ATP SYNTHESIS DRIVEN BY A K^+-VALINOMYCIN-INDUCED CHARGE IMBALANCE ACROSS CHLOROPLAST GRANA MEMBRANES*

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1. Introduction

Recent reports of the stimulation and inhibition of ATP synthesis by the imposition of K^+ ion gradients across the grana membranes of valinomycin-treated chloroplasts [1–3] have strengthened the concept that a membrane potential may be a component of the energy conservation system of this organelle.

The experiments of Uribe [1] and Uribe and Li [3] have shown that the imposition of a K^+ gradient across valinomycin-treated grana fragment membranes simultaneous with a pH transition causes a directional modulation of the membrane-dependent acid-induced ATP synthesis. These experiments also indicate that ATP synthesis could be achieved when a K^+ gradient from the outside/in was supplied simultaneously with a proton gradient of a magnitude insufficient to cause ATP synthesis. These effects on energy conversion were postulated to be due to transient membrane potentials generated by the separation of positive and negative charges by valinomycin-facilitated transport of K^+ along a concentration gradient. Schuldiner et al. [2] have independently described the stimulation of ATP synthesis in the acid–base system and also extended the observations to a K^+ gradient stimulation of post-illumination ATP synthesis under conditions where the light-generated proton gradient (X_e) was of an insufficient magnitude to drive to ATP synthesis. The experiments described above have shown that the imposition of an ionic imbalance which should lead to a transient positive internal polarity stimulates ATP synthesis. This membrane-potential-driven reaction is presumably coupled to an efflux of protons which may contribute to the energy input into the system.

This paper reports experiments which describe a system in which ATP synthesis by chloroplast grana fragments is driven solely by employing a charge imbalance (membrane potential) generated by valinomycin-facilitated potassium movement across the grana membrane in chloroplast fragments prepared by osmotic shock.

2. Methods

Spinach chloroplast fragments with a high volume to surface ratio were prepared by osmotic shock as previously described [4] by washing whole chloroplasts in a medium containing impermeant osmotic supports such as 10 mM sodium succinate and 0.1 mM tricine pH 8.0 or 10 mM sucrose containing 1 mM $MgCl_2$ and 0.1 mM sodium tricine at pH 8.0. The washed chloroplasts were resuspended in the above solutions for use in the experiments described herein. These chloroplast suspensions had a pH of 7.5.

Reactions were carried out in semi-darkness at 0°C in a two stage protocol which included: A) A preincubation of 0.5 ml chloroplast fragments containing 200 μg of chlorophyll with 200 nmoles valinomycin for 15 sec; B) Rapid transfer by syringe into a phosphorylation stage of 1.3 ml volume which contained the following components in μmoles: sodium tricine, pH 8.2, 100; DCMU, 0.027; magnesium succinate, 5; Na_2HPO_4, 2; NaADP, 0.2; NaOH, 10 and $1-5 \times 10^6$ cpm ^{32}P [P_i]. In certain experiments 5 μmoles $MgCl_2$

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and 0.5 μmoles Na_2HPO_4 were substituted. The potassium gradients (outside/in) were generated by including an appropriate potassium salt (usually potassium succinate) in the phosphorylation stage of the reaction at a final concentration of 28 mM unless otherwise noted. Dinitrophenol (DNP) was added to the phosphorylation stage where indicated at a concentration of 0.26 mM. The chloroplast fragments were allowed to remain in the phosphorylation stage for 15 sec; then the reaction was stopped by making it up to 2% in trichloroacetic acid. ATP synthesis was measured by the method of Avron [5]. Chlorophyll was determined as described by Arnon [6]. Valinomycin and dinitrophenol were purchased from the California Corporation for Biochemical Research.

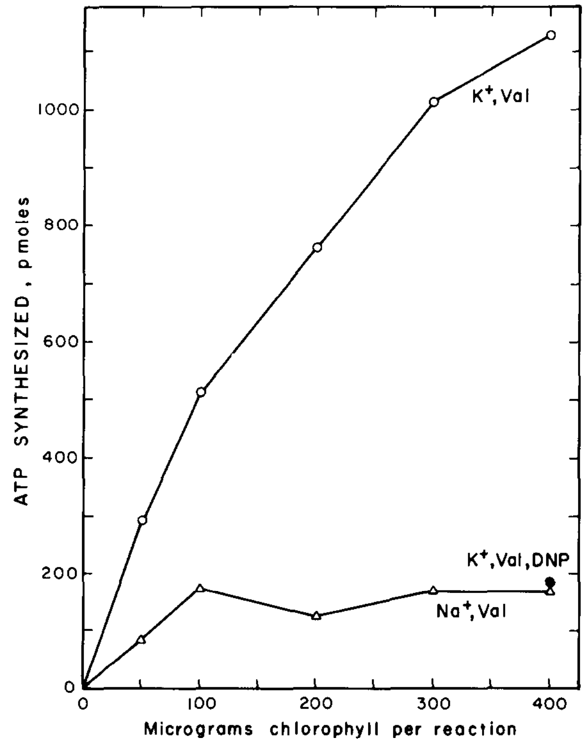

Fig. 1. Effect of chloroplast concentration on ATP synthesis. Reactions were run as described in Methods using sodium-succinate-washed fragments in increasing concentration and constant valinomycin:chlorophyll ratio.

3. Results and discussion

3.1. *Effect of chlorophyll concentration*

The generation of valinomycin-facilitated charge imbalance should be most favored if the anionic species present in the reaction are relatively impermeant. Permeability studies by Uribe and Jagendorf [7] and Schuldiner and Avron [8] have shown that chloroplast grana membranes are not readily permeable to certain ionic species. The most impermeable being zwitterions, dianions of dicarboxylic organic acids and certain components of the phosphorylation reaction such as ADP and inorganic phosphate. More permeable anions include inorganic species such as I^-, NO_3^-, Cl^- and $SO_4^=$.

Our preliminary experiments had indicated that chloroplast fragments isolated in the low ionic strength solutions of impermeant anions such as the sodium succinate–sodium-tricine medium could synthesize small but reproducible amounts of ATP under the conditions described in Methods. A determination of ATP yield as a function of chlorophyll concentration in the standard reaction (fig. 1) indicates that at a constant chlorophyll to valinomycin ratio one sees increasingly large amounts of ATP synthesized with increasing amounts of chloroplast fragments in the reaction mixture. The synthesis is specific for K^+-valinomycin as replacement of K^+ by Na^+ causes a slight and relatively concentration-insensitive ATP synthesis. It is important that the ATP synthesis by valinomycin-treated chloroplasts is reduced to that provided by Na^+-valinomycin by the addition of DNP to the K^+-valinomycin. The inhibition produced on the addition of DNP to valinomycin-treated fragments in the presence of K^+ is variable (see also tables 1–3); however the specificity and directionality of the inhibition is identical to that reported for the membrane-potential-stimulated acid–base ATP synthis [3]. The increase in yield, due to valinomycin and K^+ addition calculated on a chlorophyll basis, was found to be maximal at approximately 200 μg chlorophyll per reaction; thus all other experiments described in this report employed chloroplast fragments containing chlorophyll in this amount.

3.2. *Dependence of ATP yield on potassium concentration*

Synthesis of ATP in response to a membrane

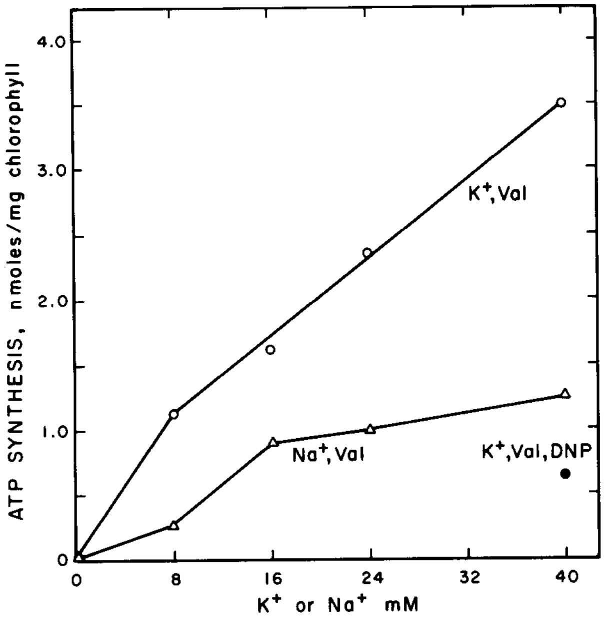

Fig. 2. Concentration dependence and specificity of K$^+$-gradient ATP synthesis. Reactions were run as described in Methods using sodium-succinate-washed fragments.

potential should be related to the magnitude of the transmembrane ionic imbalance generated and this, in turn, should be dependent on the magnitude of the gradient imposed. The data of fig. 2 indicate that the amount of ATP formed in this system is increased in a linear manner as the potassium succinate concentration is increased from 0–40 mM. Higher K$^+$ concentrations were found to result in a decreased yield possibly due to osmotic effects resulting in decreased internal volume. This was supported by the results of experiments with chloroplast grana isolated in isotonic media. These intact grana failed to synthesize ATP under the standard assay condition indicating a requirement for an internal volume into which K$^+$ can enter along its concentrations gradient. A similar requirement has been described for ATP synthesis driven by a proton gradient in spinach chloroplast fragments [9]. Replacement of potassium succinate with the sodium salt results in a slight synthesis of ATP which is not concentration-dependent and not affected by the addition of DNP. Again, the synthesis of ATP is inhibited in the presence of valinomycin, DNP and potassium, as has been demonstrated for light-driven [10] and acid-induced ATP synthesis [3].

Table 1
Cofactor requirements for K$^+$-gradient ATP synthesis.

Reaction components	ATP synthesis (pmoles/mg chlor.)
Complete	1600
$-K^+$	80
$-P_i$	0
$-ADP$	0
$-Mg^{2+}$	300
Complete +DNP	0

Reactions were carried out as described in the Methods section using sodium-succinate-washed fragments.

3.3. *Cofactor requirements*

It was important to establish the cofactor requirements of ATP synthesis driven by a membrane potential. The results of the experiment of table 1 show that the system has a requirement for ADP, P_i and Mg^{2+}. The synthesis of ATP is completely abolished by the omission of ADP or P_i; omission of magnesium leaves a residual yield of approximately 18% which was repeatedly found. This ATP synthesis may represent an activity supported by enzyme bound Mg^{2+} or residual magnesium ion not removed in the washing process. A similar low yield was seen on omission of K$^+$ ion.

3.4. *Effect of uncouplers and membrane active agents on ATP synthesis*

The response of this system to uncouplers and membrane active treatments was investigated (table 2). The effect on ATP synthesis is similar to that reported

Table 2
Effect of uncouplers on K$^+$-gradient ATP synthesis.

Reaction components and additions	ATP synthesis (pmoles/mg chlor.)
Complete	1300
Complete + 1.1 mM Methylamine	0
Complete + 37 μM *m*-Cl-CCP	0
Complete + 2.7% Triton X-100	0
Complete + DNP	0

Chloroplast fragments prepared by washing in sodium succinate were preincubated for 2 min with the compounds listed and then carried through the standard reaction sequence.

Table 3
Relation of pH of preincubation stage to ATP yield and DNP effect.

Preincubation stage (pH)	Reaction components	ATP synthesis (pmoles/mg chlor.)
7.0	Complete	1419
	Complete + DNP	86
7.5	Complete	638
	Complete + DNP	0
8.0	Complete	519
	Complete + DNP	263
8.5	Complete	127
	Complete + DNP	212

Reactions were carried out as described in Methods using sucrose-washed chloroplast fragments. Fragments were preincubated with 5 mM tricine at each pH indicated prior to initiation of the standard reaction sequence which contained 5 μmoles $MgCl_2$ and 0.5 μmoles Na_2HPO_4 in the phosphorylation stage which was at pH 8.0.

Table 4
Influence of counter ion on ATP yield and specificity.

Anionic species	ATP synthesis (pmoles/mg chlor.) Cation present K^+	Na^+
Chloride	4260	356
Nitrate	2200	142
Bicarbonate	1280	925
Succinate	5180	496
Glutamate	2620	566
Phthalate	3080	496

Reactions were carried out as described in Methods using sodium-succinate-washed fragments. Potassium and sodium ions were present at a final concentration of 28 mM.

for other phosphorylating reactions in chloroplasts. Uncouplers such as methylamine and *m*-chlorocarbonyl cyanide phenylhydrazone (*m*-Cl-CCP) are very effective in inhibiting ATP synthesis in this system. Treatment of the chloroplast fragments with a membrane active agent such as the nonionic detergent Triton X-100 is also very effective in abolishing ATP synthesis in this reaction. This was not unexpected as the yield of ATP in this system should depend on the relative permeability of the grana membrane fragments to the anionic and cationic species present in the reaction.

3.5. *Effect of pH differential on ATP yields*

A previous report [2] has indicated that the stimulation of ATP synthesis by K^+ ion gradients leading to the generation of membrane potentials is maximal when applied simultaneously with a suboptimal proton gradient; however, the magnitude of stimulation of ATP synthesis achieved in the acid–base system was found to be independent of a proton differential between Δ pH values of 4.4 and 2.4 units [3]. This prompted us to re-examine the relation of the magnitude and direction of the proton gradient to the amounts of ATP synthesized on the imposition of a K^+ gradient. The data of table 3 indicate that there is a detactable membrane-potential-driven synthesis of ATP at all pH differentials between 7.5 and 8.2; however the ATP yield decreases as the pH of the preincubation and phosphorylation stages become more equal. When the pH of the preincubation stage is equal to or higher than that of the phosphorylation stage a minimal amount of ATP synthesis occurs. More importantly, the sensitivity to DNP as an inhibitor of ATP synthesis in the presence of valinomycin and K^+ is lost as the pH of the preincubation stage is made equal to or higher than that of the phosphorylating stage. These data support the concept that the K^+ ion gradient generates a membrane potential which works in concert with the H^+ diffusion potential when there is an internal source of protons. This energy-conserving system can be abolished by DNP, whereas the ATP synthesis driven by a membrane potential is not DNP-sensitive.

3.6. *Effect of the counter anion on ATP synthesis*

The yields of ATP realized in a reaction dependent on a membrane potential produced articially or as a result of electron transport will be influenced by the relative permeabilities of the cationic and anionic species used to generate the potential. Thus, an increased resistance of the membrane to permeation by the anionic species in the reaction should be expressed as increased yields of ATP under valinomycin-enhanced permeation of K^+. Indeed, if there is sufficient differential in permeability between the cationic and anionic species then one should be able to achieve a transient membrane potential in the absence of valinomycin-facilitated transport. Some evidence for such an occurrence may be afforded by ATP synthesis in the presence of valinomycin and a Na^+ gradient (figs. 1, 2). The results of table 4 show the effect of various counter anions on ATP synthesis in the K^+-dependent reaction. The greatest yields are obtained with K^+ in combination with the impermeant anion e.g. succinate and phthalate. Somewhat reduced yields are seen in the presence of potassium glutamate, decreased but substantial yields are provided by the potassium salts of nitrate and bicarbonate. It is of interest that a high yield is obtained with potassium chloride in view of the fact that chloride is more permeant than the organic dianions [8, 11]. All the ion pairs tested showed selectivity for potassium in the presence of valinomycin and a sensitivity to the addition of DNP in the standard assay. These data indicate that the yields of ATP realized are affected by the nature of the anionic species in a manner which seems to be not entirely related to permeability properties of the anions as reported in the literature. This may be due to the presence of sufficient anionic contaminants in the chloroplast fragment preparations to affect the expected results. Another possibility is that the reflection coefficients of the phosphorylating reagents themselves may exert an effect on the membrane potential developed. These data also show that slight yields of ATP may be generated by a sodium gradient in the presence of valinomycin. This strongly supports the concept that the synthesis of ATP depends on charge separation irrespective of the nature of the ionic species. Indeed, we have observed ATP synthesis in response to a sodium succinate gradient alone indicating that differential anion and cation permeabilities independent of valinomycin may provide a sufficient ionic imbalance to lead to the synthesis of small amounts of ATP. The properties of this type of ATP synthesis are currently under investigation.

These experiments suggest that the synthesis of ATP by spinach grana fragments can be driven by an artificially produced ion imbalance (membrane potential) in the absence of a hydrogen ion gradient. The synthesis of ATP in this system is increased by the presence of internal protons which presumably contribute to the energy input as their electrochemical gradient is enhanced by the applied membrane potential. Under these conditions the system is sensitive to DNP while the synthesis of ATP driven by a membrane potential in the absence of a proton gradient is unaffected by the addition of this compound. While the data are consistent with the synthesis of ATP solely as a result of an applied diffusion potential they cannot exclude the possibility that the synthesis may be due, in part, to an increase in the electrochemical gradient of protons bound to internal membrane components with pK's in the range of pH used in the phosphorylation stage of these reactions.

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