THE EFFECT OF SOME PROTON CONDUCTING SYSTEMS ON PROTAMINE INHIBITED RESPIRATION

J.POPINIGIS, W.RZECZYCKI and J.ŚWIERCZYŃSKI
Department of Biochemistry, Medical School, Gdańsk, Poland

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1. Introduction

The present paper reports studies on the mechanism of the inhibitory action of the basic protein protamine on succinate (+ rotenone) oxidation in isolated rat liver mitochondria. The data obtained suggest that this protein inhibits respiration by affecting proton re-entry into the mitochondria.

2. Materials and methods

Rat liver mitochondria were prepared according to Weinbach [1] in 0.25 M sucrose with 3 mM tris chloride pH 7.3. Mitochondria depleted of "high-energy intermediates" were obtained by the method of Graafmans and coworkers [2], by preincubation of freshly prepared mitochondria with dicoumarol followed by the binding of dicoumarol with albumin.

Sonicated submitochondrial particles were prepared from liver mitochondria according to Bronk and Kielley [3]. Protein was estimated by the biuret method [4].

Protamine sulphate ex herring, pure (Koch-Light) was used after adjusting to pH 7.3 with tris. All anions examined were used as solutions (pH 7.3) of the K salts except isocitrate which was used as the Na salt.

3. Results

Protamine did not inhibit citrulline synthesis in mitochondria which had been depleted of "high-energy intermediates", when exogenous ATP (+ oligomycin) was used as energy donor. In contrast to the action of antimycin or oligomycin, protamine did not prevent inhibitory action of succinate or ATP on this synthesis (table 1).

Table 1
The effect of protamine on exogenous ATP-dependent citrulline synthesis in mitochondria depleted of "high-energy intermediates". Citrulline synthesis was assayed by incubation of mitochondria depleted of "high-energy intermediates" (3.5 mg protein) in 1 ml of complete medium, final pH 7.3, consisting of: 15 mM KCl, 50 mM tris chloride, 5 mM $MgSO_4$, 5 mM potassium phosphate, 10 mM ornithine, 20 mM NH_4HCO_3, 10 mM ATP, 10 mM succinate, 4 μg rotenone, 10 μg oligomycin, 5 μg antimycin and 25 mM sucrose. After 30 min of incubation at 25° the reaction was stopped by $HClO_4$ and citrulline was determined according to Archibald [5].

Additions to, or omissions from complete medium	Citrulline synthesis (μmoles)
–	1.4
+ protamine 300 μg	1.4
– oligomycin	0.2
– oligomycin + protamine	0.2
– antimycin	0.2
– antimycin + protamine	0.2
– antimycin – succinate	1.4
– antimycin – succinate + protamine	1.4
– antimycin – succinate – oligomycin	0.2
– antimycin – succinate – oligo. + protam.	0.2

The data shown in table 2 indicate that protamine inhibited respiration of isolated mitochondria, but did not affect respiration of submitochondrial particles obtained by sonication (exp. no. 16).

Table 2

The effect of protamine on respiration. The respiration was measured with a Clark electrode in 3.5 ml medium of pH 7.3 containing: 15 mM KCl, 50 mM tris chloride, 5 mM $MgSO_4$ and 5 mM potassium phosphate (P_i). Differences in oxygen consumption with time (expressed as natoms O/min/mg protein), after addition of 8 mg protein of intact mitochondria (m), or 1 mg protein of sonicated particles (s.p.). Other additions when indicated in table: 4 μg rotenone (r), 300 μg protamine (prot), 10 μg oligomycin (oligo), 3 μg gramicidin (gram), 3 μmoles ADP and anions in a final concentration of 10 mM isocitrate (isocit), 10 mM citrate (cit), 10 mM succinate (succ) and 0.1 mM DNP.

		Time of exp. (min)											
Exp. No.	medium	0	1		2		3		4		5		6
		additions and oxygen uptake											
1	r, m	succ	20	ADP	70	prot	12		12		12		12
2	r, m	succ	22	ADP	75	prot	12	DNP	35		35		35
3	r,m	succ	20	ADP	75	prot	10	cit	35		35		35
4	r, m	succ	20	ADP	75	prot	10	isocit	30		30		30
5	r, m, -P_i + ADP	succ	25	ADP	25	prot	10	cit	10	Pi	35		35
6	r, m, -P_i + ADP	succ	25	ADP	25	prot	10	DNP	35		35		35
7	r, m	succ	22	ADP	75	prot	12	DNP	35	DNP	35		35
8	r, m	succ	20	ADP	70	prot	12	DNP	30	cit	55		55
9	r, m	succ	23	ADP	70	prot	11	oligo	11	DNP	35		35
10	r, m	succ	22	ADP	70	prot	13	oligo	13	cit	13		13
11	r, m	succ	20	ADP	70	prot	13	oligo	13	isocit	13		13
12	r, m	succ	20	ADP	70	prot	12	oligo	12	DNP	30	cit	60
13	r, m	succ	20	ADP	70	prot	12	gram	12		12		12
14	r, m	succ	20	ADP	70	prot	10	gram	10	DNP	10	cit	10
15	r, m	succ	20	ADP	70	prot	12	gram	12	cit	12	DNP	12
16	r, s.p.	succ	60	prot	60	prot	60		60	prot	60		60

Inhibition by protamine of succinate (+ rotenone) oxidation by intact rat liver mitochondria was partially reversed by additions of DNP*, citrate, and isocitrate, but not of gramicidin. Citrate did not affect protamine inhibited respiration in the absence of phosphate. Oligomycin prevented restoration of the protamine inhibited respiration by citrate and isocitrate, but did not affect the action of DNP. In spite of the presence of oligomycin, the reversing effect of DNP was increased by citrate.

When protamine was used together with gramicidin irreversible inhibition of respiration was observed.

* DNP: 2,4-dinitrophenol.

4. Discussion

The data obtained on citrulline synthesis showed that protamine, in contrast to antimycin or oligomycin, does not prevent formation of the H^+/OH^- potential across the mitochondrial membrane, generated during succinate oxidation or ATP hydrolysis. This conclusion is based on the findings of Graafmans and coworkers [2], that exogenous ATP-dependent citrulline synthesis is inhibited by "high-energy intermediates".

It is possible that protamine, a non penetrant polycation, reacts with the mitochondrial membrane when the inner part of mitochondrion is negatively charged by the action of respiratory chain, thereby preventing backdiffusion of protons and inhibiting other exchange-diffusion carriers. In these conditions, a very

high pH gradient would be built up until the passage of electrons along the respiratory chain becomes inhibited.

When the polarity of membrane is reversed, as in submitochondrial particles obtained by sonication, the direction of proton movement is the opposite to that observed with intact mitochondria [6] and protamine does not affect respiration.

On the basis of these experiments, an explanation of the reversing effect of some proton-conducting systems on protamine-inhibited succinate (+ rotenone) oxidation in intact rat liver mitochondria may be proposed (fig. 1).

It is probable that DNP, citrate, and isocitrate, in the presence of protamine, penetrate the mitochondrion only in the nonionised form (A^-/H^+ symport of Mitchell [6]), thereby conducting the protons into the mitochondrion. These protons react with OH^- ions generated by respiration or by ATP. These "conditionally" accumulated anions may be "fully" accumulated when counter cations are also accumulated or they may leave the mitochondrion again, in unionised forms by the A^-/H^+ symport, after reaction with protons generated by respiration, endogenous ATP hydrolysis, or by both.

Our data suggest that the conditions required for the output of anions from mitochondria are different for the various groups of anions examined. Output of DNP requires protons from respiration or ATP. The

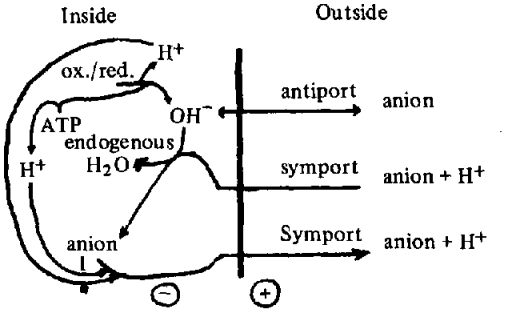

Fig. 1. Proposed mechanism for anion transport into, or accumulation in, mitochondria. Protamine inhibits anion transport by the A^-/OH^- antiport, but some anions may be transport by A^-/H^+ symport. This form of transport is Mg^{2+}-dependent and is decreased when permeability of the mitochondrial membrane to alkali cations is increased.

tricarboxylic anions require protons from ATP and probably respiration. In spite of the presence of oligomycin (table 2, exp. 12) the overall effect of citrate and DNP suggests that DNP can replace ATP as proton donor in the output of citrate from mitochondria.

The fact that the observed effects of anions are related to their ability to pass across the mitochondrial membrane may be seen in experiments 5 and 6 (table 2); these indicate that citrate affects protamine-inhibited respiration only when the conditions required for its passage through the mitochondrial membrane are present [7]. This view is also supported by other experiments not presented here, in which the reversing effect of ketoglutarate (+ NH_4Cl) was inhibited by aspartate; the latter compound is known to be a potent inhibitor of ketoglutarate entry into mitochondria [8].

Experiments with gramicidin support the view that in the presence of protamine a pH gradient across the mitochondrial membrane might be built up. Gramicidin, which is known to stimulate respiration and to uncouple oxidative phosphorylation by increasing alkali cation accumulation [9, 10] did not affect respiration in the presence of protamine. It has been demonstrated recently that addition of valinomycin, which has a gramicidin-like action, to mitochondria increases proton ejection and an apparent shift of internal pH in the alkaline direction is observed [11]. It may be seen from our experiments that when protamine and gramicidin were used together, irreversible inhibition of respiration occurred. This is probably due to a very high alkalisation inside the mitochondria which causes serious damage to the mitochondrial structure. This is visible on electronmicrographs (to be published elsewhere).

The proposed mechanism for anion transport is speculative but it is in accord with the inhibitory effect of anions on the H^+/O quotient [12], and explains the recently observed stimulatory effect of oligomycin and inhibitory effect of DNP on respiration-dependent citrate accumulation [13], as well the inhibitory effect of citrate on K^+ accumulation in liver slices [14].

The experiments presented here fit in reasonably with the chemiosmotic hypothesis of oxidative phosphorylation, and suggest an important role for the intramitochondrial pH in the penetration or accumulation of anions.

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