 shows the binding curve. The Hill (1910) treatment of the data, shown in the insert, indicates that spermine binds submitochondrial particles in a co-operative way, with a Hill coefficient, h , of 2 and a K_d of $8 \mu\text{M}$.

Fig. 3. Binding of $[^{14}\text{C}]$ spermine to submitochondrial particles

Submitochondrial particles were incubated with various concentrations of $[^{14}\text{C}]$ spermine as indicated in the Materials and methods section. Insert: Hill analysis of binding data.

Spermine interaction with trypsin-treated submitochondrial particles

Trypsin-treated submitochondrial particles were tested to evaluate the binding of $[^{14}\text{C}]$ spermine. The amount of spermine bound was drastically decreased (to 5–8% of untreated control values), but the binding parameters were unchanged (results not shown).

Under these experimental conditions ATPase was no longer stimulated by the addition of

spermine to the assay mixture (Table 1). These data seem to indicate that spermine exerts its action on ATPase activity through a protein-mediated effect.

When sonicated vesicles (ETP_H) are incubated with trypsin, the proteolytic action is exerted on the ATPase complex, inducing removal of the

natural protein ATPase inhibitor (Racker, 1963; Horstman & Racker, 1970) and on the liaison between F_1 and the membrane sector F_0 . Proteolysis of oligomycin-sensitivity-conferring protein and F_6 has been reported (McLennan & Tzagoloff, 1968; Kanner *et al.*, 1976; Mairouch & Godinot, 1977). Thus the polyamines may act on the F_0/F_1 interface and/or on the mode of interaction of the inhibitor with F_1 , and, in order to define which of the two known trypsin-sensitive regions of ATPase was involved in the binding of spermine, inhibitor-depleted particles were prepared by ammonia and Sephadex treatment (Racker & Horstman, 1967).

Spermine interaction with inhibitor-depleted particles

As shown in Table 1 the ammonia-Sephadex particles showed activation of ATPase activity by $25\text{ }\mu\text{M}$ -spermine, suggesting that the ATPase inhibitor is not involved in the activation mechanism. This is further supported by the observed unchanged ability of the inhibitor-depleted particles to bind [^{14}C]spermine; in fact the binding parameters and the total amount of labelled spermine were the same as for the untreated particles (results not shown).

Discussion

At micromolar concentrations, spermine stimulated ATPase activity of bovine heart submitochondrial particles. The stimulation should not be due to a Mg^{2+} -like effect on the substrate ATP. In fact it has been reported that the formation constant for the spermine-ATP complex is approx. 40% lower than for Mg-ATP (Nakai & Glinsmann, 1977). Within the spermine concentration range stimulating the enzyme ($25\text{--}50\text{ }\mu\text{M}$), the amount of spermine-ATP complex formed would be much lower than the Mg-ATP concentration in the assay mixture. The hypothesis that spermine might act as a generic organic cation is not tenable, as similar studies in the presence of putrescine, spermidine and also the synthetic amine triethylenetetramine did not evoke any stimulation at any concentration tested. Consequently spermine did not simply act as a non-specific polycation, but rather the stimulation effect was due to its molecular characteristics *in toto*: number and relative positions of the charges in the molecule, and highly flexible structure.

As the V of the enzyme in the presence of spermine was almost twice that in the absence of the polyamine, and as the apparent K_m seemed unaffected by the polyamine, the difference in enzymic activity could be due to an increased ability of the enzyme to break the phosphate-ester bond, or indeed spermine could influence a step after ATP binding more than the formation of the enzyme-substrate complex.

The binding studies showed that spermine binds submitochondrial particles in a co-operative way with a dissociation constant of $8\text{ }\mu\text{M}$. Binding to the co-operative site is characterized by an interaction exponent of 2, which is large enough to indicate a rather significant degree of interaction. In order to explain the mechanism of this type of interaction, two models have been proposed: one in which the acceptor molecule undergoes a concerted transition between two structural forms as it preferentially binds ligand to one of these forms (Monod *et al.*, 1965), and a 'sequential' model in which binding of one ligand effects a conformational change, which eases the binding of the next ligand (Koshland *et al.*, 1966). The present data cannot discriminate between these possibilities.

It is known that, owing to their polybasic nature, polyamines bind to cellular polyanions; then binding to the negatively charged phospholipid groups on the membrane surface is conceivable. Spermine's inability to bind submitochondrial particles after the trypsin treatment, however, hinted at a protein-mediated binding. The loss of ATPase stimulation after trypsin treatment in the same experimental conditions suggested a correlation between the binding and the activating effect. Trypsin exerts its proteolytic action on the ATPase complex at the F_0/F_1 interface and at the inhibitor- F_1 level. Considering that the inhibitor-depleted particles were activated and bound to spermine to the same extent as did the untreated ETP_H , the trypsin-modified coupling factors oligomycin-sensitivity-conferring protein and F_6 at the F_0/F_1 interface might be involved in spermine binding. The molecular mechanism through which the binding of spermine affected ATPase activity might involve a direct interaction of spermine with the trypsin-modified polypeptides of the ATPase complex or the binding of spermine to acidic phospholipids, which indirectly would influence the conformation of the trypsin-modified polypeptides. The increased catalytic activity of the enzyme might be a consequence of this spermine-induced conformational change. However, this modification appeared unable to affect the ATPase sensitivity to oligomycin.

The physiological role of spermine on mitochondrial functions seen *in vitro* must be related to the polyamine concentration in the mitochondria as well as in the cell *in vivo*. However, not much is known about the subcellular distribution of polyamines. Preliminary data obtained in our laboratory indicated that coupled mitochondria stripped of the polyamines bound to the outer surface of the outer membrane contain $33 \pm 7\text{ }\mu\text{M}$ -spermine (Tadolini *et al.*, 1983). This spermine concentration is of the same order of magnitude as that exerting the stimulatory effect on ATPase.

The physiological role of spermine at 10 μM concentrations on mitochondrial functions cannot yet be conclusively stated with the data available at present. However, the lack of spermine effect on the ADP phosphorylation and the marked increase of the ATP-hydrolysis rate induced by slight changes in spermine concentration are compatible with a possible involvement of the amine in the maintenance of the correct membrane potential ($\Delta\Psi$), the ATP-hydrolysis rate being increased when $\Delta\Psi$ decreases and the ATP/ADP ratio is high. In this respect our data match the results of Toninello *et al.* (1983) indicating a synergistic effect of spermine and ATP in restoring the collapsed membrane potential of mitochondria and a lack of effect when ADP substitutes for ATP.

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