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CONTROL OF PROTON TRANSLOCATION INDUCED BY ATPase ACTIVITY IN CHLOROPLASTS

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SUMMARY

1. Proton uptake was induced by ATP in the dark following light triggering of ATPase activity in chloroplasts. The accumulated protons were released when ATPase activity was inhibited by the energy transfer inhibitor DIO-9.

2. Approximately two protons were taken up for each ATP hydrolyzed at pH 8. A drop in H^+/ATP ratio was caused by uncouplers such as NH_4Cl and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone. These uncouplers caused an increase in the rate of ATP hydrolysis without a corresponding increase in proton uptake.

3. The energy transfer inhibitor dicyclocarbodiimide inhibited both ATPase activity and the rate of proton uptake without changing the H^+/ATP ratio.

4. The antibiotic valinomycin caused an increase in the rate of both proton uptake and ATP hydrolysis without altering the ratio of H^+/ATP. The H^+/ATP ratio varied with changes in the external pH. The results were discussed in view of the chemiosmotic theory of oxidative and photosynthetic phosphorylation.

INTRODUCTION

In oxidative and photosynthetic phosphorylation energy-linked proton movement across a coupling membrane was assumed to generate an electrochemical gradient. The electrochemical gradient is comprised of a ΔpH and a membrane potential [1]. Light-dependent electron transport in chloroplasts is associated with proton uptake [2] and with the formation of ΔpH [3]. Light-induced chromic changes in the photosynthetic pigments were suggested to indicate a formation of a membrane potential which precedes proton uptake [4]. The formation of a membrane potential was also deduced from the effect of the antibiotic valinomycin, which was shown to act as a carrier of potassium across biological membranes allowing potassium to proton counter fluxes [5]. The rate of light induced proton uptake and the ratio of protons taken up per electrons in chloroplasts were increased in the presence of valinomycin [6, 7]. These observations indicate that in the absence of valinomycin proton uptake

Abbreviations: FCCP, *p*-trifluoromethoxyphenylhydrazone; DCCD, dicyclocarbodiimide.

was limited by the backpressure of a membrane potential which was formed by the protons taken up.

An NH_4^+ uptake associated with light-triggered ATPase activity was assumed to indicate an ATPase-driven proton uptake in chloroplasts [8]. In a preliminary report we have demonstrated that ATP hydrolysis by ATPase in chloroplasts induced proton uptake [9]. The finding of a ratio of two protons taken up per one ATP hydrolyzed was confirmed by measurements of radioactive amines uptake associated with ATP hydrolysis in chloroplasts [10]. In the present experiments we have demonstrated the reversibility of the process of proton translocation induced by ATP hydrolysis in chloroplasts. The possible control of the rate of ATPase activity by proton flux and by membrane potential was concluded from the effect of uncouplers on ATPase activity.

METHODS

Chloroplast fragments were prepared either from two-week-old pea seedling leaves or from spinach leaves as previously described [2] in a medium containing 0.2 M sucrose, 20 mM NaCl, 50 mM tricine/NaOH (pH 7.8), 13 mM sodium ascorbate and 1 mg/ml bovine serum albumin. The chloroplasts were washed with 10 mM KCl and finally suspended in a medium containing 10 mM KCl and 10 mg/ml bovine serum albumin. Chlorophyll was determined in a 80 % acetone extract of chloroplasts [11].

The pH changes were assayed in a reaction mixture containing 50 mM KCl, 3 mM $MgCl_2$, 5 mM dithiothreitol, 30 μM phenazine methosulphate, 0.4 mM K_2HPO_4 and chloroplasts containing 100 μg/ml chlorophyll in a total volume of 3 ml at pH 8 and at 24 °C. When addition of ATP is indicated, a solution containing 250 nmol ATP, 250 nmol $MgCl_2$ (pH 8) in a volume of 5 μl was used. In several experiments 2 mM phosphoenol pyruvate and 1 I.U./ml pyruvate kinase were added to the reaction mixture. Pyruvate kinase was freed from $(NH_4)_2SO_4$ by dialysis of 0.2 ml of the enzyme against 2 l of 5 mM tricine–NaOH (pH 8) and 2 M KCl for 24 h at 4 °C. Pyruvate kinase activity was assayed by measuring pH changes [12].

ATPase activity was triggered by illumination with a 500-W slide projector through a RG 610 Schott filter. The chloroplast suspension was illuminated for 2 min in the absence of ATP. Magnesium-ATP was added between half to one min after light was turned off. The rate of ATPase activity was measured as a change in pH of the medium [9].

In some experiments ATPase activity was assayed by measuring the release of radioactive phosphate from [^{32}P]ATP. The reaction was started by addition of [^{32}P]ATP (10^5 cpm/μmol) instead of non-radioactive ATP. Aliquots were withdrawn and deproteinized with cold 3 % trichloroacetic acid. The $^{32}P_i$ content was assayed according to the isobutanol/benzene extraction procedure [13]. Highly labelled [^{32}P]ATP was prepared by phosphorylation of ADP with the same chloroplasts and isolation by the charcoal-adsorption method [14].

Proton uptake and proton release during ATPase activity were measured as a change in pH in a rapidly stirred medium by a G202B and a K401 Radiometer electrodes connected to a model 26 Radiometer pH meter and recorded on Rikadenki recorder.

RESULTS

The hydrolysis of ATP at pH 8 is accompanied by release of protons as indicated in the following scheme:

Scheme I: $Mg\text{-}ATP^{2-} + H_2O \rightarrow Mg\text{-}ADP^{1-} + P_i^{2-} + nH^+$.

A value of $n = 0.957$ ($n = \Delta H^+/P_i$) was calculated for the hydrolysis of ATP at pH 8 in the presence of Mg^{2+} [15]. The slow rate of decrease in pH on addition of ATP to a chloroplast suspension in the dark (Fig. 1A) indicated a low rate of ATPase

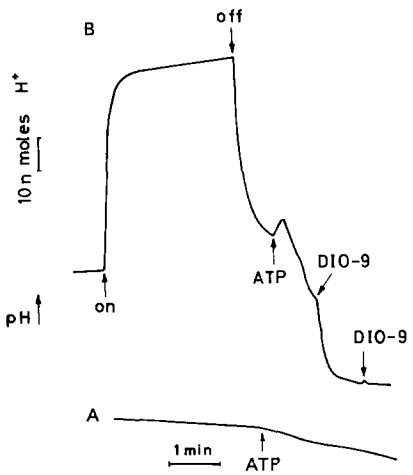

Fig. 1. The effect of DIO-9 on pH changes induced by ATP following light triggering of ATPase activity. The pH of the stirred chloroplast suspension was monitored by a recording pH meter. Reaction mixture was as described in Methods containing lettuce chloroplasts however pyruvate kinase and phosphoenol pyruvate were not included. A. The pH changes were measured in a chloroplast suspension in the dark. When indicated a solution of magnesium-ATP was added. B. Light was turned on for 2 min in order to trigger ATPase activity. Half a minute after the end of illumination magnesium-ATP was added. A final concentration of 10 μg/ml DIO-9 (pH 8) was added when indicated.

activity catalyzed by the chloroplasts. A great increase in the rate of ATP hydrolysis was observed following light triggering of ATPase activity in the presence of dithioerythritol (Fig. 1B). The light-induced proton uptake [2] is indicated in this figure as an increase in pH when light was turned on. During this illumination period ATPase activity was triggered. ATP was added approximately half a minute after light was turned off when the pH returned to its preillumination value. The drop in pH at steady state represented ATP hydrolysis and was used for the measurement of the rate of ATPase activity. This drop was preceded by a pH rise initiated by the addition of ATP in the dark. The increase in pH was assumed to be a result of proton uptake energized by ATPase activity minus protons released during ATP hydrolysis as seen in Scheme 1. Therefore the initial rate of proton uptake was calculated by adding the rate of proton released during steady state to the apparent rate of proton uptake.

On addition of the energy transfer inhibitor DIO-9 a fast drop in pH was observed. The drop was following by levelling off of the pH change which indicated an

inhibition of ATPase activity. It was assumed that the inhibition of ATPase activity by DIO-9 stopped the influx of protons. The passive release of the protons which were accumulated during ATP hydrolysis was indicated by a fast drop in the pH. A second addition of DIO-9 did not cause further decrease in pH indicating that once ATPase was inhibited the reagent itself did not cause any change in pH.

The rephosphorylation of ADP by phosphoenol pyruvate at pH 8 involves an uptake of protons from the medium according to Scheme 2:

Scheme 2: Phosphoenol pyruvate^{3-} + Mg-ADP^{1-} + nH^{+} → Mg-ATP^{2-} + pyruvate^{1-}.

Therefore the rephosphorylation of the ADP which was released during ATPase activity by pyruvate kinase with phosphoenol pyruvate as the phosphate donor could neutralize the pH changes caused by ATP hydrolysis (see Scheme 1). In the presence of phosphoenol pyruvate and pyruvate kinase the addition of ATP following light triggering caused a rise in the pH which was not followed by a drop (Fig. 2). From

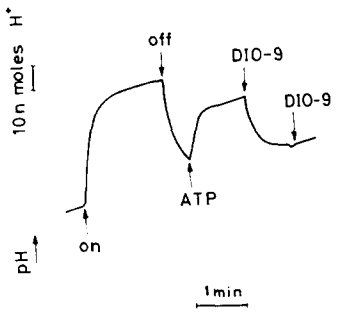

Fig. 2. The effect of DIO-9 on ATP induced pH changes. The reaction mixture included lettuce chloroplasts, pyruvate kinase and phosphoenol pyruvate as described in Methods. When indicated DIO-9 was added to give a final concentration of 10 μg/ml.

the p*K*a values for Mg-ATP^{2-}, Mg-ADP^{1-} and phosphoenol pyruvate [9] a value of $n = 0.992$ ($n = \Delta H^{+}/ATP$) was calculated for the phosphorylation of ADP at pH 8. The small increase in pH observed at steady state can be explained by a difference of 0.035 in the n value between ATP hydrolysis and ATP synthesis.

The initial rate of proton uptake was measured as the rate of pH rise minus the rate at steady state. The measurement of the initial rate in the presence of pyruvate kinase and phosphoenol pyruvate required only small correction since the rate at steady state was much smaller than the initial rate. The initial rates of proton uptake measured in the presence of pyruvate kinase were found to be in agreement with those measured without it. The pH rise caused by ATP in the presence of pyruvate kinase and phosphoenol pyruvate fell rapidly from steady state to the value which existed prior to the addition of ATP. The drop could result from the release of protons which accumulate during ATP hydrolysis. Addition of DIO-9 during light-induced proton uptake did not a cause decrease in pH (not shown).

Effect of pyridine and pH

In the presence of pyridine the amount of protons taken up at steady state

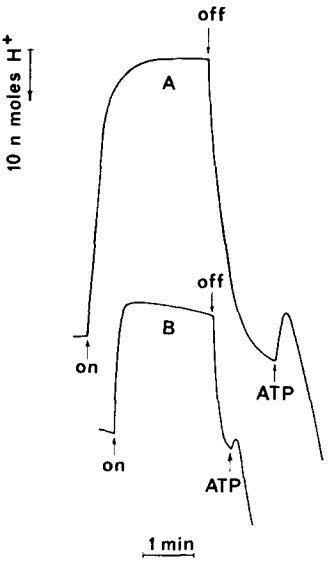

Fig. 3. The effect of pyridine on light and ATP induced pH changes. Reaction mixture was as described in Methods including lettuce chloroplasts but without phosphoenol pyruvate. A. Pyridine, 5 mM was included in the reaction mixture. B. No pyridine added.

during ATP hydrolysis was increased (Fig. 3). As was suggested for the light induced proton uptake [16], the increase was probably due to augmented internal buffer capacity brought about by the pyridine which penetrated inside the thylakoid membrane. The increase in the amount of protons taken up did not alter the stoichiometry between protons taken up and ATP hydrolyzed by the ATPase. However, H^+/ATP ratio increased when the pH was lowered from 8 to 7 (Table I). A decrease in the rate

TABLE I

pH DEPENDENCE OF THE H^+/ATP RATIO

The apparent initial rate of proton uptake was measured from the pH rise induced by the addition of magnesium-[^{32}P]ATP following light triggering of ATPase activity in the presence of phosphoenol pyruvate and pyruvate kinase as seen in Fig. 2. The rate of ATPase activity was assayed from the release of $^{32}P_i$ from γ-labelled [^{32}P]ATP as described under Methods.

pH	Rate of activity (μmol · mg chlorophyll^{-1} · h^{-1})		H^+/ATP
	ATP hydrolyzed	H^+ uptake	
8	62.2	106	1.7
7	35.7	137	3.8

of ATPase activity accompanied by a faster rate of proton uptake resulted in a higher H^+/ATP ratio at pH 7. This increase cannot necessarily be correlated to the increase in the internal buffer capacity which occurs at lower pH [3]. It seems that the internal

buffer capacity effects the amount of proton influx. However the apparent H^+/ATP ratio is altered with changes in the external pH.

Effect of uncouplers

The uncoupler *p*-trifluoromethoxyphenylhydrazone (FCCP) caused an increase in the rate of ATPase activity without a corresponding increase in proton uptake. As a result a decrease in the H^+/ATP ratio was observed (Table II). NH_4Cl had a similar effect at low concentrations however, at a concentration of 1 mM NH_4Cl the rate of proton uptake was lower while the rate of ATPase activity was

TABLE II

EFFECT OF UNCOUPLERS ON PROTON TRANSLOCATION INDUCED BY ATPase ACTIVITY

The rate of ATPase activity was measured from the pH changes at steady state following the addition of magnesium ATP. The apparent initial rate of proton uptake was calculated from the sum of the rates of pH rise induced by ATP plus the rate of pH decrease at steady state. When indicated uncouplers were added between the end of the light triggering and the addition of ATP. Spinach chloroplasts were used under the experimental conditions and in the reaction mixture as indicated under Fig. 1.

Additions	Rate of activity (μmol · chlorophyll^{-1} · h^{-1})		H^+/ATP
	H^+ uptake	ATP hydrolyzed	
None	125	85	1.46
FCCP, 0.5 M	125	106	1.18
FCCP, 1 μM	137	137	1.00
NH_4Cl, 0.5 mM	130	144	0.90
NH_4Cl, 1 mM	69	140	0.50
DCCD, 0.2 mM	72	47	1.54

higher than in the control experiment. Unlike uncouplers the energy transfer inhibitor dicyclocarbodiimide (DCCD) inhibited both ATPase activity and the associated proton uptake without altering the H^+/ATP ratio.

In an attempt to correlate proton movement to the rate of ATPase activity the effect of the uncoupler gramicidin was tested on light- and ATP-induced proton translocation as well as on the rate of ATPase. Between the concentrations of 10^{-9} and 10^{-8} M gramicidin the apparent rates of both light-induced proton uptake and the rate of ATPase activity were increased (Fig. 4). At concentrations higher than 10^{-7} M gramicidin both rates decreased. The amount of protons taken up in the light at steady state declined with increasing concentrations of gramicidin starting from 10^{-9} M. It seems that the rate of ATPase activity could be best correlated to the changes in the rate of proton uptake but no strict stoichiometry could be found.

The stimulation of ATPase activity which was caused by increasing concentrations of gramicidin up to 10^{-8} M was followed by stimulation of ATP-driven proton uptake (Fig. 4). As a result only a small change in H^+/ATP ratio was observed (Fig. 5). At gramicidin concentrations higher than 10^{-8} M, there was a faster decline in the rate of proton uptake than in the rate of ATPase activity with a concomitant drop in H^+/ATP ratio. The possibility that the effect of gramicidin at low concentrations

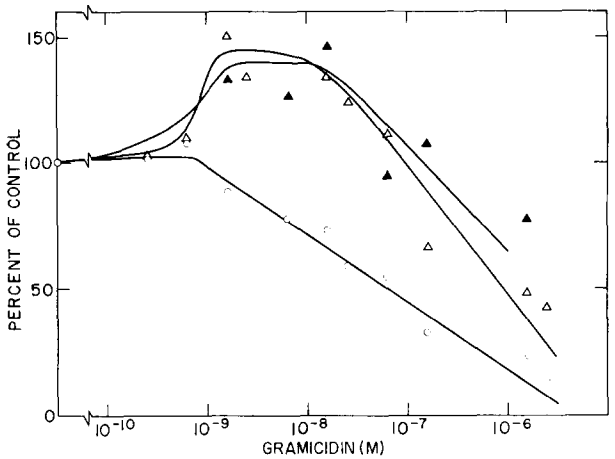

Fig. 4. The effect of gramicidin on light-induced pH changes and on light-triggered ATPase activity. The apparent initial rate of light-induced proton uptake (△) and the amount of protons taken up at steady state (○) were measured from recording of pH changes similar to those seen in Fig. 1 and under the same experimental conditions. The values recorded in the presence of gramicidin at the indicated concentrations were calculated relatively to the activity recorded in the absence of the uncoupler which was given the value of 100. The uncoupler was added after the illumination period which triggered ATPase activity. The rate of ATPase activity (▲) was calculated from the pH decrease at steady state which was reached in the dark following the addition of magnesium-ATP.

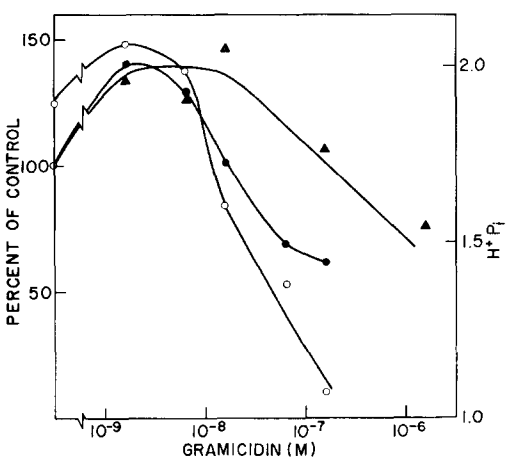

Fig. 5. The effect of gramicidin on the rates of ATP hydrolysis and the accompanying proton translocation. The apparent initial rate of ATP induced proton uptake (▲) was calculated as a sum of the pH increase which followed the addition of magnesium-ATP and the pH decrease at steady state. The rate of ATPase activity (●) was calculated from the rate of the pH decrease at steady state. The rate obtained without gramicidin was given the value of 100. The H^+/P_i ratio (○) was calculated by dividing the apparent initial rate of ATP-induced proton uptake by the rate of ATP hydrolysis at the indicated gramicidin concentration under the experimental conditions as described under Fig. 1. The uncoupler was added after light triggering of ATPase activity and before the addition of ATP.

was mainly due to an increase in the permeability of the membrane to potassium was verified by testing the effect of valinomycin.

Effect of valinomycin

Valinomycin stimulated the rates of ATPase activity and of the associated proton uptake (Table III). The effect was specific for K^+ as seen from the experiment

TABLE III

EFFECT OF VALINOMYCIN ON ATP-DRIVEN PROTON UPTAKE

Experimental conditions and reaction mixture were as indicated under Table II except for the changes in the monovalent cation as indicated.

Salt	Valinomycin (μM)	Rate of activity (μmol · mg chlorophyll^{-1} · h^{-1})		H^+/ATP
		H^+ uptake	ATP hydrolyzed	
50 mM NaCl, 1 mM KCl	—	96	67	1.43
50 mM NaCl, 1 mM KCl	1.3	121	87	1.40
50 mM KCl	1.3	153	102	1.50
50 mM KCl	2.0	170	112	1.53

in which K^+ replaced Na^+ as the major ion. Unlike gramicidin which also increases the permeability of membranes to protons, valinomycin did not cause a decline in the rate of ATPase activity and of proton uptake even at high concentrations. Thus the ratio of protons to ATP remained the same as in the control in spite of the increase in proton uptake induced by valinomycin.

DISCUSSION

The presence of a reversibly bound ATPase system which translocates protons across the coupling membrane during ATP hydrolysis is required according to the chemiosmotic hypothesis [1]. Protons which are translocated will be expected to be released down the diffusion-potential gradient when the energizing system is inhibited. Reversibility of the pH increase induced by ATP could indicate that the system supports proton translocation. The fast drop in the pH which followed the inhibition of ATPase activity by DIO-9 could be a result of a release of protons which were accumulated inside the thylakoid membrane during ATP hydrolysis. The direction of this fast pH change was opposite to the direction of the change observed on initiation of ATP hydrolysis. The extent of the reversed pH change was similar to that of the pH rise induced by ATP. This could be seen clearly in the experiments where the pH decrease caused by ATP hydrolysis was neutralized by the rephosphorylation of the released ADP with phosphoenol pyruvate and pyruvate kinase. Here it was shown that ATP hydrolysis caused a pH increase which levelled off after about 1 min. The pH change reached a steady state at this point as is evident from the fact that inhibition of ATPase activity by DIO-9 reversed the pH to its initial value.

The light induced pH changes were increased in the presence of pyridine. It was suggested that an increase in the internal buffer capacity caused by the pyridine

which penetrated the chloroplast membrane caused this increase. If this were the case it can be suggested that the increase in the ATP induced pH changes in the presence of pyridine indicated that protons were translocated across the membrane. The formation of a ΔpH during ATP hydrolysis [17] supports this suggestion.

The rate of proton translocation depended on the rate of ATP hydrolysis giving a stoichiometry of H^+/ATP approaching a ratio of 2 at pH 8. Increasing the permeability of the membrane to protons by the uncoupler FCCP did not decrease the apparent rate of proton uptake while stimulating ATPase activity. It can be assumed that even in the presence of the uncoupler the ratio of H^+/ATP was two. However, because of the much faster release of protons caused by the increase in the permeability of the membrane the instrumentation was not sensitive enough to measure the actual initial rate which could have been faster than the apparent rate. The overall effect of an uncoupler was to increase the turnover of protons and to increase the rate of ATPase activity. An apparent decrease in the rate of proton uptake resulted probably from even faster release of protons caused by 1 mM NH_4Cl while ATPase activity reached a faster rate.

The rate of ATP induced proton uptake was also controlled by a formation of a membrane potential. Such a potential is expected to be formed when proton influx is faster than the influx of an accompanying anion or of the efflux of a counter cation. The stimulation of the rate of proton uptake by valinomycin in a medium containing K^+ indicated the possible formation of a membrane potential which exerted back pressure on proton uptake. Valinomycin which specifically increases the permeability of membranes to K^+ probably facilitated an efflux of those ions that decreased the membrane potential which was apparently formed by proton uptake. Valinomycin has a similar effect on light induced proton uptake [6].

The rate of ATPase activity was stimulated both by uncouplers which increased the permeability of membranes to protons and by valinomycin which carries K^+ across the membrane. These effects may be interpreted to indicate the control of ATPase activity by both ΔpH and membrane potential. These properties are compatible with a reversible ATPase which catalyzes ATP formation in a system where both ΔpH and membrane potential can contribute to ATP synthesis [18]. Gramicidin at low concentrations acted as a K^+-specific ionophore while at higher concentrations it increased the permeability of membranes to both monovalent cations and to protons [6]. The effect of gramicidin on ATPase activity was consistent with these properties. At low concentrations it increased the rates of both ATPase activity and proton uptake without a considerable change in the H^+/ATP ratio. At higher concentrations the H^+/ATP ratio was decreased. The inhibition of ATPase activity at high concentrations was probably due to the decay of the triggered state of ATPase caused by uncouplers as we have previously shown [19].

Some caution should be exercised during the evaluation of the values of H^+/ATP ratio. It should be remembered that the calculations were based on the assumption that the initial rate of ATP hydrolysis was similar to the rate which was observed at steady state during the recording of pH changes. It is possible however that the rate was faster than that which was observed at steady state giving values higher than the real H^+/ATP ratios. To overcome this problem the initial rate of ATPase activity was measured as a release of $^{32}P_i$ from [^{32}P]ATP while proton uptake was measured as changes in pH of the reaction mixture in the presence of pyruvate kinase and phos-

phoenol pyruvate. The precision of this method was limited by the radioactive assay. In various experiments variations were observed in the H^+/ATP ratio however there was good agreement between the results obtained by the two methods. At each experiment the H^+/ATP ratio was found to be higher at pH 7 than at pH 8.

The increase of the H^+/ATP ratio at pH 7 could be explained if it is assumed that this ratio varies with the ratio of forces [20]. In this system H^+/ATP will depend on the ratio of phosphate potential to ΔpH and the membrane potential. It is possible that at pH 7 the ΔpH formed across the membrane is smaller than at pH 8, therefore a higher H^+/ATP ratio is obtained. These results point out the difficulties in the determination of the stoichiometry in this coupled system.

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