SC 2214

Cation requirements for the formation of adenosine triphosphate from a high-energy intermediate of oxidative phosphorylation*

The requirements for Mg^{2+} and other cations in oxidative phosphorylation in mitochondrial fragments[1-3] and in bacterial particles[4,5] have been reported previously. Metal ions have been implicated in the binding of coupling factors to oxidizing systems[2,3,5] and in activation of exchange reactions as well as in the promotion of coupled phosphorylation[1,4]. The isolation of a high-energy intermediate which combines with P_i and ADP to form ATP permits a study of the metal requirements for the final steps in the phosphorylation reaction (Fig. 1, Reactions 2 and 4).

The high-energy intermediate from the first phosphorylation site, *i.e.*, the phosphorylation from the DPN–protein high-energy intermediate DPN~E (see Fig. 1)[6,7], was prepared from *Alcaligenes faecalis* particles by incubation with DPNH in the absence of P_i and ADP as previously described[8]. After centrifugation, the supernatant solution (S_{DPNH}) containing the intermediate was dialysed overnight against several changes of 0.5 mM EDTA to remove free pyridine nucleotides and metal ions. The S_{DPNH} was then tested for phosphorylation in the presence of ADP and P_i and varying concentrations of cations using $^{32}P_i$ incorporation into the organic fraction as previously described[5]. Protein was determined by SUSKIND's modification[9] of the LOWRY[10] procedure. The efficacies of various cations in the reaction were then compared at their optimal concentrations with the results shown in Table I. Magnesium appears to activate phosphorylation quite specifically.

TABLE I

EFFECT OF CATIONS ON PHOSPHATE UPTAKE IN THE PRESENCE OF S_{DPNH} ASSAYED BY $^{32}P_i$ INCORPORATION

$2.5 \cdot 10^{-2}$ M glycylglycine buffer (pH 7.4), 0.5 mM potassium phosphate buffer (pH 7.4), $2.5 \cdot 10^5$ counts/min $^{32}P_i$, 0.3 mM ADP, $2.5 \cdot 10^{-2}$ M glucose, and metal chlorides as indicated. 0.3 mg S_{DPNH} protein in a total volume of 1 ml incubated 20 min at 24°.

Cation and optimum concn.	*Specific activity (mμmoles/mg protein)*	*Mg^{2+} activity (%)*
None	10.4	6.0
8 mM Mg^{2+}	174.0	100.0
3 mM Mn^{2+}	101.0	58.0
2 mM Co^{2+}	12.1	7.0
2 mM K^+	18.0	10.4
4 mM Ba^{2+}	14.5	8.4
2 mM Sr^{2+}	18.3	10.5
2 mM Ca^{2+}	7.2	4.2
1 mM Zn^{2+}	5.2	3.0
1 mM Fe^{3+}	2.4	2.0
1 mM Fe^{2+}	10.3	6.0

In order to rule out other reactions, such as $^{32}P_i$–ATP exchange reactions, by which $^{32}P_i$ might be incorporated into the organic fraction, ATP was trapped as glucose-6-*P* and assayed spectrophotometrically using TPN^+ and glucose-6-*P* dehydrogenase (EC 1.1.1.49). Since the S_{DPNH} preparations contain adenylate kinase

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(EC 2.7.4.3) which is also activated by Mg^{2+} (ref. 11), the contribution of this enzyme to ATP formation was estimated by running the assay in the presence of arsenate instead of phosphate. All the ATP formed in the presence of arsenate under phosphate-less conditions should come from the action of adenylate kinase[7]. Since at pH 7.4 turbidity of the reaction mixture interfered with the measurements of absorbancy,

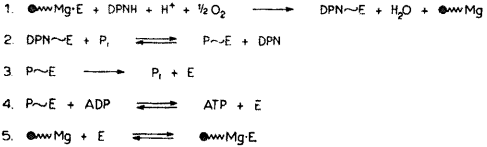

Fig. 1. Proposed mechanism of oxidative phosphorylation in *Alcaligenes faecalis*[7]. In Reaction 1, phosphorylating particles, consisting of a particulate oxidase (●), Mg^{2+}, polynucleotide (〰)[5,12] and a coupling protein (E), oxidize DPNH, forming a high-energy intermediate (DPN~E) and incompetent (*i.e.* non-phosphorylating) particles. Reactions 2 and 4 are the ATP-forming steps studied here. Reaction 3 accounts for ATPase activity, and in Reaction 5 the coupling protein is reassociated with the incompetent particles, completing the microcycle of coupled phosphorylation[7].

TABLE II

EFFECT OF CATIONS ON ATP FORMATION FROM S_{DPNH} MEASURED BY GLUCOSE-6-*P* ASSAY

pH of buffers 7.0, other conditions same as in Table I except for the omission of $^{32}P_i$, inclusion of 0.4 enzyme unit of hexokinase (EC 2.7.1.1), and substitution of arsenate for phosphate as indicated. After incubation 0.8 μmoles of TPN^+ and 1 enzyme unit of glucose 6-*P* dehydrogenase were added. TPNH was measured spectrophotometrically when a constant absorbancy at 340 mμ was reached.

Cation addition	*Specific activity (mμmoles ATP/mg protein)*	
	0.5 mM phosphate	*0.5 mM arsenate*
None	0	0
8 mM Mg^{2+}	100.0	14.3
3 mM Mn^{2+}	38.4	0

a pH of 7.0 was used for these assays. At this pH the solutions were quite clear and the enzymes used in assaying ATP were not activated by Mg^{2+} or inhibited by arsenate to any great extent. The results of these experiments are shown in Table II, from which it can be seen that the ATP assay gives close agreement with the $^{32}P_i$ data and that the major part of the ATP formation from S_{DPNH} is arsenate sensitive and thus not due to adenylate kinase. Again Mg^{2+} is far more active than Mn^{2+}, and it is concluded that Mg^{2+} specifically activated the reaction or reactions involved in producing ATP from the non-phosphorylated high-energy intermediate of the first phosphorylation site (DPN~E) (Fig. 1, Reactions 2 and 4). Magnesium therefore has a dual role in oxidative phosphorylation, binding the coupling protein to oxidizing particles[5] and promoting the formation of ATP from the high-energy intermediate as has been shown here.

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Chloroplast storage with retention of photosynthetic activities

Chloroplasts from spinach and other higher plant leaves possess the capacity for light-dependent TPN^+ reduction and ATP synthesis. While market spinach provides an easily prepared chloroplast fraction of relatively constant specific activities in regard to the Hill reaction and photophosphorylation, occasionaly variations are encountered. Further, when chloroplasts are subjected to various treatments designed to extract active components, the degree of extraction and subsequent restoration may prove quite variable. Thus it seemed desirable to devise a method whereby large quantities of chloroplasts of uniform activity might be stored without loss of activity.

Chloroplasts were prepared from market spinach according to the procedure of AVRON *et al.*[1]. Chlorophyll was determined by the method of ARNON[2]. Photophosphorylation activity was measured under the circumstances described by AVRON AND JAGENDORF[3] and the Hill reaction, using 2,3',6-trichlorophenolindophenol as oxidant, was measured as described by JAGENDORF[4].

It was immediately apparent that rapid freezing and thawing are necessary to avoid loss in chloroplast activities. Furthermore, resuspension of chloroplasts in 0.4 M sucrose solution prior to freezing proved superior to the NaCl and dextrin solutions occasionally used to maintain tonicity. To accomplish rapid freezing, 1 ml of a chloroplast suspension containing 1 mg chlorophyll is pipetted into a 2-dram screw-cap vial and the vial mounted vertically on the shaft of a high-speed stirring motor. Adhesive tape wrapped around the shaft of the motor provides an adequate friction fitting to hold the vial in place. When the motor is turned on, the chloroplast suspension is driven against the wall of the vial to give a thin film containing little or no trapped gas. A beaker of acetone containing dry ice is raised so as to immerse the spinning vial and quickly freeze its contents. The vial is then capped and stored in a liquid-nitrogen deep freeze (obtained from the Linde Corporation of New York, New York). Care must be taken to use a tightly fitting cap lest liquid nitrogen leak into the sample and explode the vial on subsequent warming. On thawing the chloroplast sample, the vial is remounted on the stirring motor, and, while rotating at top speed, is immersed several times in a beaker of water at 35° for 5-sec intervals until thawed.