Steady-state kinetics of photophosphorylation: Limited access of nucleotides to the active site on the ATP synthetase

Claude Aflalo and Noun Shavit

Department of Biology, Ben-Gurion University of the Negev, 84105 Beer-Sheva, Israel

Received 10 February 1983

Abstract not received

Chloroplast thylakoid *Hypotonic treatment* *Immobilized enzyme* *Occluded nucleotide* *Diffusion control* *Inhibitors of photophosphorylation*

1. INTRODUCTION

The molecular mechanism of ATP synthesis in energy-transducing membranes has been extensively investigated. Studies on the dependence of the steady state rate of phosphorylation on the concentration of substrates, including the effects of energy input [1–4], substrate analogs [5,6], and specific inhibitors [6], have implicitly assumed that the kinetic behavior of the ATP synthetase does not greatly depart from that of a soluble enzyme. Thus, changes in the apparent affinity for substrates were interpreted in terms of enzyme conformational changes or interaction between multiple substrate binding sites on the enzyme. When energy input was lowered by varying the rate of electron transport by chloroplast membranes, the apparent affinity for ADP increased when light intensity was decreased [3,4], while with uncouplers the apparent affinity decreased [4]. These results suggest a rather complex mechanism for the regulation of the ATP synthetase, involving dual modulation of both the catalytic ability (V_{max}) and the affinity for substrates (K_m and/or V_{max}/K_m) [7,8].

Abbreviations: BSA, bovine serum albumin; CF_1, coupling factor 1, the ATP synthetase/hydrolase of chloroplast membranes; EDTA, ethylenediaminetetraacetic acid; Tricine, *N*-tris (hydroxymethyl)methylglycine

We proposed that a free species of newly formed ATP diffuses slowly from a space near the ATP synthetase and is not accessible to hexokinase [9]. Analysis of the kinetic behavior of enzymes immobilized on or within solid supports, as observed from macroscopic measurements, has shown the importance of slow translocation of substrates, products and effectors to or from the site of enzymic reaction [10–12]. Here, we show that the steady state rate of phosphorylation may be limited by mass transfer of nucleotides to and/or from a space surrounding the membrane bound ATP synthetase.

2. MATERIALS AND METHODS

2.1. *Chloroplast thylakoid preparation*

Chloroplast thylakoids were isolated from fresh market lettuce by conventional procedures [9], washed once with 0.4 M sucrose, 1 mM Tricine–NaOH (pH 8.0) and resuspended in the same medium (control thylakoids). Hypotonically treated thylakoids were obtained by adding 100 vol. 0.5 mg/ml bovine serum albumin (BSA), 1 mM Tricine–NaOH (pH 8.0) to 1 vol. once-washed, drained thylakoid pellets. After homogenization and centrifugation at 20000 × *g* for 10 min, the particles were resuspended in 0.1 mgBSA/ml, 1 mM Tricine–NaOH (pH 8.0). Heavy particles (~10–30%) were removed by cen-

Published by Elsevier Science Publishers
00145793/83/0000–0000/$3.00

trifugation at 4000 × *g* for 90 s and discarded; the remaining hypotonically treated thylakoids were collected (20000 × *g*, 10 min) and resuspended in 0.4 M sucrose, 1 mM Tricine–NaOH (pH 8.0). The CF_1 content of both preparations, as estimated by heat-activated Ca^{2+}-ATPase activity [13], was similar. Chlorophyll was estimated as in [14].

2.2 *Phosphorylation assays*

The standard reaction mix contained in 1 ml the following components (μmol); Tricine–NaOH (pH 8.0) 50; sucrose, 140; EDTA–NaOH, 1.5; MgO–Tricine, 6.5; $NaKH^{32}PO_4$, 10 (containing 6–20 × 10^6 cpm); glucose, 20; ADP, 0.005–0.2; phenazine methosulfate, 0.04; BSA, 0.5 mg; hexokinase, 20 units; and thylakoids containing 8–12 μg chlorophyll. The complete reaction mixes were preincubated in the dark for 1 min in a thermostatted (22°C) water bath before illumination with four 300 W lamps (white light at ~0.2 W/cm²). The reactions were terminated in the dark by adding 0.5 ml 10% trichloroacetic acid, and the radioactivity incorporated into the organic phosphate fraction was determined [9]. In experiments in which hexokinase was not saturating (non-linear time course), both the initial and steady state rates of total $^{32}P_i$ incorporation were determined and plotted *vs* initial or steady-state [ADP] (calculated as in [9]), respectively.

3. RESULTS

The properties of thylakoids submitted to a transient hypotonic shock were compared with those of control thylakoids washed in isotonic medium. Their phosphorylating activity was determined, using a hexokinase trap to provide a constant [ADP] under steady state conditions. Fig. 1 shows Lineweaver-Burk plots of the dependence of $^{32}P_i$ incorporation on [P_i] or [ADP] at steady state, with control or treated thylakoids. The hypotonic treatment does not appear to affect the catalytic ability (V_{max}) of the thylakoids (fig. 1A,B) or the apparent affinity for P_i (fig. 1A). However, it causes a significant increase in their affinity for ADP (fig. 1B), as indicated by a decrease in the apparent K_m and increase in the limiting pseudo second-order rate constant (V_{max}/K_m). This conclusion is supported by the pseudo first-order

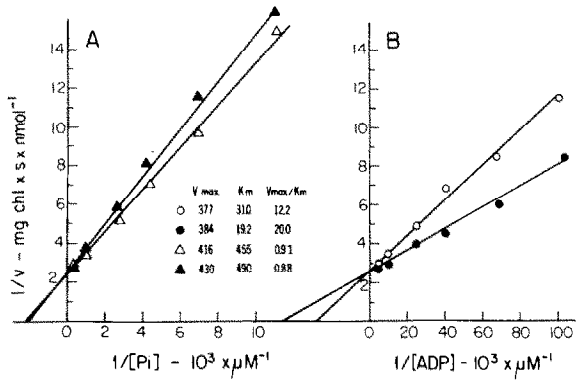

Fig. 1. Dependence of phosphorylation on [P_i] and [ADP] with control and hypotonically treated thylakoids. Experimental conditions as in section 2. Control (△,○) and treated thylakoids (▲,●) contained 9 μg chl. (A) [ADP], 0.5 mM; (B) [P_i], 10 mM. The calculated parameters are also given.

kinetics of the phosphorylation of low amounts of ADP with both preparations in the absence of hexokinase (not shown). The increase in the apparent affinity for ADP in hypotonically treated thylakoids was observed in ~30 expt, done with thylakoids prepared and assayed in different media, although sometimes we also observed a lower ~10–40%) catalytic ability. This may have been due to damage of the membranes during hypotonic treatment.

AT low [ADP], the velocity of P_i incorportation in the presence of hexokinase might also be determined by the capacity of hexokinase to steadily resupply ADP to the phosphorylating system. With treated thylakoids, at low [ADP] and saturating light intensity, the supply of ADP by the hexokinase reaction is strongly limiting (fig. 2). In contrast, with control thylakoids, the phosphorylation rate is lower and hexokinase is much less limiting (saturation is reached at a lower enzyme concentration). Since we have no reason to assume that the activity of hexokinase is different with either thylakoid preparation, these results suggest that the supply of newly formed ATP to the hexokinase present in the bulk medium is more efficient with hypotonically treated thylakoids. Thus, the hypotonic treatment seems to remove a kinetic diffusion barrier for ATP.

The effect of light intensity on phosphorylation as a function of [ADP] is given in fig. 3. With hypotonically treated thylakoids a family of

straight lines with similar slopes is obtained, which indicates that the rate-limiting step is indepenent of light intensity. However, with control thylakoids, we observe a non-linear dependence on [ADP] showing that light intensity limits the reaction rate only at high [ADP]. In the inset to fig. 3, we compare the normalized phosphorylation velocities with both preparations at two light intensities. At low light intensity and high [ADP], the reaction in both preparations shows the same dependence on [ADP]. However, at high light intensity or at low [ADP], the reaction in control thylakoids appears to depend more on [ADP]. Similar results were also obtained when thylakoids were prepared and assayed at relatively high ionic strength.

The rate of phosphorylation was also controlled with uncouplers (fig. 4). The presence of nigericin and K^+ decreases both the apparent V_{max} and K_m in control and hypotonically treated thylakoids. However, with the treated thylakoids the apparent K_m is much less affected (fig. 4B and inset). We obtained similar results with ammonium chloride and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

At saturating light intensity and [P_i], the phosphorylation rate at low [ADP] was consistently higher with treated than with control thylakoids. Thus, we compared the degree of stimulation by hypotonic treatment, at low and high [ADP], with the catalytic ability when the rate of phosphorylation was controlled by varying the light intensity or [P_i], or by adding phlorizin, an energy transfer inhibitor (fig. 5). At high [ADP] the rate is not

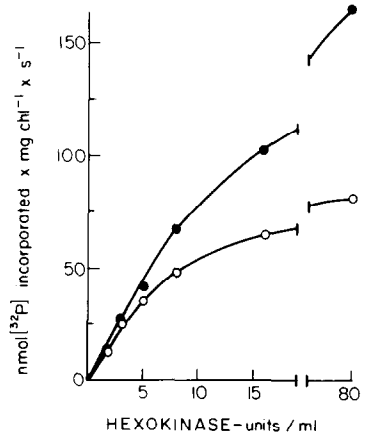

Fig. 2. Effect of hexokinase on the steady state rate of phosphorylation at low [ADP]. The values given for the linear rates obtained betweeen 5–35 s of illumination and at 18 μM ADP. Control (○) or treated (●) thylakoids contained 94 μg chl. Control experiments showed that light intensity and [P_i] were saturating.

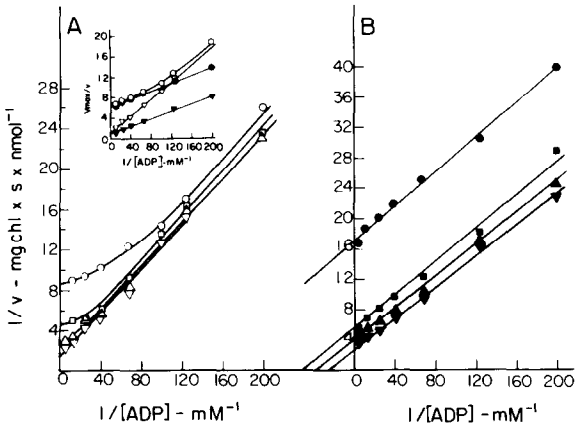

Fig. 3. Effect of light intensity on the steady state rate of phosphorylation. Experimental conditions as in fig. 1. Light intensities were: 0.20 (▽,▼), 0.11 (△,▲), 0.07 (□,■), and 0.04 (○,●) W/cm^2. (A) Control (12 μg chl); (B) treated thylakoids (7 μg chl). Inset: normalized double reciprocal plots with both preparations at the lowest and highest light intensities.

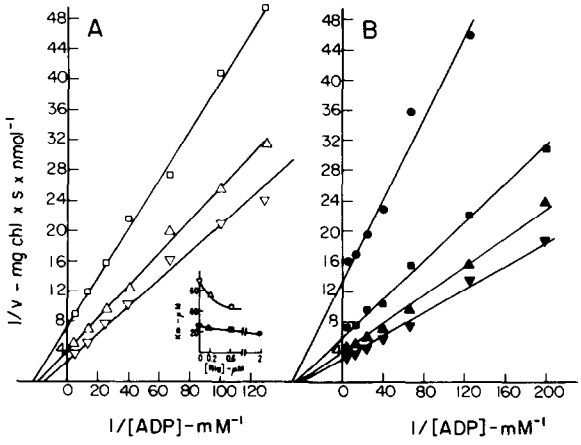

Fig. 4. Effect of nigericin and K^+ on the steady state rate of phosphorylation. Experimental conditions as in fig. 1. [K^+] was 30 meq/l and nigericin concentrations (μM) were: 0 (▽,▼); 0.2 (△,▲); 0.67 (□,■) and 2.0 (●). (A) Control (6 μg chl); (B) treated thylakoids (10 μg chl). Inset: apparent K_m values obtained by non-linear regression of the data shown in (A,B). For control thylakoids, the velocities with 2 μM nigericin were too low to permit an accurate analysis.

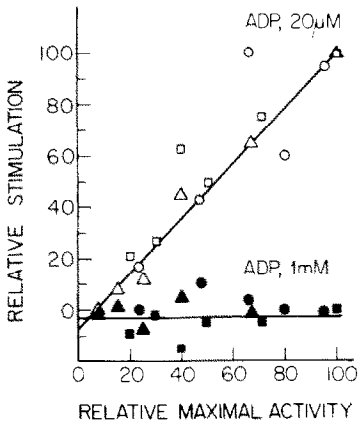

Fig. 5. Correlation between the catalytic ability and the observed stimulation at low [ADP] following hypotonic treatment. Rate of phosphorylation was controlled in each experiment by varying: (○,●) light intensity, 0.04–0.2 W/cm^2; (□,■) [P_i], 0.1–10 mM; or (△,▲) phlorizin, 0–2 mM. Stimulation was calculated as the increase in velocity ($v_{treated} - v_{control}$) divided by the velocity for the control at low (open symbols) or high [ADP] (solid symbols). The maximal activity was taken as the velocity of control thylakoids at high [ADP]. The largest stimulations were 52, 58 and 73% for light intensity, P_i and phlorizin experiments, respectively. The highest maximal activities (mmol.mg chl^{-1}.s^{-1}) were 230, 320 and 360 in the same order. Other conditions were as in fig. 1.

stimulated in treated thylakoids, while at low [ADP] the stimulation is directly proportional to the catalytic ability of both preparations.

4. DISCUSSION

The above results can be explained by a simple model in which phosphorylation, catalyzed by broken chloroplasts prepared in an isotonic medium, is kinetically limited by the transfer of nucleotides from the bulk medium to the ATP synthetase, and/or vice versa. Reactions catalyzed by enzymes immobilized on a surface or in solid matrices appear to be strongly limited by the mass transfer of substrates to the enzyme [10–12]. When the rate of catalysis is higher than the rate of diffusion of substrate to the immobilized enzyme, the concentration of substrate in the enzyme environment is lower than in the bulk medium. Such a diffusion effect lowers the reaction velocities observed with immobilized enzymes at non-saturating substrate concentrations. Derivation of K_m and V_{max}/K_m from the dependence of the reaction rate on the medium (rather than the local) substrate concentration gives apparent higher K_m and lower V_{max}/K_m values than those inherent to the enzyme. However, V_{max} estimated at saturating substrate concentration is not related to diffusion.

The ATP synthetase bound to the thylakoid membrane surface, under optimal conditions, can phosphorylate ADP efficiently (high V_{max}/K_m) at very high rates (k_{cat} = 300–500 s^{-1}). Since the environment of the membrane-bound ATP synthetase is probably not an ideal aqueous solution, in which solutes diffuse rapidly, the diffusion of nucleotides to the enzyme active site might limit the steady-state velocity of the process. Under our experimental conditions, when hexokinase is used to provide a constant [ADP], any limitation imposed by slow diffusion of solutes will be magnified due to the limited diffusion of both the substrate (ADP) and the product (ATP).

Hypotonic treatment and washing of normally-prepared thylakoids may be expected to remove existing diffusion layers by opening the grana stacks. Thus, in normally-prepared thylakoids, the diffusion of solutes may limit the rate of reaction under certain conditions, a conclusion based upon the following results:

(1) The catalytic ability (V_{max}) is not affected by the hypotonic treatment, but the apparent affinity for medium ADP is markedly increased (fig. 1);
(2) The apparent better accessibility of the newly formed ATP to hexokinase (fig. 2) indicates that a higher steady-state concentration of free ATP may accumulate in the near environment of the enzyme in normally prepared membranes [9];
(3) The relative stimulation of phosphorylation (at low [ADP]) caused by the treatment is decreased when the catalytic ability is artificially lowered (fig. 5), suggesting that the limitation due to diffusion is relieved.
(4) With P_i, which is utilized much less efficiently (low V_{max}/K_m), the apparent affinity is not affected (fig. 1) [6].

Light intensity, which strongly limits V_{max} in both preparations, has much less effect on the activity of control thylakoids at low [ADP]. Assuming that the slope of the curves obtained in double

reciprocal plots represents the substrate-dependent rate limiting step [7,8] allows one to differentiate between two microscopic steps involving ADP (essentially independent of light intensity), which may limit the reaction rate in control thylakoids (fig. 3). One step probably represents a property inherent to the membrane-bound ATP synthetase, since it also limits the reaction with treated thylakoids. This limitation is observed at low light intensity and becomes predominant at high [ADP]. The second is a relatively slow step which becomes predominant at low [ADP] and therefore may represent the limitation imposed by the mass transfer of nucleotides from the bulk medium to the ATP synthetase. The relative limitation by each step on the utilization of ADP will be determined by the balance between the rates of mass transfer and enzymatic catalysis. In contrast to light intensity, uncouplers strongly affect the rate limiting step of the reaction both with control and treated thylakoids (lower V_{max}/K_m), in addition to the inhibition of their catalytic activity (fig. 4). Thus, uncouplers may affect both of the above-mentioned steps involved in ADP utilization. This complex relation between energy input and ADP association is being studied further.

ACKNOWLEDGEMENTS

We wish to thank Mrs Zvia Conrad for excellent technical assistance. This work was carried out in partial fulfillment of the requirements for the PhD degree of C.A.

REFERENCES

[1] Kayalar, C., Rosing, J. and Boyer, P.D. (1976) Biochem. Biophys. Res. Commun. 72, 1153–1159.
[2] Hatefi, Y., Yagi, T., Phelps, D.C., Wong, S.Y., Vik, S.B. and Galante, Y.M. (1982) Proc. Natl. Acad. Sci. USA 79, 1756–1760.
[3] Bickel-Sandkötter, S. and Strotmann, H. (1981) FEBS Lett. 125, 188–192.
[4] Vinkler, C. (1981) Biochem. Biophys. Res. Commun. 99, 1095–1100.
[5] Selman, B.R. and Selman-Reimer, S. (1981), J. Biol. Chem. 256, 1722–1726.
[6] Magnusson, R.P. and McCarty, R.E. (1975) J. Biol. Chem. 250, 2593–2598.
[7] Cleland, W.W. (1970) in: The Enzymes (Boyer, P.D. ed) vol. 2, pp. 1–65, Academic Press, New York.
[8] Segel, I.H. (1975) in: Enzyme Kinetics, pp. 751–757, Wiley, New York.
[9] Aflalo, C. and Shavit, N. (1982) Eur. J. Biochem. 126, 61–68.
[10] Engasser, J.-M. and Horvath, C. (1976) in: Applied Biochemistry and Bioengineering (Wingard L.B. et al. eds) vol. 1, pp. 127–220, Academic Press, New York.
[11] Goldstein, L. (1977) Methods Enzymol. 44, 397–443.
[12] Laidler, K.J. and Bunting, P.S. (1980) Methods Enzymol. 64, 227–248.
[13] Farron, F. and Racker, E. (1970) Biochemistry 9, 3829–3836.
[14] Arnon, D.I. (1949) Plant Physiol. 29, 1–15.