Spermine binding to submitochondrial particles and activation of adenosine triphosphatase

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(Received 26 October 1983 | Accepted 7 November 1983)

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 $V_{\text{max}}$ of the enzyme, but the $K_m$ for ATP was virtually unaffected. Binding studies of $[^{14}\text{C}]$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 modulating 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$\mu$mol of Tris/acetate, pH 8.5, 25$\mu$mol of potassium acetate, 0.3mmol of sucrose, 2$\mu$mol of MgCl$_2$, 0.2$\mu$mol of NADH, 0.5$\mu$mol of phosphoenolpyruvate, 2.5 units of lactate dehydrogenase, 2.5 units of pyruvate kinase and 1$\mu$g of rotenone in
a final volume of 1 ml ETP\textsubscript{H} suspension (100 \mu g of protein) was added to the mixture equilibrated at 30°C and the reaction was started with 2 \mu mol of ATP (pH 7). The decrease in NADH, which is a measure of ATP 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 \mu mol of Tris/HCl (pH 8.5) and 7.5 \mu mol of MgCl\textsubscript{2} in a final volume of 1.5 ml. ETP\textsubscript{H} suspension (400 \mu g of protein) was added to the mixture and the reaction was started by the addition of 5 \mu mol of ATP (pH 7.5). The reaction was stopped after 5 min by addition of 0.5 ml of 20% (w/v) trichloroacetic acid. \textsubscript{Pi} released was determined colorimetrically (Fiske & Subbarow, 1925).

ATP formation was determined by disappearance of \Pi in a glucose/hexokinase trap system. Submitochondrial particles (2 mg), inhibited with rotenone (2 \mu g), were incubated aerobically by shaking with 0.25 M sucrose, 20 mM-glucose, 2 mM-MgCl\textsubscript{2}, 0.5 mM-EDTA, 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 \Pi 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 \mu g 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 100000 g for 40 min and resuspended in 0.25 M sucrose/10 mM-Tris/HCl, pH 7.5, before assay of the ATP\textsubscript{ase} activity.

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

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

The binding of [\textsuperscript{14}C]spermine to ETP\textsubscript{H} was performed under the conditions of the ATP\textsubscript{ase} assay in method (b). To the incubation mixture various concentrations of [\textsuperscript{14}C]spermine (122 mCi mmol\textsuperscript{-1}) (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

\textbf{Polyamine interaction with submitochondrial particles}

Putrescine, spermidine and spermine were tested to evaluate their effect on oligomycin-sensitive ATP\textsubscript{ase} activity of submitochondrial particles.

Although spermine activated the hydrolytic activity of oligomycin-sensitive ATP\textsubscript{ase}, putrescine and spermidine showed a slight inhibitory effect at the concentrations tested (10–100 \mu M). As shown in Fig. 1, at pH 8.5 the rate of ATP hydrolysis increased as spermine concentration was increased up to 30 \mu M. 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 ATP\textsubscript{ase} activity, as did the other polyamines tested (Fig. 1). Moreover, addition of comparable concentrations of Mg\textsuperscript{2+} to the reaction mixture could neither replace the polycation nor modify the stimulatory effect of spermine (Table 1).

The kinetic constants of ATP\textsubscript{ase} were derived from studies in the substrate concentration range 0.03–0.5 mM by using the ATP-regenerating system

\begin{table}[h]
\centering
\begin{tabular}{|l|c|}
\hline
Treatment & ATP hydrolyase activity (\mu mol/min per mg of protein) \\
\hline
None & 1.50 \\
+ 25 \mu M-spermine & 2.56 \\
+ 1 mM-Mg\textsuperscript{2+} & 1.60 \\
+ trypsin & 3.72 \\
+ trypsin + 25 \mu M-spermine & 3.60 \\
Ammonia–Sephadex particles & 4.06 \\
Ammonia–Sephadex particles + 25 \mu M-spermine & 4.98 \\
\hline
\end{tabular}
\caption{Effects of spermine on the specific activity of ATP\textsubscript{ase} in submitochondrial particles after various treatments}
\end{table}

Experimental conditions were as described in the text. The enzyme activity was assayed by determining colorimetrically the \Pi released.

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

The hydrolysis rate was measured by determining P,

\[
\text{specific activity (\text{umol of ATP hydrolysed/min per mg})}
\]


![Graph showing the effect of polyamines on ATPase activity.](image)

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 \(\mu\)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 \(\mu\)M.

**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\textsubscript{1} and the membrane sector F\textsubscript{0}. Proteolysis of oligomycin-sensitivity-conferring protein and F\textsubscript{0} has been reported (McLennan & Tzagoloff, 1968; Kanner et al., 1976; Mairouch & Godinot, 1977). Thus the polyamines may act on the F\textsubscript{0}/F\textsubscript{1} interface and/or on the mode of interaction of the inhibitor with F\textsubscript{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 μM-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 [14C]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\textsuperscript{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 (25–50 μM), 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\textsubscript{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 μM. 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\textsubscript{0}/F\textsubscript{1} interface and at the inhibitor-F\textsubscript{1} level. Considering that the inhibitor-depleted particles were activated and bound to spermine to the same extent as did the untreated ETP\textsubscript{H}, the trypsin-modified coupling factors oligomycin-sensitivity conferring protein and F\textsubscript{0} at the F\textsubscript{0}/F\textsubscript{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 $33 \pm 7 \mu M$-spermine (Tadolini et al., 1983). This spermine concentration is of the same order of magnitude as that exerting the stimulatory effect on ATPase.
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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 (ΔΨ), the ATP-hydrolysis rate being increased when ΔΨ 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.

This work was supported by Consiglio Nazionale delle Ricerche (Roma). We are grateful to Professor C. A. Rossi for his interest in this research and for providing all the facilities.

References

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