Photophosphorylation as Function of ADP Concentration at Varying Transmembrane Proton Gradients

Georg Heinen and Heinrich Strotmann

Institut für Biochemie der Pflanzen, Heinrich-Heine-Universität Düsseldorf, Universitätsstraße 1,
D-4000 Düsseldorf, Bundesrepublik Deutschland

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Dedicated to Professor Achim Trebst on the occasion of his 60th birthday

Isolated Spinach Chloroplasts, Photophosphorylation, Proton-Motive Force,
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Rates of photophosphorylation were measured at constant saturating phosphate concentration, varying ADP concentration, and varying light intensity. As the transmembrane proton gradient is decreased by phosphorylation to different extents depending on the concentration of ADP, rates of ATP formation obtained at the different ADP concentrations were plotted *versus* the actual steady state ΔpH (in the absence of Δψ) during the course of the reaction. ΔpH was monitored by the calibrated 9-aminoacridine fluorescence technique. In secondary plots phosphorylation as function of ADP concentration at different constant ΔpH values were obtained. The results indicate Michaelis-Menten kinetics. The true K_m for ADP is virtually unaffected by ΔpH whereas V_{max} (at ADP saturation) strongly depends on ΔpH. The results are discussed in the framework of a simple enzyme kinetic model which considers the intrathylakoidal proton (at constant external pH) as a third substrate for ATP formation. The model is capable of explaining the reported results as well as a variety of important results from the literature.

Introduction

Photophosphorylation is catalyzed by a proton-translocating ATPase of the F_0F_1 type which is a constituent of the thylakoid membrane of the chloroplast. The mechanism of $\Delta\tilde{\mu}_{H^+}$-coupled phosphoryl transfer to ADP is a subject of intensive investigation. As the catalytic site is located on β subunit or at the interface between α and β [for review see 1–3], three catalytic entities must be assumed to be present per one ATPase molecule because the number of α and β copies per enzyme is three [4–5]. Nevertheless Michaelis-Menten kinetics are usually observed when phosphorylation is measured as function of ADP or phosphate concentration.

This fact may be explained within the framework of the three-site binding change mechanism proposed by Boyer and his coworkers [6]. This hypothesis assumes that the three catalytic sites change their properties in a sequential mode, so that at a given moment only one of them is in a condition to take up the substrates. Substrate binding to one site facilitates product release from another site. At very low ADP concentration (< 1 μM), however, a deviation from monophasic Michaelis-Menten behaviour was observed [7], a result which was interpreted to indicate the operation of a single site in this concentration range of ADP. Although important for the elucidation of the catalytic mechanism, single site catalysis can be disregarded in a physiological substrate concentration range.

Manyfold determination of K_m for ADP are reported in the literature with rather diverging results. A systematic investigation showed that K_m increases with increasing light intensity [8–10], decreases with increasing concentration of electron transport inhibitors like DCMU [8], increases or decreases in the presence of an uncoupler depending on the concentration employed [9, 12]. Different conclusions were drawn from these experimental findings. Davenport and McCarty [11] were right in criticizing that all these measurements suffered from uncontrolled experimental conditions with regard to $\Delta\tilde{\mu}_{H^+}$ during the phosphorylation reaction. $\Delta\tilde{\mu}_{H^+}$ is a function of the rate of electron transport, as well as the rates of passive and productive proton efflux. With increasing substrate concentration we can expect a progressive decrease of the electrochemical proton gradient even at constant light intensity, since the rate of phosphorylation is dependent on the concentrations of

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Abbreviations: Chl, chlorophyll; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; PMS, phenazine methosulfate; Tricine, N-[tris(hydroxymethyl)methyl]glycine.

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the substrates. As electron transport is controlled by intrathylakoidal pH [12, 13] varying extents of acceleration of electron transport must be taken into account, and the rate of passive proton leakage which is a function of the internal proton concentration will be changed, too. Analysis of the effect of ADP [10] and phosphate concentrations [14] on the magnitude of steady state ΔpH indeed verified these predictions. The goal of this study is the determination of the kinetic constants of the H^+-ATPase at controlled ΔpH (in the absence of $\Delta\psi$) and to give an answer to the question whether the true K_m is constant or variable with ΔpH. This decision has an important implication for the understanding of the enzymatic mechanism.

The experimental approach includes measurements of photophosphorylation at varying light intensity or uncoupler concentration, respectively, and varying ADP concentration. By simultaneous registration of 9-aminoacridine fluorescence which was calibrated for the employed experimental conditions [15, 16], the rates of ATP formation could be related to the actual ΔpH. K_m and V_{max} at constant actual ΔpH were ascertained by secondary plots.

Methods

Thylakoids were isolated from spinach leaves as in ref. [17].

All measurements were conducted in a cylindrical glass cuvette which was placed in a self-constructed fluorometer [16]. The experimental set-up permitted easy addition of substrates and taking of aliquots for analysis. During the whole experiment the fluorescence of 9-aminoacridine was recorded as a measure of ΔpH.

The final cuvette volume was 2.5 ml, the temperature 20 °C. The medium consisted of 25 mM Tricine buffer, pH 8.0, 50 mM KCl, 5 mM MgCl, 10 mM dithiothreitol, 50 μM PMS, 50 nM valinomycin, 10 mM glucose and 30 units/ml hexokinase (salt-free, Sigma). After addition of thylakoids corresponding to 25 μg chlorophyll/ml, 5 μM 9-aminoacridine was injected. Then full light was given for 2 min to bring the ATPase in its thiol-modulated state.

After dark relaxation of the fluorescence signal, the indicated concentrations of ADP were added. Since ADP caused some instantaneous artificial fluorescence quenching [15], the fluorescence emission obtained after ADP addition minus basal fluorescence (absence of 9-aminoacridine) was used as standard (Φ_0) for calculation of ΔpH.

Subsequently, light of varying intensity was turned on to produce different initial ΔpH values. 2 min later, after reaching a steady fluorescence, photophosphorylation was started by the addition of ^{32}P-labeled phosphate. Initiation of ATP synthesis causes a decrease of ΔpH. The steady state fluorescence during phosphorylation (Φ) was used for calculation of the actual ΔpH. For analysis of the formed ^{32}P-labeled glucose-6-phosphate 0.15 ml samples were taken 15, 30, 45 and 60 s after addition of $[^{32}P]P_i$ and deproteinized by $HClO_4$ (0.5 M final concentration). Organic ^{32}P was separated from inorganic ^{32}P by precipitation [18] and measured in a scintillation counter.

The calculation of ΔpH was based on the calibration performed in ref. [16] under the same experimental conditions. Because of the observed linear relationship between $\log(\Phi_0-\Phi)/\Phi$ and ΔpH in the relevant ΔpH range between 2.5 and 3.5 [15, 16], the equation by Schuldiner *et al.* [13] could be employed. From the calibration curve [16] an apparent internal thylakoid volume of 30 μl/mg chlorophyll was estimated. The empiric formula for calculation of ΔpH under the employed experimental conditions was

$$\Delta pH = \log(\Phi_0-\Phi)/\Phi + 3.12.$$

Results

Fig. 1 shows rates of photophosphorylation as a function of the actual ΔpH at 6 different ADP concentrations ranging from 10 to 152 μM. The data are taken from a large number of single independent experiments; in every individual experiment up to 20 points were gained. As the activity of phosphorylation varied between the experiments, the rates were normalized by relating them to mean maximal rates. For every ADP concentration in every experiment V_{max} values at saturating ΔpH were estimated by extrapolation. For this purpose reciprocal rates ($1/v$) were plotted *versus* reciprocal internal proton concentration to the power n ($[H^+_{in}]^n$). The exponent n was chosen, so that the coefficient of determination (r^2) of a linear regression attained its maximum. Usually n was between 2.5 and 3. In order to ensure comparability of the ΔpH curves at different ADP concentrations, ΔpH was varied in one experiment with not less than two ADP concentrations in an overlapping mode.

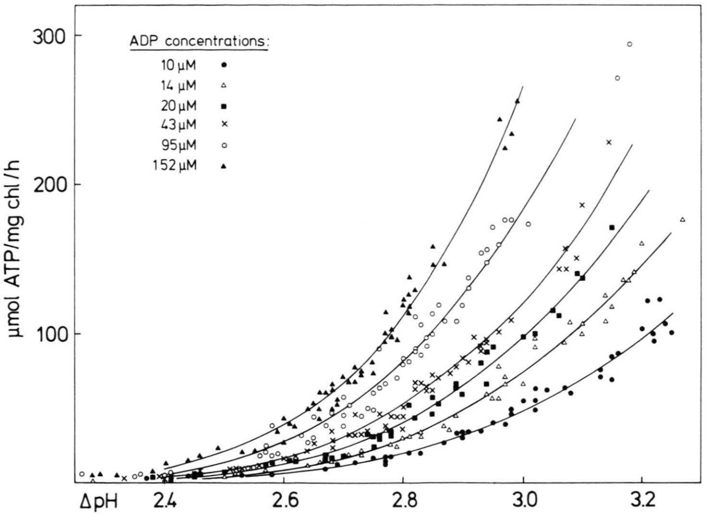


Fig. 1. Rate of photophosphorylation at 6 different ADP concentrations as function of the actual steady state ΔpH measured during the course of the reaction.

ΔpH was also changed by addition of varying concentrations of the uncoupler nigericin at constant (maximal) light intensity. The results (not shown) indicate that these data fit into the curves of Fig. 1. This finding which could be expected on the basis of the chemiosmotic theory, demonstrates that solely the magnitude of ΔpH is critical, not the method of its manipulation.

Rates of ATP formation as a function of ADP concentration at constant ΔpH values are shown in Fig. 2. The points were either taken directly as single or mean values from Fig. 1 or gained by linear interpolation between vicinal experimental points. Because of the low rates, a fair evaluation of data below pH = 2.6 is impossible. In a reasonable ΔpH range the plots of phosphorylation rate *versus* ADP concentration are essentially hyperbolic; hence Michaelis-Menten kinetics may be presupposed. In order to determine $V_{\max}$ and K_m the graphs were linearized according to Lineweaver-Burk (1/*v versus* 1/[ADP]), Eadie-Hoffstee (*v versus v*/[ADP]) and Hanes-Woolf ([ADP]/*v versus* [ADP]). The three modes of evaluation give different emphasis to the single experimental points, depending on the ADP concentration and the obtained rates. Therefore experimental scattering affects the determination of the kinetic parameters by the three methods in different ways. As the confidence of the estimates is restricted by the relatively low number of ADP concentrations employed, the evaluation by different plots may give additional certainty. To exclude any subjectivity in preparation of the plots, the graphs were computed from the experimental data by linear regression. The results are summarized in Table I. As expected, $V_{\max}$ increases progressively with rising ΔpH. Between ΔpH 2.6 and 3.0 maximal velocity increases by a factor of about 7. On the other hand, K_m is virtually unaffected by the magnitude of ΔpH in this range.

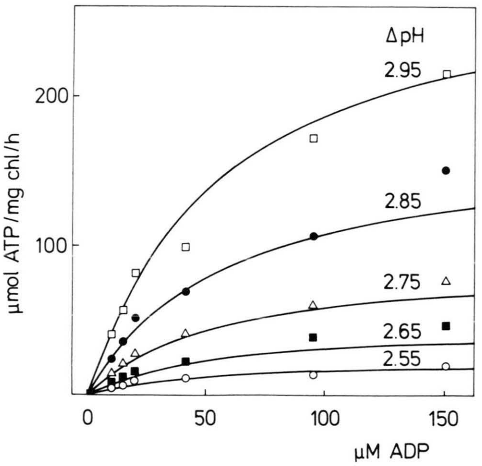


Fig. 2. Rate of photophosphorylation at different actual steady state ΔpH values as function of ADP concentration. The experimental points were taken from Fig. 1. The curves were computed by the model presented in the Discussion.

Table I. Determination of V_{max} and K_m(ADP) for photophosphorylation at different actual ΔpH values. The data were taken from Fig. 1 and plotted according to Lineweaver-Burk (A), Eadie-Hoffstee (B), and Hanes-Woolf (C). The kinetic parameters were gained from linear regression analysis of the experimental points.

Actual	V_{max} [μmol ATP/mg Chl per h]				K_m(ADP) [μM]			
ΔpH	A	B	C	mean	A	B	C	mean
2.60	44	37	46	42	58.3	43.6	65.0	55.6
2.65	62	63	71	66	60.9	62.5	76.1	66.5
2.70	67	76	88	76	48.1	56.9	75.2	60.3
2.75	94	96	102	97	49.3	51.3	56.8	63.5
2.80	131	123	169	141	54.4	46.9	84.1	61.8
2.85	209	184	217	203	72.9	59.6	79.8	70.8
2.90	196	205	238	213	47.9	50.9	67.6	55.5
2.95	270	247	303	274	55.4	45.7	67.3	56.1
3.00	265	277	351	298	42.1	43.1	67.4	50.9

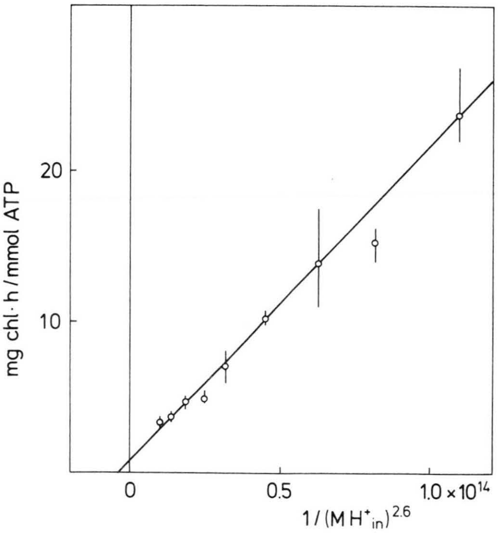


Fig. 3. Lineweaver-Burk plot of V_{max} (saturating ADP concentration) *versus* internal H^+ concentration. The proton concentration is expressed in $M^{2.6}$ (see text). The results were taken from Table I. The points are mean values of V_{max}, the bars indicate the limits of variation.

The single estimates of K_m obtained by the three different plots vary by a factor of about 2. However, they reveal no clear-cut increasing or decreasing tendency in dependence of ΔpH. Hence we conclude that ΔpH in the indicated range from 2.6 to 3 does not affect the Michaelis constant significantly.

The reciprocal V_{max} values of Table I were plotted *versus* the reciprocal internal H^+ concentrations to the power n, $[H^+_{in}]^n$. The value of n was chosen so that the plot gave a linear approximation. Linear relationships were found at values of n between 2.5 and 3, Fig. 3 shows a fit performed with $n = 2.6$. n is identical with the Hill coefficient for H^+_{in} which can be classified as the third substrate of photophosphorylation according to

$$ADP + P_i + n\,H^+_{in} \rightarrow ATP + H_2O + n\,H^+_{out}.$$

The reaction can be written as a unidirectional process, because ATP is recycled to ADP by the subsequent hexokinase reaction. The maximal velocity obtained at saturation of ADP and P_i as function of $[H^+_{in}]$ can be expressed by the Michaelis-Menten equation as

$$V_{max} = [E_t] \cdot k_c \cdot [H^+_{in}]^n / ([H^+_{in}]^n + K(H^+_{in})),$$

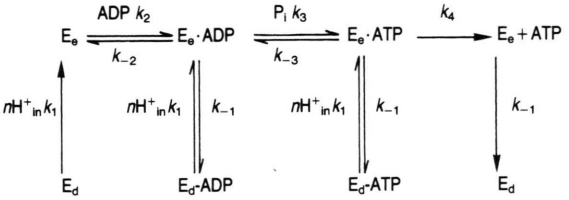
where $[E_t]$ is the total enzyme concentration, k_c is the rate-limiting velocity constant and $K(H^+_{in})$ means an equilibrium constant for the internal proton with the dimension $(mol/l)^n$. Theoretically n is equal to the stoichiometry of H^+ translocated through the ATPase complex per ATP formed. The approximation towards 3 is in agreement with determinations of H^+/ATP stoichiometry obtained by different experimental approaches [15, 19–22]. The plot $1/V_{max}$ *versus* $1/[H^+_{in}]^n$ permits the estimation of the maximal rate of ATP formation at saturation of all involved substrates. This parameter (V_{abs}) was determined as 1000 to 2000 μmol/mg chlorophyll per h, depending on the chosen value for n, which agrees well with the reported turnover number of the enzyme [23]. In

Fig. 3, V_{abs} was extrapolated to 1250 μmol/mg chlorophyll per h. The received value of $K(H^{+}_{in})$ permits the calculation of a half-maximal ΔpH of about 3.1.

Discussion

The here reported results show that the Michaelis constant for ADP is largely independent of ΔpH in a range from 2.6 to 3.0 when measured at phosphate saturation and controlled thylakoid energization, *i.e.* at a constant actual pH gradient during variation of the ADP concentration. The evaluation proposes Michaelis-Menten type kinetics, an assumption which is based on the hyperbolic dependence of velocity on ADP concentration in the employed range. We have to admit considerable experimental error due to scattering in ΔpH determination and the utilization of results from diverse independent experiments. Nevertheless, the large number of data and their treatment by different secondary plots confers some confidence on the determination of the kinetic parameters. The evaluated ΔpH range from 2.6 to 3.0 appears rather small; however, in this range the activity of phosphorylation increases by almost one order of magnitude, which should be sufficient to ensure possible substantial changes in K_m. Since no clear-cut tendency of an increase or decrease of K_m could be detected in dependence of ΔpH, it appears highly probable that the true K_m is ΔpH-independent. The same presumption was made by Quick and Mills [10] in explaining the results on apparent K_m (ADP) at uncontrolled ΔpH on the basis of a reasonable theoretical model. In variance to their assumption (K_m = 12 μM), however, our data indicate a true K_m which is 5 times higher (59 μM).

In a formal enzyme kinetic model the internal H^+ can be considered as a kind of substrate in phosphorylation when the external H^+ concentration is kept constant as in the here conducted experiments. Invariability of K_m(ADP) at V_{max} being increased by raising $[H^{+}_{in}]$ may be interpreted by conceiving H^{+}_{in} as an "inverse" non-competitive inhibitor. In analogy to the formalism of non-competitive inhibition, H^{+}_{in} would act on a deenergized free enzyme to create an energized enzyme which is ready to bind substrate ADP, as well as on a deenergized form of the enzyme with bound ADP to create the energized enzyme-ADP complex. The active enzyme-ADP complex interacts with phosphate in a reversible reaction to form the enzyme-product complex. In the model shown below, it is assumed that the active enzyme-ATP complex can also undergo transfer to a deenergized complex and that the reverse reaction depends on $[H^{+}_{in}]$, too. The final reaction, the dissociation into the free enzyme and free ATP, can be written as an irreversible step since the product ATP is trapped by hexokinase. As a consequence of product release, the energized state of the enzyme is discharged. The model is a different view of a conformational coupling hypothesis which is basically not far from the mechanism proposed by Boyer and his colleagues [6].



The respective energized and deenergized forms are designated by the indices e and d, the number of protons reacting from the inner phase of the thylakoid is *n*. The value of *n* is assumed to be 3. Computation of the model yields for the parameters V_{max} and K_m(ADP):

$$V_{max} = E_t \cdot [H^{+}_{in}]^n \cdot k_1[P_i]k_3k_4/ \\ ([H^{+}_{in}]^n \cdot k_1 + k_{-1})([P_i]k_3 + k_{-3} + k_4)$$

and

$$K_m(ADP) = (k_{-2}k_{-3} + k_{-2}k_4 + [P_i]k_3k_4)/ \\ (k_2[P_i]k_3 + k_2k_{-3} + k_2k_4).$$

The model which is similar but not identical to a scheme proposed by Quick and Mills [10, 14] is certainly incomplete. So, it does not include site cooperativity and explains ATP synthesis but not hydrolysis without making additional assumptions. Nevertheless, the model is capable of explaining a variety of important results reported in the literature as well as the findings of the present study.

(1) The independence of the true K_m(ADP) as well as the dependence of V_{max} on the actual ΔpH or $[H^{+}_{in}]$, respectively, is fulfilled by the model. As in the scheme of Quick and Mills [10], variability of the apparent K_m obtained at different light intensities or uncoupler concentrations can be explained, too.

(2) Energy of the electrochemical gradient is not invested directly in the chemical reaction at the

catalytic site [6]. Here the transformation of "deenergized" into "energized" enzyme forms is the only site of energy input. This assumption was also made by Schumann [24] in a comprehensive hypothesis of the H^+-coupled chloroplast ATPase. The deenergized species may be conceived as enzyme molecules characterized by "closed" *i.e.* unavailable catalytic sites, whereas the energized forms have "open" catalytic sites which are accessible to the substrates of the medium. If a closed site is containing a nucleotide molecule, this ligand therefore appears "tightly bound". In variance with the "energy-linked binding change mechanism" [6] the formation of tightly bound ADP or ATP would be a side reaction rather than an obligate intermediate step of the catalytic sequence. The ratio of ATPase molecules with exchangeable nucleotides to those with tightly bound nucleotides depends on the energy state of the membrane [8, 25] and the level of tightly bound nucleotides changes inversely with the activity of photophosphorylation as function of energy input [26]. When light is turned off, the level of tightly bound nucleotides increases to a maximum of one per ATPase when ADP or ATP is present in the medium [27]. Tight binding of ADP is related with inactivation of the enzyme [28]. On the other hand, the tightly bound nucleotides are released or exchanged upon reillumination, a reaction which is related with enzyme reactivation [28]. Under certain conditions the initial rate of nucleotide release matches the rate of ATP formation [29, 30]. All these results can be easily explained by the model. Hence, the reactions related with enzyme activation are basically identical with the energy transfer reactions involved in the catalytic process and deactivation is a compulsory consequence of relaxation of the proton gradient leading to closure of the catalytic sites. From the standpoint of this model it is unnecessary to assume extra nucleotide binding sites for nucleotide-dependent regulatory processes. This view is in line with the recent finding that tightly bound ADP and ATP in darkened thylakoids are on catalytic sites, a result which was obtained by the photoreactive nucleotide analogue 2-azido-ADP [32]. The general structural identity of the sites containing tightly bound nucleotides with those performing catalysis had been suggested before on the basis of photolabeling experiments with the same analogue [31]. Although up to 6 nucleotide-binding sites were identified in isolated CF_1 [33], the participation of all of them in catalysis and control of the membrane-bound enzyme has never been proven.

(3) It was shown by ^{18}O exchange that decrease of substrate concentration increased the number of reversible cycles of ATP formation at the catalytic site before the product ATP is released [34]. This experimental fact was concluded to indicate catalytic site cooperativity, *i.e.* release of the product is facilitated by binding of substrates to a second catalytic site. For the reason of simplicity, site cooperativity was omitted, but could be integrated in the scheme without changing the general features significantly. The same effect on ^{18}O exchange was reported to occur when light intensity was reduced [35] and this result was taken as evidence for $\Delta\tilde{\mu}_{H^+}$ dependence of product release. In contrast, our model proposes that release of ATP from the energized enzyme is a spontaneous reaction. This conclusion which is derived from the formal enzyme kinetic interpretation of the reported results, is in line with the earlier finding that the affinity of the energized enzyme for ATP is by one to two orders of magnitude lower than for ADP [36]. On the energized enzyme, ATP acts as a competitive inhibitor of phosphorylation with a K_i as high as 4 mM [36], indicating that the dissociation equilibrium is far on the side of free ATP. In the framework of the model, the effect of low light intensity on ^{18}O exchange may be interpreted by assuming that the cycles of reversible ATP hydrolysis occur in the state of the enzyme where ATP is tightly bound (E_d-ATP). Actually the exchange of $[^{18}O]H_2O$ with tightly bound ATP was demonstrated experimentally [37]. If $k_{-1} > k_4$, the equilibrium may be on the side of E_d-ATP rather than free ATP at low ΔpH, thus permitting repetitive ^{18}O exchange with medium $[^{18}O]H_2O$.

(4) The "kinetic competence" of tightly bound ATP is an unsolved problem connected with the energy-linked binding change mechanism. In this respect conflicting results were reported [38, 39]. It is quite certain that the exchange of at least part of the tightly bound ATP is too slow to be a step in the catalytic process [37]. In our model the exchange of tightly bound nucleotides is not necessarily in pace with the catalytic cycle. In this context it should be noted that for sake of simplicity the rate constants k_1 and k_{-1} were assumed to be identical for the interconversion of all deenergized to energized enzyme forms. They could well be different for the reversible transitions of E_d, E_d-ADP and E_d-ATP, respectively,

to the corresponding energized forms. Independence of K_m on ΔpH would be fulfilled if the ratios of the forward and backward constants were the same.

The curves drawn in Fig. 2 were computed with the model by chosing the following constants: $E_t \cdot k_4 = V_{abs} = 1250$ µmol/mg chlorophyll per h (Fig. 3), $K_m(ADP) = 59\ \mu M$ (Table I), $(k_{-3} + k_4)/k_3 = K_m(P_i) = 500\ \mu M$ [40], and $k_{-1}/k_1 = 2000\ \mu M^3$. The latter value was calculated from the ΔpH at half-maximal phosphorylation rate which was found to be 3.1 (Fig. 3):

$$k_{-1}/k_1 = (10^{(3.1-8)} \cdot 10^6)^3 = 2000\ \mu M^3.$$

It should be mentioned that this value of course relies on the correct quantitative determination of ΔpH in our experiments. The problems related with calibration of the 9-aminoacridine fluorescence method by the phosphate potential in energetic equilibrium are discussed elsewhere [16].

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