THE PROBLEM OF PERMEABILITY BARRIER IN MITOCHONDRIAL RESPIRATION: DUAL EFFECT OF PROTAMINE ON SUCCINATE (+ ROTENONE) OXIDATION

Jerzy POPINIGIS
Department of Biochemistry, Medical School, Gdańsk 80210, Poland

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1. Introduction

The inhibitory effect of protamine on the respiratory rate of mitochondria in an isotonic medium is well documented [1–4]. In the present paper a stimulatory effect of protamine on mitochondrial succinate (+ rotenone) oxidation is described. Stimulation was observed in the medium containing high concentration of sucrose (in the presence of citrate and phosphate). In the same medium supplemented with halothane protamine had an inhibitory effect on respiration.

A possible explanation of the action of protamine as an agent affecting anion transport by influencing positively charged groups of the inner mitochondrial membrane (including adenine nucleotide binding sites) is discussed.

2. Materials and methods

Rat liver mitochondria were prepared and suspended in 0.25 M sucrose + 3 mM Tris chloride (pH 7.3), according to Loewenstein et al. [5], with the omission of the last digitonin step. Oxygen uptake and pH were measured as described previously [6].

Protamine sulphate (ex herring, Koch-Light) was used after it had been brought to pH 7.0 with Tris. Polyadenylic acid, (potassium salt) was from British Drug Houses Ltd. Sucrose (Analar) was from Koch-Light. Halothane (Fluothane) was from Imperial Chemical Industries Ltd., distributed by LEK Ljubljana.

Detailed experimental conditions are described in the legends to the figures.

3. Results

It is shown in fig. 1, that the response of mitochondrial respiration to protamine added is dependent on the composition of the medium.

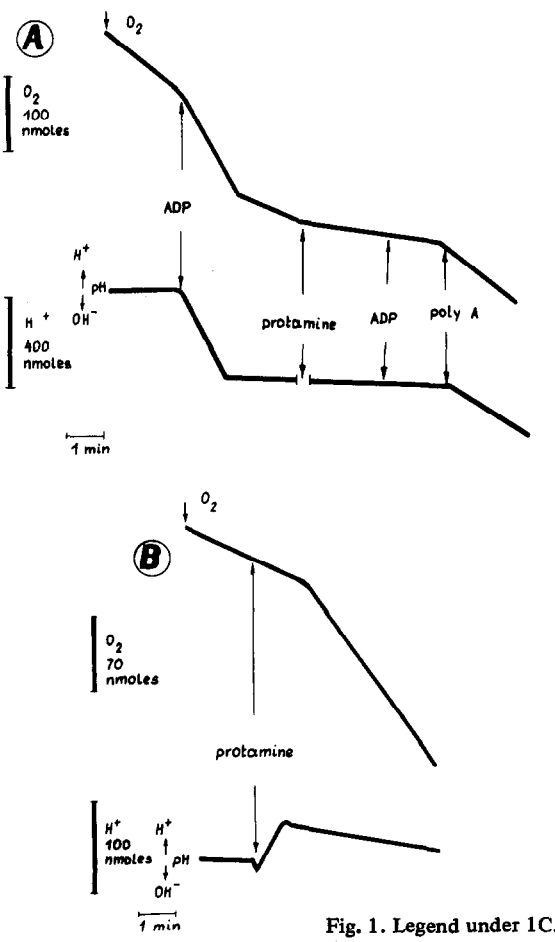

Fig. 1. Legend under 1C.

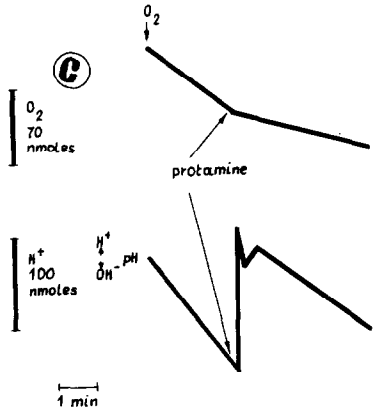

Fig. 1. Response of mitochondrial respiration to added protamine in media of different composition. Rat liver mitochondria (4 mg of protein in 0.05 ml of 250 mM sucrose + 3 mM Tris chloride) were added into 2 ml of the pH 7.0 media containing: In the Exp. A (medium A) – 170 mM sucrose, 10 mM Tris chloride, 3 mM $MgCl_2$, 0.01 mM EGTA, 5 mM potassium phosphate, 5 mM Tris succinate and 4 μg of rotenone. In the Exp. B (medium B) – 500 mM sucrose, 1.25 mM Tris citrate, 0.5 mM potassium phosphate, 2.5 mM Tris succinate and 10 μg of rotenone. In the Exp. C (medium C) – 500 mM sucrose, 1.25 mM Tris citrate, 0.5 mM potassium phosphate, 2.5 mM Tris succinate, 10 μg of rotenone and 10 mM halothane added in 20 μl of ethanol. After the mitochondria had been preincubated in the above media for 1 min, oxygen uptake and changes in proton concentration were recorded simultaneously. The pH electrode was calibrated by the addition of 400 or 100 nmoles of protons as a HCl solution at the end of experiments. Additions: 0.5 μmoles of ADP, 150 μg protamine, 300 μg polyadenylate (poly A).

In the isoosmotic medium A protamine inhibited the rate of state 4 mitochondrial succinate (+ rotenone) oxidation and prevented ADP induced transition of the state 4 to the state 3 in both oxygen and proton uptake. This inhibition could be reversed by the introduction of polyadenylate into the incubation medium (fig. 1, Exp. A).

When protamine at the same concentration was added to the mitochondria suspended in the medium B, containing 0.5 M sucrose, (but also phosphate and citrate) – the rate of succinate (+ rotenone) oxidation significantly increased (fig. 1, Exp. B). Simultaneous measurements of proton concentration in the medium indicate that the protamine-induced proton ejection from the mitochondria was followed by a slow phase of proton reuptake. Stimulatory effect of protamine on respiration was observed with some delay, and occurred when the proton ejection phase was completed.

On the other hand, when medium B was supplemented with halothane (medium C), protamine inhibited mitochondrial respiration (fig. 1, Exp. C).

Protamine was found to stimulate mitochondrial respiration only if, in addition to a high concentration of sucrose in the medium, both phosphate (fig. 2, Exp. A) and citrate (fig. 2, Exp. B) were present. This protamine-induced succinate (+ rotenone) oxidation was found to be inhibited by dinitrophenol (DNP) + ADP (fig. 2, Exp. C), benzene 1,2,3 tricarboxylate (fig. 2, Exp. D) and polyadenylate (fig. 2, Exp. E).

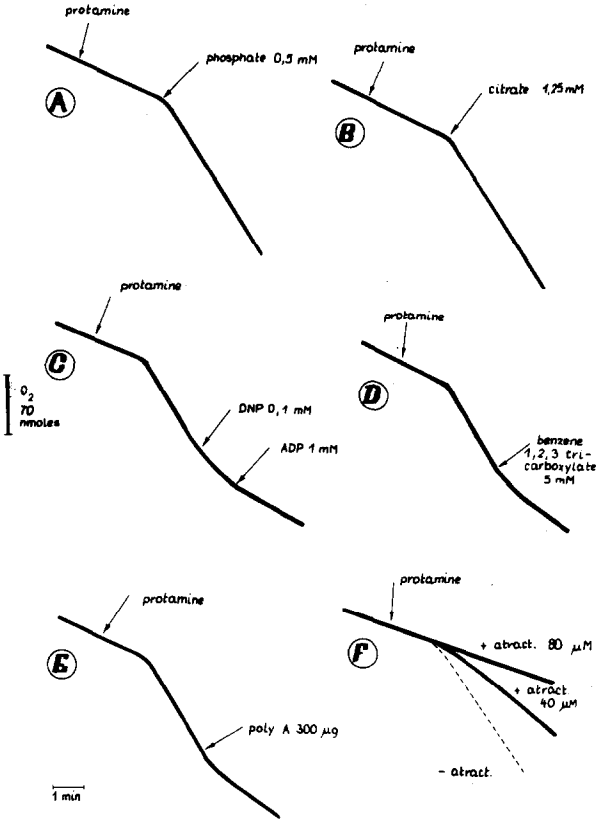

Fig. 2. Protamine-stimulated succinate (+ rotenone) oxidation. Experimental conditions as described in the legend to fig. 1, Exp. B with the exceptions that: In Exp. A phosphate was omitted; In Exp. B citrate was omitted; In Exp. F before mitochondria were added, the medium had been supplemented with atractyloside (atract.) in concentrations as indicated. Additions: 150 μg protamine, other as indicated.

The stimulatory effect of protamine on mitochondrial respiration in the described experimental conditions was found to be lowered by a prior addition of atractyloside (fig. 2, Exp. F).

4. Discussion

Although it is known since 1941, that colloidon membranes become 'anion selective' upon incorporation of protamine (for review see [7]), no special attention was paid to the possibility to correlate this observation with the effect of basic proteins on mitochondrial respiration. Only recently, data available from electron microscopy [8,9] and observations that protamine inhibited mitochondrial succinate (+ rotenone) oxidation was restored by the addition of citrate + benzene 1,2,3 tricarboxylate [6], revealed that the effect of protamine on mitochondrial respiration may be related to the transitions in membrane permeability to anions.

This aspect of protamine action was investigated in the present paper. The effect of protamine on mitochondrial succinate (+ rotenone) oxidation was measured:

– in medium A, routinely used for oxidative phosphorylation study,

– in medium B, in which the rate of mitochondrial respiration is belived to be limited by anion penetration, and

– in medium C, with the same composition as medium B, but supplemented with halothane.

It has been found, that protamine inhibited mitochondrial respiration in medium A and C, whereas, in medium B, a stimulatory effect was observed.

It is the author's contention that the action of protamine on mitochondria in all media studied occurs through the following sequential stages: 1) Neutralization of fixed negative charges on membranes; 2) An increase in the amount of positively charged groups; 3) An increase in the membrane permeability to anions; 4) New Donnan distribution of ions and water; 5) Transitions in mitochondrial ultrastructure.

Depending on the experimental conditions, this action may result in an increase or decrease of the rate of mitochondrial respiration:

I. In the medium A protamine exerts its known inhibitory effect on mitochondrial respiration. (fig. 1, Exp. A). The protamine effect may be counteracted by simple direct interaction between added polycation and polyanion as first described by Person and Fine [10]. The reversing effect of polyadenylate (fig. 1, Exp. A) on protamine inhibition seems to occur by this mechanism.

The same counteraction may be achieved by neutralization of positively charged groups of the mitochondrial inner membrane (including adenine nucleotide binding sites) by the anion present in the intermembrane space of mitochondria. This may occur only if the ability of other mitochondrial proteins to bind (or adsorb [11]) anions is reduced. An example of such action is the reversing effect of DNP + ADP [12] or citrate + benzene 1,2,3 tricarboxylate [6], on protamine inhibited respiration.

II. The stimulatory effect of protamine on the rate of mitochondrial respiration in the medium B, containing high concentration of sucrose, seems to reflect the transition of mitochondrial membrane from an 'anion impermeable' to 'anion permeable' state. The low rate of mitochondrial respiration in the medium containing high concentration of sucrose [13], is belived to be due to limited penetration of anions. According to Chappell and Haarhoff [14], sucrose exerts a direct inhibitory effect on a 'carrier system' involved in penetration of anionic substrates into the mitochondria. Green and Harris [15] postulated that osmotic compression of cristae, which occurs in the medium containing high concentrations of sucrose may create an electronegative electrostatic barrier to anion binding.

Observations that protamine stimulated respiration only in the presence of phosphate and citrate (fig. 2, Exps. A and B), and that this protamine-induced respiration was sensitive to benzene 1,2,3 tricarboxylate (fig. 2, Exp. D) indicate that movement of anions in these conditions occurs through the known 'anion–anion exchange' reactions. Moreover, agents which were found to counteract the inhibitory effect of protamine, e.g., DNP + ADP or polyadenylate, also counteract protamine-stimulated respiration (fig. 2, Exps. C and E).

Recently Shug et al. [16] have demonstrated close interrelationship between adenine nucleotide translocase and tricarboxylate carrier system of inner mitochondrial membrane. They found that anion

transport through both the adenine nucleotide translocase and the tricarboxylate carrier could be inhibited by atractyloside. In experimental conditions described here, atractyloside was found to lower the stimulatory effect of protamine on respiration (fig. 2, Exp. F). Since adenine nucleotide binding sites are responsible for ultrastructural transitions of mitochondria [17–20] it can be considered that these positively charged constituents of mitochondrial membrane may regulate both structural and permeability properties of the membrane. Therefore, it is possible that the postulated induction of membrane permeability to anions by protamine occurs at the level of a 'contractile system' [19–21], consisting of adenine nucleotide binding sites [19,20].

III. When medium B was supplemented with halothane (medium C), in spite of the presence of high concentration of sucrose (+ phosphate + citrate) the effect of protamine on mitochondrial respiration was inhibitory (fig. 1, Exp. C). Woźniak in our laboratory has found that the osmotic barrier of mitochondrial membrane to sucrose can be abolished by the introduction of halothane into the suspending medium [22]. Based on this observation a supposition is possible that in the absence of an osmotic factor, protamine-induced new Donnan distribution of ions and water across the mitochondrial membrane may lead to the transition in mitochondrial structure into the form in which electron transport is inhibited.

In this paper, the action of protamine was considered to occur mainly at the level of permeability of mitochondrial membrane to anions. Since the osmotic factor may alter not only the permeability properties of the membrane, other interpretations of the data presented are also possible.

On the other hand, observations presented here seem to suggest that the rate of mitochondrial respiration may be controlled also by permselectivity of the membrane to anions. This regulation which is dependent on osmosis, involves the action of adenine nucleotide binding sites and includes a role of polycations and polyanions as effectors, may be of a great significance in the intact cell.

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