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REVIEW

Monovalent Cations in Mitochondrial Oxidative Phosphorylation

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Introduction

The purpose of this paper is to review work that relates to the effect of inorganic and organic monovalent cations on oxidative phosphorylation. The extensive research on the transport of cations across the mitochondrial membrane, which undoubtedly effects oxidative phosphorylation, will not be discussed, except in those cases in which it is not clear whether modifications of oxidative phosphorylation are a consequence of monovalent ion transport or of a direct action of a cation on the reactions that lead to ATP formation.

Effect of K^+ on Oxidative Phosphorylation

Pressman and Lardy [1, 2] reported in 1952 and 1955 that aged mitochondria had higher respiratory and phosphorylating rates in mixtures that contained K^+ than in those that contained Na^+, and they concluded that the action of K^+ resided at the level of the phosphorylation reaction. However, Opit and Charnock [3, 4] were unable to confirm the findings of Pressman and Lardy. Although the cause of this discrepancy was not explored, Krall et al. [5], employing an impure mitochondrial preparation from brain, found higher state 3/state 4 ratios in the presence of 3–10 mM K^+ and, in short incubation times, an enhancement of P:O

ratios by K^+. In apparent agreement with these findings, Blond and Whittam [6] concluded that respiration of kidney mitochondria was stimulated by K^+, but their data showed that choline similarly to K^+ also activated respiration. Thus the picture is not clear, and Smith and Beyer [7] reported that in bovine heart mitochondria K^+ induced a small and non-reproducible favorable effect on oxidative phosphorylation.

These controversial reports raise the question as to whether K^+ is indeed involved in oxidative phosphorylation. On the a priori assumption that K^+ is necessary for oxidative phosphorylation, it is likely that in intact mitochondria an effect of added K^+ would be difficult to detect, mainly because the high K^+ content of the mitochondria would maintain the process at a maximal efficiency. Thus an effect of added K^+ would be noted only in mitochondria with a level of K^+ low enough so as to be rate limiting in oxidative phosphorylation. This possibility has been explored.

Gómez-Puyou et al. reported that mitochondria with less than 15 nmoles of K^+ per milligram of protein possess extremely low rates of electron transport and P:O ratios that are increased to a maximum by added K^+ [8]. Interestingly, the effect of K^+ depletion is observed only with NAD-dependent substrates; with succinate as substrate, K^+ depletion does not affect oxidative phosphorylation. However, Judah et al. [9] reported that K^+-depleted mitochondria carry out oxidative phosphorylation at normal rates, but the level of K^+ in their preparation [9] was approximately 40–50 nmoles of K^+ per milligram of protein, much higher than in the preparation of Gómez-Puyou et al. [8].

In K^+-depleted mitochondria, K^+ also enhances the state 3/state 4 ratios with NAD-dependent substrates [19, 11], but this observation is difficult to evaluate since K^+ facilitates the influx of substrates into the mitochondria [12–16]. Therefore, it would seem that a possible direct action of K^+ and other cations on oxidative phosphorylation could be better analyzed in submitochondrial particles. These preparations are vesicles of inverted inner membranes [17–20] which lack permeability barriers to NADH and succinate, as well as to ADP and phosphate.

In contrast to whole mitochondria, a lowering of P:O ratios is the most striking feature of the action of K^+ in submitochondrial particles [21, 22]. These findings might be challenged by Smith and Beyer [7], who reported that K^+ did not affect oxidative phosphorylation, but their data indicate that with NADH, K^+ induces a slight lowering of the P:O ratios. Nevertheless, the experiments on the detrimental action of K^+ on oxidative phosphorylation in submitochondrial particles are controversial. According to Papa et al. [21], with β-hydroxybutyrate the diminution of P:O ratios by K^+ is due to a lowering of the phosphorylation rate, and apparently the effect of K^+ is competitive with ADP. On the other hand,

the uncoupling action of K^+ described by Christiansen et al. [22] with NADH is due to both an increase of the respiratory rate and a lowering of the phosphorylation rate; with succinate, K^+ only induces a decrease of the phosphorylating rate. These results point out the complexities involved in evaluating the action of K^+. As shown below the discrepancies are probably due to two separate, but superimposable, actions of K^+, i.e., one on the electron transport system and another on the ATPase complex.

Effects of K^+ on Electron Transport

In the early experiments of Pressman and Lardy [1, 2], K^+ induced higher rates of respiration in the presence of uncouplers, which would suggest that K^+ acts at the level of the respiratory chain. Also, K^+ is known to stimulate the respiration of brain mitochondria [23]. Along the same line, it is known that during loss of K^+ from mitochondria, the oxidation of certain substrates is inhibited [13, 24]. Moreover, mitochondria depleted of K^+ show low respiratory rates with NAD-dependent substrates (but not with succinate) [24], which can be increased to a maximum by K^+ [10, 11]. These findings would be consistent with the idea that the internal concentration of K^+ controls the rate of respiration, but they could also indicate that K^+ movements across the membrane regulate substrate influx and thus the rate of electron transport [12–16]. Accordingly it would seem that experiments on submitochondrial particles would be easier to interpret.

In phosphorylating and nonphosphorylating systems, the rate of oxidation of NADH, but not of succinate by submitochondrial particles, is importantly enhanced by K^+ [22, 25, 26]. These observations would agree with the reported effect of K^+ in K^+-depleted mitochondria. In the latter K^+ enhances the rate of oxidation of NADH-dependent substrates, whilst the oxidation of succinate is not affected by K^+ depletion or by added K^+ [10, 11].

Therefore, it is concluded that K^+ is necessary for maximal rates of electron transport from NADH to ubiquinone. Its precise site of action is unknown, but it is to be noted that a large number of cations, not only K^+, stimulates electron transport [27]. Accordingly, it is possible that the enhancement of respiration is an effect of ionic strength, but a direct interaction of K^+ or other cations with components of the NADH–ubiquinone segment cannot be excluded. It should be acknowledged that the aerobic oxidation of NADH by rotenone-insensitive submitochondrial particles from *Saccharomyces cerevisiae* which lack site I phosphorylation is also stimulated more than twofold by K^+ [28].

Effect of K^+ on the ATPase Complex

Lardy and Wellman in 1953 [29] showed that the uncoupler-activated ATPase of mitochondria is larger in mixtures that contain K^+ or Na^+. This finding has been confirmed in many laboratories in mitochondria from a wide variety of tissues [30–38]. The question that arises from these observations is whether, in mitochondria, K^+ or other cations influence ATPase activity of intact mitochondria by interacting with the ATPase complex of the mitochondria (for review see [39, 40]) or by facilitating the influx of ATP into the mitochondria. As ATP^{4-} enters the mitochondria in exchange for ADP^{3-}, electrical neutrality could be maintained by influx of a cation, and this would result in stimulation of ATPase activity. Efflux of phosphate cannot charge compensate the ATP–ADP exchange since phosphate movements occur as a neutral exchange of phosphate for OH^-. Moreover, it has been shown that K^+ facilitates the exchange of adenine nucleotides [41, 42]. Interestingly enough, in some mitochondrial preparations electron transport is required for optimal rates of ATPase activity [32, 37, 38, 43–45]. In agreement with these findings, Brierley [46] has shown that K^+ influx in heart mitochondria requires electron transport.

Cereijo-Santaló [35] found that ATPase activity diminishes as K^+ is lost from the mitochondria and concluded that internal cations are critical in the functioning of the ATPase complex. In this respect, K^+ depleted mitochondria exhibit a low uncoupler-stimulated ATPase activity [32] and low rates of ^{32}P–ATP exchange, which are importantly enhanced by promoting the influx of K^+ [38]. These experiments suggest, but do not prove, that K^+ exerts a direct effect on the ATPase complex.

Unfortunately not many reports exist on the effect of cations on the ATPase activity of submitochondrial particles, which theoretically would be more useful to evaluate an action of K^+ on the ATPase complex. Lehninger et al. [47] reported that KCl inhibited the ATPase activity of digitonin particles, but the concentration employed was high, i.e. 0.4 M. It is not known whether this effect is related to the dissociating action of salts on F_1 [48].

More recently, Adolfsen and Moudrianakis [49] found that K^+ stimulates the ATPase activity of the F_1 components of *Alcaligenes faecalis* and heart mitochondria. This stimulating action of K^+ has been confirmed in submitochondrial particles and in the soluble F_1 component from heart mitochondria [50]. The action of K^+ on soluble F_1 is highly specific; Na^+ and Li^+ do not stimulate ATPase activity, while Cs^+ exerts a slight stimulating action [50]. It has not been explored whether this effect of K^+ on F_1 is related to the competitive inhibiting action of K^+ with ADP in

oxidative phosphorylation of submitochondrial particles as described by Papa et al [21].

In the light of this evidence, it may be affirmed that K^+ is involved in the functioning of the ATPase complex. In whole mitochondria, however, many of the reported favorable effects of cations on ATPase activity [30–38] could be related to their action on adenine nucleotide translocation mainly because the action of cations in mitochondria is unspecific, i.e., Na^+ as well as K^+ enhance ATPase activity, whilst the ATPase activity of F_1 and of submitochondrial particles is stimulated by K^+ and not by Na^+ [50].

In conclusion, the available data indicate that potassium ions are directly involved in oxidative phosphorylation. Effects of K^+ are observed both at the level of the respiratory chain in the NADH ubiquinone span, and also at the level of the F_1 component of the ATPase complex, but its role in oxidative phosphorylation is not clear.

Effect of Na^+

The fast decay of proton pulses in mitochondrial mixtures that contained Na^+ was taken as evidence by Mitchell and Moyle [51, 52] for postulating that mitochondria possess a rapid H^+/Na^+ antiport. In addition important ion movements occur when mitochondria are incubated with Na^+, phosphate, oxidizable substrate, and EDTA [53–56], simultaneously there is an enhancement of the respiratory rate and a partial uncoupling of oxidative phosphorylation [57]. Weiner and Lardy [58] studied the spectroscopic changes that occur in kidney mitochondria incubated with Na^+ and glutamate-malate. In contrast to the oxidation of pyridine nucleotides that occurs upon the addition of ADP to mitochondria incubated with K^+, ADP induced *reduction* of pyridine nucleotides in mitochondria incubated with Na^+. It has not been ascertained whether this ADP-induced reduction of pyridine nucleotides is a consequence of Na^+ movements across the membrane, of interaction of Na^+ with some component of the respiratory chain, or of loss of K^+ that occurs in the presence of Na^+ [55, 56] and which is known to diminish electron transfer in the NADH–ubiquinone span [10, 11].

The effect of Na^+ described by Weiner and Lardy [58] is difficult to explain since, in submitochondrial particles, Na^+ and K^+ stimulate electron transfer through the NADH–ubiquinone segment to the same extent [22, 27]. The above-mentioned effects of K^+ on oxidative phosphorylation of submitochondrial particles reported by Papa et al. [21] and Christiansen et al. [22] are reproduced almost exactly by Na^+. If the

effect of these two cations is related to oxidative phosphorylation, it may be concluded that K^+, not Na^+, is the active cation, since the concentration of Na^+ in mitochondria is much lower than that required to affect respiration and phosphorylation.

Effect of Organic Cations on Oxidative Phosphorylation

This section will review the effect of organic cations on oxidative phosphorylation. It includes the action of guanidines, since at the pH in which most studies on mitochondria are conducted, guanidines exist mainly as positive charged ions. Moreover, there is important evidence that indicates that guanidines interfere with cation-dependent processes of a wide variety of biological systems [59–67]. In addition, some lipophilic cations such as tetraalkylammonium salts modify mitochondrial functions similarly to alkylguanidines [68].

Hollunger [69] was the first to show that guanidines inhibited the ADP-stimulated oxygen uptake. Pressman [70] found that their effectiveness was proportional to the length of their alkyl chain; he also showed that the octylguanidine-induced inhibition of state 3 respiration was gradually reversed by 2,4-dinitrophenol and that it did not inhibit the 2,4-dinitrophenol-stimulated ATPase activity. Simultaneously Chappell [71] studied the action of galegine (4-methyl-3-butenylguanidine), and by monitoring redox changes of pyridine nucleotides he concluded that galegine (and hexylguanidine) inhibited the phosphorylation that occurs in the NADH–cytochrome-*b* span. One of the most interesting actions of alkylguanidines is that they inhibit the coupled oxidation of NAD-dependent substrates, but not that of succinate [70–73]. A higher sensitivity of site I phosphorylation has been shown also for tetrabutylammonium [68] and for diphenyliodonium [74]. Recently, however, it was reported that octylguanidine does inhibit the coupled oxidation of succinate, but at concentrations significantly higher than those which inhibit site I phosphorylation [75].

Diphenyliodonium exerts some interesting effects on mitochondria. This compound catalyzes a OH^-/Cl^- antiport [76]. In the presence of Cl^-, it inhibits the oxidation of succinate and of NAD-dependent substrates, but in the absence of Cl^- only the oxidation of NAD-dependent substrates is prevented [74]. In submitochondrial particles, diphenyliodonium inhibits the aerobic oxidation of NADH, but not that of succinate [74]. Therefore, it would seem that diphenyliodonium, similarly to octylguanidine [27], inhibits electron transport in the NADH–ubiquinone segment, but this does not explain why in the presence of Cl^-, diphenyliodonium inhibits the coupled oxidation of succinate in

mitochondria [74, 76]. Since ΔpH controls the uptake of anions [77], it is proposed that as diphenyliodonium induces a Cl^-_{out}/OH^-_{in} antiport, a diminution of ΔpH across the membrane would result in inhibition of succinate influx and respiratory activity.

The effects of biguanidines on mitochondria have also been explored extensively. Falcone et al. [78] reported that phenethylbiguanide inhibited the oxidation of glutamate, β-hydroxybutyrate, and succinate, as well as the $^{32}P_i$–ATP and the P_i–H_2O^{18} exchange reactions. Spectroscopic studies on cytochrome *b* indicate that phenethylbiguanide acts mainly on site II phosphorylation [79]. Some controversy exists, however, as to the effect of phenethylbiguanide on site I. Schafer found inhibition of NAD-linked respiration by phenethylbiguanide [80], but Haas reported that it does not affect the phosphorylation that accompanies NADH oxidation by fumarate [73].

The effect of aliphatic biguanides on oxidative phosphorylation is different from that of phenethylbiguanide; they inhibit the coupled oxidation of glutamate at concentrations that induce uncoupling in the presence of succinate [81].

Another interesting guanidine derivative is decamethylene diguanidine (Synthalin). It inhibits the coupled respiration of heart mitochondria from monkeys [63], as well as the phosphorylation of ADP supported by ascorbate + TMPD in liver mitochondria without affecting significantly the respiratory rate [72]; apparently it is without effect on site I phosphorylation [72]. It is also a powerful inhibitor of the ADP–ATP exchange reaction [72] and of the ATPase activity of yeast mitochondria [82].

One of the most intriguing properties of lipophilic cations is their ability to interfere with one specific phosphorylating site. For most of the "site-specific" agents it is not clear if the preference for a given phosphorylating site is due exclusively to modification of the activity of ATPase complex or to the sum of various independent actions on various components of the inner membrane. This question is of particular importance, because of the implications it might have on the current hypothesis of energy-coupling [83–85].

At least for octylguanidine, the evidence indicates that its preference for inhibiting site I phosphorylation is due to its effect on the NADH–ubiquinone segment and not to its effect on ATPase. According to Schatz and Racker [86], inhibition of electron transfer in the NADH–CoQ span occurs at concentrations of octylguanidine lower than those that inhibit phosphorylation. These concentrations correspond to those that inhibit state 3 respiration with NAD-linked substrates [75]. Moreover octylguanidine at similar concentrations inhibits the ATP-supported NAD reduction by succinate, but not the ATP-supported NADH–NADP

transhydrogenation [87]. Clearly octylguanidine acts on the NADH–CoQ span, most probably by interacting with the locus where K^+ exerts its action [27].

Finally Papa et al. [75] found that inhibition of state 3 respiration *with succinate* occurs at concentrations that inhibit ATPase activity. Thus when octylguanidine modifies the functioning of the ATPase complex, it loses its "site-specific" action. This set of findings would be entirely consistent with the basic concepts of the chemiosmotic hypothesis [85] and it is emphasized that, in the absence of data with other "site-specific" agents, it is difficult to accept that lipophilic cations exert their "site-specific" actions by affecting the ATPase complex.

With respect to the mechanism of action of lipophilic cations, it is recognized that these compounds exert a multiplicity of actions. For instance tetrabutylammonium, at low concentrations, induces H^+ ejection, transient increases in ATPase activity and respiration, and swelling [88], and at higher concentrations it inhibits respiration [68]. Aliphatic biguanides may act either as uncouplers or as inhibitors of oxidative phosphorylation [81] and there is evidence that shows that lipophilic cations must be accumulated by the mitochondria in order to affect oxidative phosphorylation [63, 89]. Schafer et al. [90, 92] have shown that certain guanidine derivatives bind to mitochondrial phospholipids and proposed that the alteration of the surface charge accounts for their action on oxidative phosphorylation. Finally, certain guanidines interact with membrane enzymes such as F_1 [50–93] as well as with nonmembranal enzymes [62]. Thus the effect of a lipophilic cation would depend both on its rate of translocation and/or on its interaction with the hydrophobic phase of the membrane or with some of its protein components. Moreover, experiments in which uncouplers and lipophilic cations have been combined [70] must be taken with caution, since uncouplers may collapse the membrane potential with a resulting release of the lipophilic cation [63, 89]. Accordingly, octylguanidine does not affect the DNP-stimulated ATPase activity of intact mitochondria [70], but it inhibits the ATPase activity of submitochondrial particles [50, 75].

Regardless of the complexity in their mechanism of action, the molecular properties of these cations in particular alkylguanidines are unique to study the molecular events involved in membrane function. Hille varied the structure of the guanidine moiety to probe the structure of the Na^+ channel in nerve [65, 66] and Schaffer and Bojanowski have made a correlation of the action of biguanides with different alkyl chains in bilayers and mitochondria [90]. Also by attaching decamethylene diguanidine to sepharose, the site of action of actylguanidine has been localized to the inner part of the mitochondrial membrane [94]. Therefore, it is conceivable that under strictly controlled conditions, the study of the

interaction of properly labeled guanidines (either with fluorescent or spin labeled groups or by NMR techniques) with mitochondrial membranes would yield considerable insight into the molecular events involved in oxidative phosphorylation.

The Question of the Natural ATPase Inhibitor

In this review, we would also like to call attention to the natural inhibitor of ATPase activity. This peptide was originally isolated by Pullman and Monroy from heart mitochondria; many workers have confirmed their findings [96–98], and Satre et al. isolated it from yeast mitochondria [99]. Even though the amino acid composition of the inhibitor from yeast differs from that of heart [97, 99] both contain an important number of basic aminoacid residues.

The inhibitor diminishes the ATPase activity of submitochondrial particles and soluble F_1 [95, 96], as well as all the ATP-driven processes of submitochondrial particles [100], but no effect on the forward reaction has been found. Nevertheless, Asami et al. reported an experiment in which the inhibitor induced a tighter coupling of oxidative phosphorylation in the presence of succinate [100].

The reason for including the inhibitor is because some similarities exist between octylguanidine and the inhibitor [50, 93], i.e., inhibition of ATPase activity of soluble F_1 and submitochondrial particles and protection of F_1 against cold-inactivation. Thus it is probable that octylguanidine mimicks the action of the inhibitor, which on this basis probably acts through its cationic groups.

It is interesting that the binding of the inhibitor to F_1 is maximal with $ATP + Mg^{2+}$ and significantly impaired at high ADP/ATP ratios [101]. By the same token the action of K^+ on the ATPase complex critically depends on the concentration of ADP and ATP [102]. Moreover, according to Papa et al. [21], K^+ decreases the affinity of phosphorylating particles for ADP. These observations together with the finding that K^+ displaces the inhibitor from F_1 [96] suggest that the ADP/ATP ratio, K^+, and the inhibitor may be involved in oxidative phosphorylation through a shuttle of the inhibitor and K^+ to and from F_1. Unfortunately, the available data are not enough to make a more critical evaluation of this possibility.

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