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KINETICS OF ATP FORMATION AND PROTON EFFLUX BY ACID–BASE TRANSITION IN CHLOROPLASTS

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SUMMARY

The relationship between the kinetics of ATP formation and proton release in chloroplast suspensions by acid–base transition were studied by means of a stopped-flow spectrophotometer. The time course of ATP synthesis shows two-phase kinetics, fast and slow, corresponding to the two-phase efflux of protons from the chloroplasts. Under certain conditions of the experiments, about 50% of the H^+ gradient is constantly utilized for ATP formation in both phases. However, the ratio of ATP formed to the amount of protons leaked out, changes depending on the rate constants of proton efflux.

INTRODUCTION

Exposing broken chloroplast fragments first to pH 4 media, containing permeant organic acid such as succinate and then to base at pH 8, is known to cause a transient high-energy state which results in ATP formation[1]. Energy in this acid–base transition experiment was suggested to be supplied by the H^+ gradient across the thylakoid membranes[2].

In the previous papers[3,4], the kinetics of proton efflux in chloroplast suspensions by the acid–base transition were studied by means of a stopped-flow apparatus. Rapid recording with bromocresol purple as a pH indicator showed that the time course of proton release consisted of two phases, fast and slow[4]. The fast phase was suggested to be due to the rapid release of protons from the matrix of thylakoid membranes, and the slow phase to the efflux through the membrane of organic acid (and/or protons) previously absorbed from the initial acid medium. This phase was followed by the additional slower component of proton loss[3,4].

The time course of phosphorylation by the acid-base transition has been followed chemically by stopping the reaction at appropriate intervals[1]. However, the relationship between the time course of ATP formation and the protein efflux from the chloroplasts has not yet been precisely established. In this report, the time course of pH changes was followed to observe the above relationship by means of a stopped-flow spectrophotometer with bromocresol purple, which has been shown not to be bound to membrane[5,6], as a pH indicator.

Abbreviation: DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea.

METHODS

Chloroplasts were isolated from market spinach as described earlier[7], and finally suspended at about 0.4 mg of chlorophyll per ml in 10 mM NaCl. 0.2 ml of this suspension was mixed with 0.2 ml of a solution at pH 4.0 containing 25 mM succinic acid, 10 mM $MgCl_2$, and 75 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in a reservoir of the stopped-flow apparatus maintained at a constant temperature (usually 3.5–4.0 °C). The acidified chloroplasts were incubated for 15–20 s, 0.25 ml of which were then rapidly picked up into one of the two syringes in the apparatus. The other syringe contained an equal volume of 30 μM bromocresol purple, 8 mM Na_2HPO_4, and NaOH solution sufficient to neutralize the succinic acid and bring the final pH to about 8. When phosphorylation was to be followed, 0.4 mM ADP was added in the latter syringe. No buffer was used in the base stage. Variations of the reaction mixture components are indicated in the figure. Chlorophyll was determined by the method of Arnon[8].

The stopped-flow spectrophotometer SPS-1 used was the same as before[4], and the changes in the absorbance of bromocresol purple at 594 nm after mixing were recorded with the memoriscope MS-5019A[4]. Two syringes were driven by compressed N_2 at 1–2 kg/cm^2 for mixing and the dead time[4] was under 2 ms, usually 1–1.2 ms.

The pH changes of the chloroplast suspension were calculated from the absorbance–pH curves of bromocresol purple at 594 nm; absorbance changes of more than 0.02 could be detected per 0.1 pH unit at the pH used. Also, the changes in H^+ concentration (ΔH^+, μequiv/mg chl) were calculated from the pH values according to direct titrations of the buffer capacities of the chloroplast suspension (the reaction mixture components present) with acid and with NaOH at each time of the experiments.

To follow the time course of ATP formation in rapid reactions such as those in the present experiments, it is difficult to determine ATP yield directly by luciferase assay and by incorporation of phosphate. Jagendorf and Uribe[1] showed that the major part of the observed phosphate incorporation by acid–base transition experiments had represented net synthesis of ATP when the basic reaction mixture contained ADP, P_i and Mg^{2+}. Further, in his experiments with chloroplasts, Schwartz[9] made use of the fact that at pH 8.0 one proton is consumed irreversibly in the synthesis of ATP.

$$ADP^{3-} + HPO_4{}^{2-} + H^+ \rightarrow ATP^{4-} + H_2O$$

Both theoretical and experimental considerations of this method were made by Nishimura *et al.*[10].

Thus in the present experiments the time course of ATP formation was measured, according to Schwartz, following its associated alkalinization (less acidification) of the chloroplast suspension during the course of efflux of protons after mixing when ADP was present. The presence of ADP, P_i or Mg^{2+} alone did not alter the rate of proton efflux to any significant extent.

RESULTS

As reported previously[4], the release of protons by acid–base transition showed two-phasic time courses at the low temperature used. The two phases could be discriminated from the recorded time course of the ΔH^+ changes as shown in Fig. 4 in ref. 4. In this report, the fast and the slow phases will be designated as Phase I and Phase II, respectively.

Time courses of the ΔH^+ and ATP formation

In Fig. 1 the time courses of the ΔH^+ of the chloroplast suspension after mixing either with or without the addition of ADP are compared. One syringe contained 12.5 mM succinate at pH 4.0 with chloroplasts (0.2 mg chlorophyll/ml) and 5 mM $MgCl_2$, and the other contained 30 μM bromocresol purple, 8 mM sodium phosphate, either with or without 0.4 mM ADP, and NaOH so that the final pH after mixing the contents of the two syringes could be 8.4.

As can be seen in Fig. 1, the tracing shows a sharp increase in H^+ concentration in the suspension immediately after mixing (Phase I), followed by a slow increase (Phase II) as a function of time until an equilibrium was attained in about 10 s. In the fast tracing of ΔH^+ (Fig. 1B), the transition from Phase I to Phase II could be clearly observed. When ADP was present, there was less change in H^+ concentration in both Phases I and II, indicating a consumption of H^+ for the formation of ATP[9,10]. The differences of H^+ concentration between the two curves (−ADP and +ADP) are equivalent to the concentrations of ATP formed in this process.

Fig. 2 shows an experiment in which one syringe contained the strong mineral acid, HCl, instead of succinic acid, to bring the pH of the chloroplast suspension to 4.0. In this case, practically the same increase in H^+ concentration in Phase I was

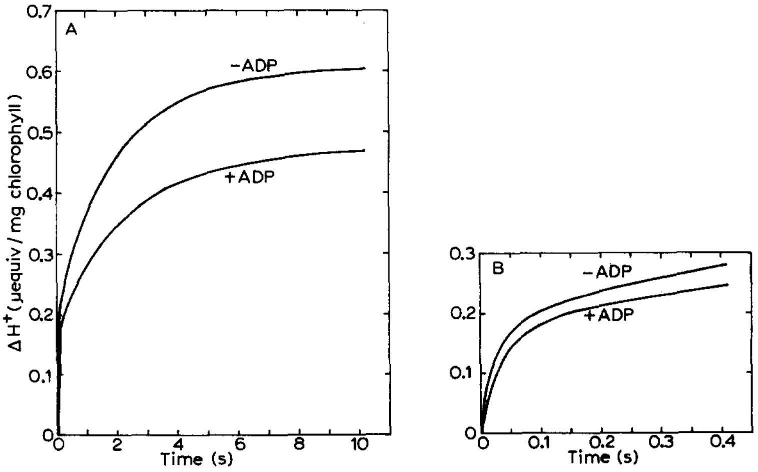

Fig. 1. Efflux of protons after mixing. The ΔH^+ curves are reproduced from the oscillograph tracing of pH change (see Methods). Initial chloroplasts at pH 4.0 with 12.5 mM succinic acid, 5 mM $MgCl_2$, and 38 μM DCMU. The base solution contained 30 μM bromocresol purple and sufficient NaOH to neutralize succinic acid to bring the final pH to 8.4 with and without ADP (0.4 mM). Chlorophyll concentration was 0.1 mg/ml after mixing. Temperature, 4 °C. (A) Slow recording. (B) Fast recording to follow transition from Phase I to Phase II.

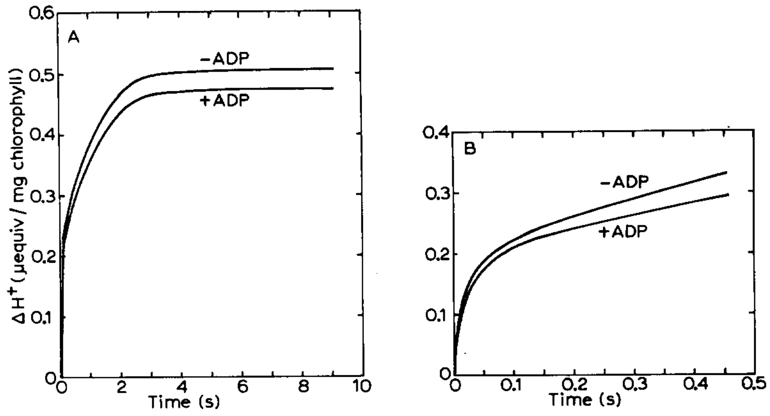

Fig. 2. Efflux of protons when HCl was substituted for succinic acid in the acid stage. Final pH 8.0. Other conditions were the same as in Fig. 1.

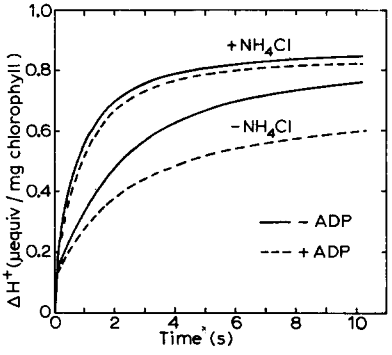

Fig. 3. Efflux of protons with and without NH_4Cl (10 mM in acid stage) together with and without ADP. Final pH 8.3. Other conditions were the same as in Fig. 1. When NH_4Cl was present, practically no difference was observed between the time courses of proton efflux with and without ADP.

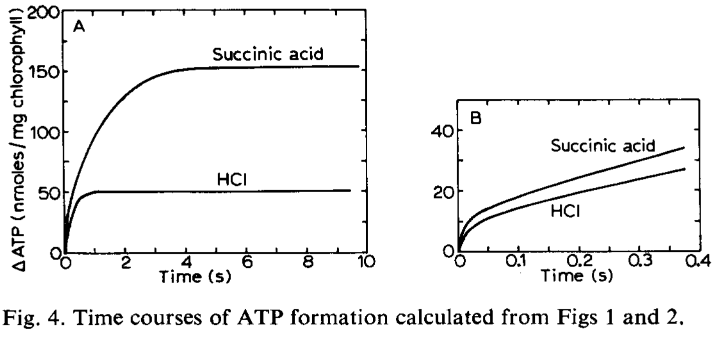

Fig. 4. Time courses of ATP formation calculated from Figs 1 and 2.

observed as in Fig. 1, but in Phase II (Fig. 2A) less H^+ was released from the chloroplasts and the equilibrium was attained much faster, that is, in about 2–3 s.

When an uncoupler such as NH_4Cl was present (added in the acid stage at

10 mM), the time course of proton efflux was practically independent of whether ADP was added or not in the base stage as shown in Fig. 3.

Time courses of ATP formation, calculated from Figs 1 and 2, are shown in Fig. 4. Identical kinetics for the ATP formation were found when HCl was used in the initial acidification in place of succinic acid immediately after mixing (Fig. 4B, Phase I). However, though the ATP formation completely stopped before 1 s had elapsed in HCl-treated chloroplasts (Fig. 4A), it continued for about 4 s and much more ATP was formed before the equilibrium was attained when succinic acid was used in the acid stage (Phase II). All of these facts are consistent with the observations that succinic acid which entered the thylakoid vesicles in the acid stage served as a proton reservoir in the base stage, resulting in a large amount of ATP formation in an acid–base experiment[2].

Considerable amounts of protons are found to have leaked out after the completion of ATP formation when ΔH^+ and ATP curves are compared in Figs 1, 2 and 4. In other words, some H^+ gradient between the inside and the outside of the thylakoid membrane still existed when ATP formation was completely stopped.

Fig. 5 gives the changes of ATP formation with temperature. At 4° C, the rate of ATP formation was less than at 20 °C. However, the increase of the ATP concentration was continued much longer (about 5 s) at 4 °C than at 20 °C (less than 2 s), resulting in a crossing of the two curves and finally more ATP was formed at 4 °C. This is consistent with the results that the rate constants of the proton release in Phase II increased markedly under higher temperatures (Table III in ref. 3 and Table I in ref. 4) and also with the fact that higher rate constants for Phase II of proton release associated with the lower yield of ATP[3].

Estimation of the efficiency of the utilization of the H^+ gradient

Both Phases I and II of proton efflux, and also the associated ATP formations, followed apparent first-order kinetics when plotted on a semi-logarithmic scale as shown in Fig. 6.

If the concurrent ATP synthesis does not affect the rate constant of the proton

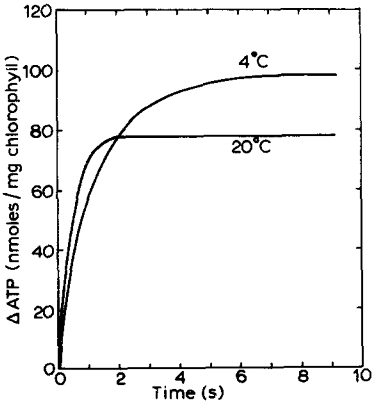

Fig. 5. Time course of ATP formation at two different temperatures (4 and 20 °C). Final pH 8.1. Other conditions were the same as in Fig. 1.

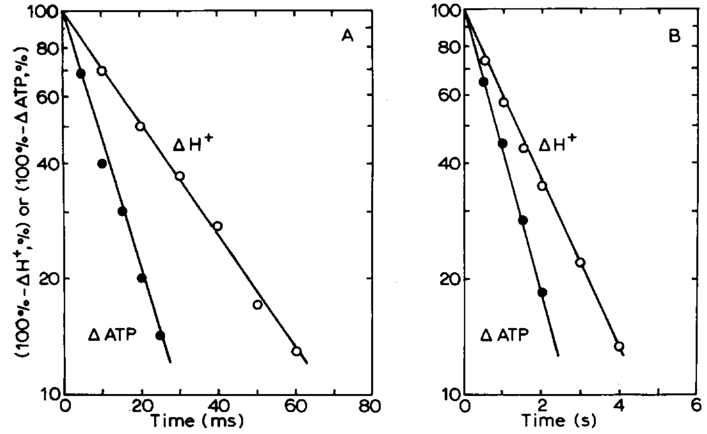

Fig. 6. Semi-logarithmic plot of proton efflux (−ADP) and ATP formation (+ADP) in Phase I (A) and Phase II (B). Ordinate shows (100%−ΔH$^+$, %) or (100%−ΔATP, %).

release in the absence of ATP formation, the processes taking place during the period of acid–base-induced phosphorylation can be assumed, as a first approximation, to be

$$0 \xleftarrow[\text{Decay}]{k_d} \textit{grad}\ \mathrm{H}^+ \xrightarrow[\text{ADP ATP}]{k_f} 0$$

where k_d and k_f represent the first-order rate constants of the two processes, efflux of protons and ATP formation, competing with each other for the common "driven force" represented by the H$^+$ gradient (*grad* H$^+$) which is established at zero time in the base stage. This is a modification of the scheme suggested by Izawa[11], and Jagendorf and Uribe[2]. In this case, the rate constant calculated directly from the apparent kinetics of ATP formation, corresponds to the combined rate constants k_d+k_f, and the rate constant k_d can be calculated from the kinetics of proton efflux without adding ADP. The efficiency of *grad* H$^+$ trapping for ATP formation is estimated at $k_f/(k_d+k_f)$. Typical data thus obtained under several conditions are summarized in Tables I and II.

TABLE I

APPARENT FIRST-ORDER RATE CONSTANTS FOR PHASE I

Experimental procedures were the same as in Fig. 1. Acid stage: 12.5 mM succinic acid, 38 μM DCMU, 5 mM MgCl$_2$, pH 4.0. Base solution: 30 mM NaOH, 8 mM P$_i$, 30 μM bromocresol purple, 0.4 mM ADP. Final pH 8.0 (Expt 1) and 8.5 (Expt 2).

Expt	*Temp. (°C)*	*ADP*	*Rate constant* (s^{-1})			$k_f/(k_d+k_f)$
			ΔH^+ (k_d)	ΔATP (k_d+k_f)	k_f	k
1	3.5	−	30.1			
		+	—	63.0	32.9	0.52
2	4.0	−	34.7			
		+	—	77.0	42.3	0.55

TABLE II

APPARENT FIRST-ORDER RATE CONSTANTS FOR PHASE II

Initial pH 4.0. Final pH 8.4 (Expts 1 and 2), 8.1 (Expt 3). Other procedures were the same as Table I.

Expt	*Acid stage*	*Temp.* (°C)	*ADP*	*Rate constant* (s^{-1})			$k_f/(k_a+k_f)$
				$\Delta H^+(k_a)$	$\Delta ATP\ (k_a+k_f)$	k_f	
1	HCl	3.5	–	1.16			
			+	—	2.31	1.15	0.50
	Succinic acid	4.0	–	0.50			
			+	—	0.87	0.37	0.43
2	Succinic acid	4.0	–	0.43			
			+	—	0.82	0.39	0.48
		20	–	0.99			
			+	—	1.82	0.83	0.46
3	Succinic acid	4.0	–	0.53			
			+	—	0.99	0.46	0.47

The efficiency of trapping of the H^+ gradient for Phase I was $k_f/(k_d+k_f)=0.54 \pm 0.06$ (four experiments) and for Phase II 0.47 ± 0.02 (five experiments). Similar efficiency (about 50%) was obtained irrespective of temperature, rate constant, and the kind of acid used in the acid stage, both in Phases I and II. These values of about 50% efficiency coincide with that of X_E (a high energy state) capture obtained by Izawa[11] in the experiments of post-illumination ATP formation. This suggests the possibility that the H^+ gradient itself in the acid–base transition experiment is identical with X_E in the post-illumination experiment.

On the other hand, the stoichiometric relationship between the amount of H^+ released and ATP formed during the processes of phosphorylation after the acid–base transition, differed appreciably in Phase I and Phase II. In Phase I the ratios of H^+ released to ATP formed were 16 (HCl in the acid stage, 160 nequiv to 10 nmoles per mg chlorophyll)–8 (succinic acid, 135 nequiv to 18 nmoles). When these ratios are corrected for the estimated efficiency of H^+ gradient utilization (54% in Phase I) the values fall to 8.6 and 4.3, respectively. In Phase II, though the ratio was 6.7 (215 nequiv to 32 nmoles) in HCl-treated chloroplasts, fairly constant values of 3.4–2.9 (385 nequiv to 135 nmoles) were obtained with succinic acid as H^+ carrier (4 °C). Corrected values for the efficiency (47% in Phase II) fall to 3.1 (HCl) and 1.6–1.4 (succinate). At higher temperatures the ratio increased, indicating a lower ATP formation than at lower temperatures. Thus the stoichiometry is suggested to be changed under different conditions.

DISCUSSION

In summarizing together the results presented in this paper and in the previous ones[3,4], the time courses for the approach to equilibrium pH in the chloroplast suspension after the acid–base transition showed apparent first-order kinetics, with three distinct components: (a) the fast phase (Phase I) of the rapid loss of protons from the matrix of thylakoid membranes having a half-life of about 20–40 ms (ref. 4), (b) the major portion of proton efflux from the inside of the thylakoid vesicles (Phase II), having a Q_{10} of 2 and a half-life of about 1.5–3 s (refs 3 and 4), and (c) a minor residual portion (Phase III) of the proton loss, having a Q_{10} of 1.2 and a half-life of about 5 s (ref. 3). As Phase III is assumed to be a simple loss of protons, having no relationship with ATP formation[3], a discussion of this is omitted here.

As shown in Figs 1, 2, 4, and 5, ATP formation started immediately after mixing, even during the process of proton dissociation from the thylakoid membrane (Phase I)[4]. The time course of ATP formation itself also has two components depending on the two-phasic time course of proton efflux (Fig. 4B). Both of the components followed apparent first-order kinetics closely depending on the first-order efflux of protons from the thylakoid vesicles both in Phases I and II (Fig. 6).

Since the production of the H^+ gradient may play a decisive role in the phosphorylation process[12], it is important to appreciate what proportion of the whole H^+ gradient may participate in the formation of ATP. The estimated efficiencies of the utilization of the H^+ gradient for the phosphorylation of ADP were 54% in Phase I and 47% in Phase II (Tables I and II). This is practically in accord with the efficiency of X_E capture (56%) obtained in the post-illumination phosphorylation experiments[11], though the Phase I component is not present in the latter. Comparable efficiency of X_E and H^+ gradient trapping under different procedures of the experiments suggests that the H^+ gradient may be identical with the X_E.

The efficiency of the utilization of the H^+ gradient is not changed when HCl is substituted for succinic acid in the acid stage in spite of the large rate constants of the H^+ efflux after the acid–base transition (Table II). The same situation is true at higher temperatures. However, the actual ratios of ATP formed to the amounts of protons released vary with the rate constants of the proton efflux. The larger the rate constants, the smaller the quantity of ATP formed in agreement with the previous observations[3]. Continued maintenance of an adequate H^+ gradient must be a necessary prerequisite for continued ATP synthesis.

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REFERENCES

1 Jagendorf, A. T. and Uribe, E. (1966) *Proc. Natl. Acad. Sci. U. S.* 55, 170–177
2 Jagendorf, A. T. and Uribe, E. (1966) *Brookhaven Symp. Biol.* 19, 215–241
3 Nishizaki, Y. and Jagendorf, A. T. (1971) *Biochim. Biophys. Acta* 226, 172–186
4 Nishizaki, Y. (1972) *Biochim. Biophys. Acta* 275, 177–181
5 Jackson, J. B. and Crofts, A. R. (1969) *Eur. J. Biochem.* 10, 226–237
6 Chance, B., Crofts, A. R., Nishimura, M. and Price, B. (1970) *Eur. J. Biochem.* 13, 364–374
7 Nishizaki, Y. and Jagendorf, A. T. (1969) *Arch. Biochem. Biophys.* 133, 255–262
8 Arnon, D. I. (1949) *Plant Physiol.* 24, 1–15
9 Schwartz, M. (1968) *Nature* 219, 915–919
10 Nishimura, M., Ito, T. and Chance, B. (1962) *Biochim. Biophys. Acta* 59, 177–182
11 Izawa, S. (1970) *Biochim. Biophys. Acta* 223, 165–173
12 Mitchell, P. (1966) *Biol. Rev.* 41, 445–502