Stimulation and Inhibition of Membrane-Dependent ATP Synthesis in Chloroplasts by Artificially Induced K⁺ Gradients*

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Abstract

The imposition of a potassium ion gradient simultaneous with a pH transition of Valinomycin treated spinach chloroplasts results in a modulation of ATP synthesis elicited by the acid-base transition. A gradient from the outside to the interior of the granum results in a stimulation of ATP synthesis. Under these conditions the synthesis of ATP is sensitive to the addition of dinitrophenol. A gradient of the opposite sense results in an inhibition of ATP synthesis and the yield is not further reduced by the addition of dinitrophenol. The data support the concept that ion imbalance caused by Valinomycin facilitated K^+ movement across the energy conserving membrane of the chloroplast can lead to membrane potential differences of sufficient magnitude to influence the yield of ATP in a membrane-dependent energy conserving system.

Introduction

The imposition of a proton gradient across chloroplast grana membranes was shown by Jagendorf and Uribe [1] to cause the synthesis of ATP in a reaction which was carried out by the same enzyme system functional in light-driven phosphorylation [2]. This synthesis of ATP was shown to

Abbreviations used include: Tricine, tris (hydroxymethyl) methyl glycine; DCMU, dichlorophenyl-1, 1-dimethylurea; DNP, 2,4-dinitrophenol.

be independent of electron transport [3] and required only membranes
capable of maintaining a proton gradient and having a residual
phosphorylation capacity [4]. This demonstration of membrane-
dependent energy conversion has supported the proposed function of a
chemiosmotic mechanism for energy conservation in chloroplasts [5].
More recent formulations by Mitchell [6], and evidence from the work
of Jackson and Crofts [7], Hauska *et al.* [8], Junge [9] and Junge *et
al.* [10] have further implicated ion movement mediated membrane
potentials ($\triangle \psi$) as a component of the free energy required for ATP
synthesis according to the equation $\triangle G = \triangle pH + \triangle \psi$.

The experiments of Jackson and Crofts and Junge *et al.*, have shown
that the imposition of a potassium gradient across chromatophore
membranes or the illumination of chloroplasts with short light flashes
induces absorbance changes in the carotenoid region of the spectrum
which may be related to membrane potentials and ATP synthesis.

Evidence questioning the significance of membrane potentials to the
energy conversion process has recently been presented. Karlish and
Avron [11] have shown that the addition of Valinomycin and K^+ to
chloroplasts carrying out photophosphorylation does not reduce ATP
synthesis under conditions which should abolish membrane potentials.
The experiments of Neumann *et al.* [12] have questioned the validity of
the 515 nm change as a specific indicator of membrane potentials related
to ATP synthesis.

The acid-base ATP synthesis system allows a direct test of the validity
of the above expression for energy input in an energy conversion
reaction which is strictly membrane dependent. The experiments
reported in this communication were undertaken to study the effect of
facilitated K^+ movement in the presence of artificially imposed K^+
gradients on an energy conversion reaction dependent on the capacity of
the grana membrane to serve as an ionic barrier.

Materials and Methods

Chloroplasts were prepared as previously described [1] and washed by
resuspending in a solution containing 15 mM NaCl, 10 mM $MgCl_2$ and
0.1 mM tricine pH 8.0. The chloroplasts were recovered by centrifuga-
tion and resuspended in the above solution. Chlorophyll was measured
by Arnon's method [13].

The kinetics of the interaction of Valinomycin with the phosphory-
lating and proton translocating systems of the chloroplast were deter-
mined by measuring the increase in the pH of illuminated reaction
mixtures in the presence and absence of ADP using a modification of the
method of Nishimura *et al.* [14] in the apparatus described previously
[15]. Acid-base ATP synthesis reactions were carried out as previously

described [1] in a three-stage protocol which involved the following steps:

1. A 15 sec chloroplast preincubation in the presence or absence of Valinomycin.

2. Rapid transferral by means of a syringe to the acid stage of the reaction.

3. Rapid transferral of the acidified chloroplasts by syringe to the base stage of the reaction.

The imposition of a potassium ion gradient simultaneous with the pH gradient was carried out by the addition of potassium salts to the appropriate stage of the protocol. A potassium gradient from the outside/in was imposed by including the appropriate potassium salt in the base stage of the reaction sequence. A gradient from the inside/out was generated by including the potassium salt in the acid stage and then diluting into a double volume of base mix. Preliminary experiments revealed that DNP inhibition of acid-induced ATP synthesis in the presence of K⁺ and Valinomycin was the same if it was included in either the acid or base stages thus it was convenient to routinely include it in the base stage. ATP synthesis was determined by the method of Avron [16].

Tricine was synthesized by Good's method [17]. Valinomycin was purchased from Calbiochem. Corporation. All other reagents were of the purest available commercial grades.

Results

Interaction of Valinomycin with the Energy Conserving Systems

The experiments of Karlish and Avron have demonstrated that Valinomycin and dinitrophenol when added separately to chloroplasts are without effect on phosphorylation or the proton pump in the presence of potassium. When they are added together with K⁺ both aspects of energy conversion are inhibited [18]. The inhibition was attributed to a K⁺/H⁺ exchange which collapsed the proton gradient and resulted in uncoupling of energy transfer. These experiments had not, however, indicated the kinetic parameters of the Valinomycin-chloroplast interaction which alters energy conversion.

The experiment of Fig. 1 was designed to test simultaneously the effect of Valinomycin interaction on both phosphorylating and nonphosphorylating energy conversion. The data confirm the specificity of the chloroplast system for uncoupling. Inhibition of energy conversion is observed only in the presence of DNP, K⁺ and Valinomycin (a, b, c). More importantly, note the instantaneous inhibition of both ATP synthesis and proton uptake on addition of Valinomycin to reactions
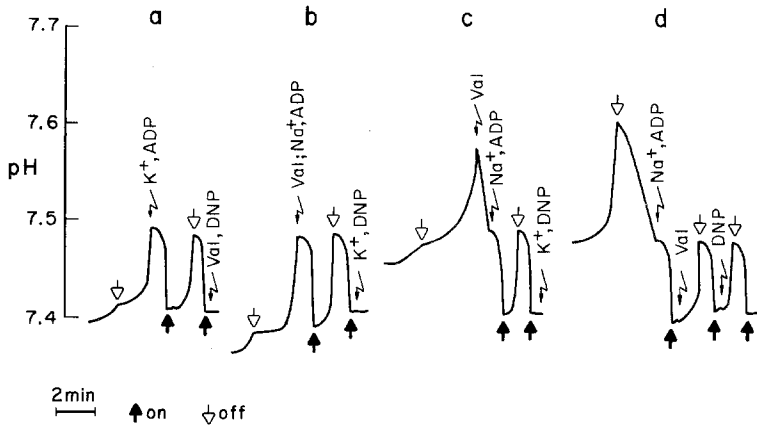
Figure 1. Interaction of Valinomycin with the phosphorylating and proton translocating systems. The reaction mixtures contained following components in μmoles in a volume of 5.0 ml: sodium tricine, pH 7.4, 0.10; NaCl, 67; Na_2HPO_4, 2.0; pyocyanine, 0.125 and chloroplasts containing 300 μg of chlorophyll. Sodium and potassium ADP were added as 0.05 ml aliquots containing 0.025 μmoles ADP and 9.0 μmoles of the cation. Aliquots of solutions containing 1.92 μmoles of DNP and 60 m μmoles of Valinomycin were added where indicated.

containing DNP and K^+ (b, c). The same result is achieved on adding K^+ to the system in the presence of Valinomycin and DNP (a). These data show that the interaction of Valinomycin with the energy conserving system is virtually instantaneous and that the K^+ movement responsible for the uncoupling of both phosphorylating and nonphosphorylating energy conversion in this system is probably from outside the chloroplast grana membrane to the inside.

Stimulation and Inhibition of Acid-base ATP Synthesis by a K^+ Gradient

The above results outlined the approach required to test the effect of artificially generated membrane potentials on acid-base ATP synthesis. Valinomycin facilitated potassium ion movement in the presence of a KCl gradient was used to impose a charge imbalance simultaneous with an acid-base transition resulting in ATP synthesis. Previous experiments had shown that the ATP synthesis reaction in this system has a half time of about 1-2 sec after the pH transition [1, 19]; thus a very rapid interaction of the ionophore with the grana membranes was essential to the experiments. It was observed that the inclusion of Valinomycin in the acid stage (pH 4.0) of the reaction was not effective in inhibiting ATP synthesis in the presence of K^+ and DNP indicating a lack of interaction at the acidic pH. When Valinomycin was included in the base

stage (pH 8.4) its effectiveness in inhibiting ATP synthesis was severely reduced as well perhaps due to insufficient time to bind and exert an effect during the brief period of proton flux and ATP synthesis. The protocol described in the methods section allowed an experimental test of the effect of Valinomycin and K^+ on the acid-base system.

In order to study the possible contribution of a membrane potential to the synthesis of ATP it was necessary to determine the effect of Valinomycin and DNP on the acid-base ATP synthesis in the routine assay. Concentration curves for the effect of these chemicals on ATP synthesis in the acid-base system showed that Valinomycin premixed with the chloroplasts as described had a variable effect. Its addition to the standard reaction mixture caused a variable reduction of ATP yield. Dinitrophenol had no effect on ATP synthesis when added alone or in the presence of Valinomycin. The acid-base system as noted in Methods is inhibited by DNP, Valinomycin and K^+ as has been shown for light-driven phosphorylation.

The initial experiments which were carried out to determine the effect of Valinomycin, DNP and K^+ on acid-base ATP synthesis had shown that the presence of K^+ in the base stage caused an increase in the amount of ATP synthesized by Valinomycin treated chloroplasts. The Valinomycin and K^+ concentration dependence of this stimulation is shown in Fig. 2. The addition of Valinomycin alone results in a decrease in the amount of ATP synthesized. ATP synthesis in the presence of Valinomycin is stimulated by the addition of K^+ to the base stage to a maximal concentration of 11-22 mM. The sensitivity of ATP synthesis in this system to the addition of DNP and K^+ is also illustrated. The presence of K^+ in the absence of Valinomycin was in some experiments found to be very slightly stimulatory to ATP synthesis as well (E. Uribe, unpublished). These data indicate that the ionic effects which result in increased yields of ATP require Valinomycin and K^+ and can be dissipated by the addition of DNP in the presence of K^+ and the ionophore.

The data of Fig. 2 indicated that an artificially imposed K^+ gradient from the *outside/in* can increase the ATP synthesis realized in an acid-base transition. It was important to know if the system showed a specificity and directionality with regard to the ionic effect. The experiments of Fig. 3 were carried out to test these points. The specificity is illustrated in insets C and D. Inset C repeats the data of Fig. 2 for comparative purposes. Note that Na^+ and Valinomycin (inset D), in contrast to K^+ and Valinomycin (inset C), are ineffective in increasing the ATP yield. The addition of DNP is also without effect on ATP synthesis when Na^+ is present. When the ion gradient is of the opposite sense (insets A, B) there is again a specific effect of Valino-mycin and potassium. An ionic gradient from the *inside/out* produces a K^+ specific inhibition of ATP synthesis which is not further enhanced by
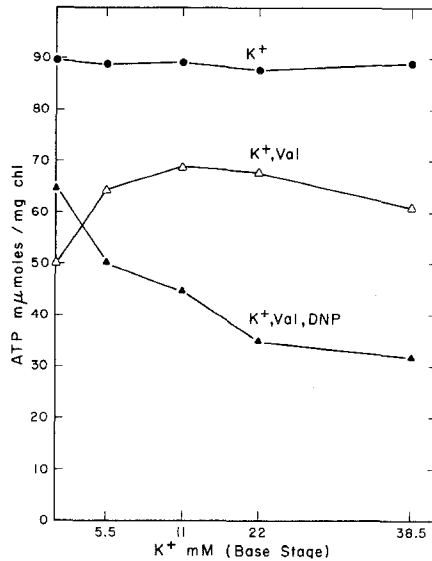
Figure 2. The stimulation of ATP synthesis by a potassium gradient. Chloroplasts containing 200 μg of chlorophyll were preincubated with either 0.01 ml ethanol or 0.01 ml ethanol containing 200 m μmoles Valinomycin for 15 sec. They were then transferred with a syringe to an acid stage mixture containing the following components in a volume of 0.4 ml: sodium succinate, pH 4.0; 10 μmoles and DCMU, 0.027 μmoles. The chloroplasts remained in the acid mixture for 15 sec and were then transferred by syringe to a base mixture of volume 0.9 ml which contained the following components in μmoles: sodium tricine, pH 8.7, 100; Na_2HPO_4, 2; $MgCl_2$, 5; NaADP, 0.2; NaOH, 50 and $^{32}P(Pi)$ containing approximately 5 x 10^5-10^6 cpm. After 15 sec the reaction was stopped by the addition of TCA. 0.48 μmoles of DNP was included in the base stage of the reaction where indicated. The potassium gradient from the outside/in was generated by including KCl in the base stage of the reaction sequence.

the presence of DNP (inset A). These results indicate that the stimulation and inhibition of ATP synthesis is dependent on the presence of Valinomycin and K^+ and that inhibition of ATP synthesis in the presence of DNP, Valinomycin and K^+ is potassium specific and depends on K^+ movement from the exterior medium to the interior of the chloroplast granum.

Stimulation of ATP Synthesis as a Function of $\triangle pH$

The data shown in Fig. 2 indicated that the stimulation of ATP synthesis by Valinomycin and K^+ could be observed with a $\triangle$ pH of 4.4 units. It was important to determine if the stimulation could be demonstrated at all pH differentials of sufficient magnitude to result in measurable ATP synthesis. The experiment of Fig. 4 shows that
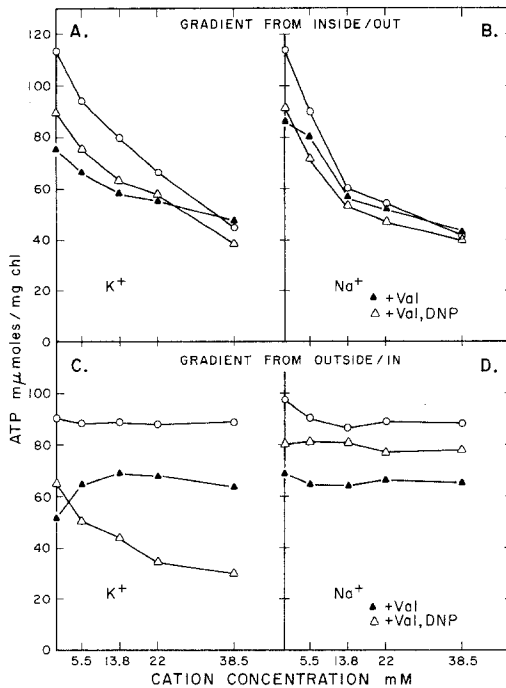
Figure 3. Specificity and directionality of the potassium effect. The protocol used for insets C and D was that described in the legend of Fig. 2 with the K$^+$ and Na$^+$ gradients being generated by the addition of NaCl or KCl to the base stage. K$^+$ and Na$^+$ gradients from the inside/out (insets A and B) were generated by including the ions as the chloride salts in the acid stage and then diluting into a double volume of base mix (1.8 ml) on pH transition. DNP addition was as for Fig. 2. The open circles represent yield obtained as a result of pH transition on the addition of the ion alone.

Valinomycin and K$^+$ stimulate ATP synthesis at all $\triangle$ pH values sufficient to drive ATP synthesis. Sodium ion in the presence of Valinomycin, in contrast, is ineffective in the stimulation of ATP yield at all pH differentials tested.

Response to Nature of K$^+$ Salt Added

The above experiments were all carried out with chloroplasts isolated in a medium containing NaCl and employed with KCl or NaCl as the cation source. The magnitude of the membrane potentials developed will depend on the permeability of the anionic species as well and since chloroplast grana are reported to be permeable to chloride [20], we wished to determine if the stimulation of ATP synthesis of the system
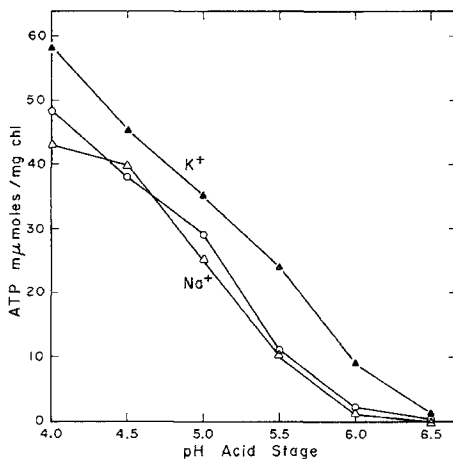
Figure 4. Stimulation of ATP synthesis as a function of $\triangle$ pH. The experiment was carried out as described for Fig. 2 (gradient from outside/in) with the acid stage being at the pH indicated. The pH after mixing the acid and base stages was 8.4. Two hundred μg of chlorophyll and 200 m μmoles of Valinomycin were used for the three curves. The open circles indicate the yield in the absence of added ions.

TABLE I. Effect of K^+ salts on acid-base ATP synthesis

Salt added	Minus Valinomycin	Plus Valinomycin	Plus Valinomycin, DNP
None	63	62	59
KCl	64	74	6
KNO_3	63	72	5
K succinate	68	73	4
$K_2 SO_4$	17	16	1

The experiment was carried out as described for Fig. 2 with the potassium salts being present in the base stage. The salt concentration after mixing the acid and basic stages was 28 mM.

was sensitive to the nature of the anionic species added. The data of Table I show that the synthesis of ATP is stimulated by a variety of potassium salts when inward transport is facilitated by Valinomycin. An exception is $K_2 SO_4$ which causes a reduction of ATP yield. This inhibition is probably due to the presence of sulfate which has been identified by Ryrie and Jagendorf as an inhibitor of photophosphorylation [21].

Discussion

The experiments described herein provide significant information regarding the interaction of membrane ion fluxes with the energy conversion process. A pertinent finding is that both phosphorylating and nonphosphorylating energy conversion by chloroplasts are simultaneously inhibited by potassium ion addition to reactions containing Valinomycin and DNP (Fig. 1). The ionic movement effective in uncoupling is apparently from the outside/in. This is supported by the directionality of the inhibition of ATP synthesis on the acid-base system (Fig. 3C) by K^+ in the presence of DNP and Valinomycin.

These experiments also indicate that the energy input provided by a proton gradient can be modulated by the simultaneous imposition of an ionic gradient in a directional manner. This modulation is dependent on presence of Valinomycin and exhibits a specificity for potassium and a directionality which causes stimulation of ATP synthesis when the K^+ gradient is from the outside/in (Figs. 2 and 3C, D). A gradient of the opposite sense produces a specific inhibition of ATP synthesis (Fig. 3A, B.). The specificity and directionality of the effect on ATP synthesis suggest that the effect is related to a Valinomycin facilitated movement of potassium ions across the grana membranes during the pH transition. This movement of potassium along a concentration gradient is postulated to provide a transient membrane potential which modulates ATP synthesis in a directional manner. The sensitivity of the acid-base ATP synthesis system to DNP, K^+ and Valinomycin is indicative that the energy conversion in the acid-base system is responding in an identical manner to treatments which facilitate K^+ movement as when the system is carrying out light driven phosphorylation and proton uptake (Fig. 1) and that the inhibition in both reactions involves K^+ movement from the suspending medium into the grana. The experiment of Table I indicates that the nature of the anion accompanying is of minor importance when the medium contains a permeant ion such as chloride. The possibility that the counter ion may influence the K^+ effect under conditions where the anion permeability is limiting remains to be determined. Such experiments are now being carried out and will be reported in a separate communication.

The data presented indicate that ionic gradients of appropriate sign and magnitude can modulate energy input in a membrane-dependent system possibly by the generation of membrane potentials. These results are consistent with the possible function of a membrane potential as a component of the energy conservation system of the chloroplast.

A preliminary account of some of the results of the experiments reported herein has been presented [22]. While this manuscript was in press a communication by Schuldiner, Rottenberg and Avron has appeared which describes similar effects in the acid-base reaction [23].

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