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ATP-Induced ΔpH Formation in Chloroplast ATP Synthase Proteoliposomes

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Summary. A procedure to reconstitute CF_0CF_1 proteoliposomes by gel filtration through a Sephadex-column pre-equilibrated with valinomycin and potassium is described. Proteoliposomes reconstituted by this procedure catalyze an ATP-induced ΔpH of 2.5 to 3.5 units. ΔpH was measured with either 9-aminoacridine or with the pH indicator pyranine trapped inside the proteoliposomes. CF_0CF_1 proteoliposomes prepared by conventional techniques catalyzed an ATP-induced $\Delta\psi$ formation, but were unable to catalyze an ATP-induced ΔpH even in the presence of valinomycin.

The ATP-induced ΔpH was sensitive to uncouplers and energy transfer inhibitors and was increased at low temperatures. It is suggested that ATP-induced ΔpH was observed in these proteoliposomes due to the efficient removal of intravesicular ammonium introduced with the CF_0CF_1 preparation. The ammonium acted as an internal buffer, and thus prevented an observable ΔpH formation.

Key Words proteoliposomes · chloroplasts · ATP-induced ΔpH · ATP synthase complex (CF_0CF_1).

Introduction

The isolated ATP synthase complex of chloroplasts (CF_0CF_1), when reconstituted into liposomes, has been shown to catalyze ATP hydrolysis, Pi-ATP exchange, acid-base dependent phosphorylation (Pick & Racker, 1979), light-dependent phosphorylation when coreconstituted with bacteriorhodopsin (Dewey & Hammes, 1981) or PS1 reaction centers (Hauska et al., 1980) and ATP-dependent $\Delta\psi$ formation (Shahak et al., 1982).

Attempts to measure ATP-induced ΔpH formation in these reconstituted proteoliposomes were unsuccessful (Dewey & Hammes, 1981; Shahak & Pick, 1983). However, an ATP-dependent ΔpH formation was observed with proteoliposomes reconstituted with a similar ATP synthase isolated from *E. coli* (Foster & Fillingame, 1979), *R. rubrum* (Schneider et al., 1980), the thermopholic bacterium *PS3* (Sone et al., 1976), pig heart mitochondria (Deleage et al., 1983) and the thermopholic cyanobacterium, *synechococcus 6716* (Van Walraven et al., 1983).

Having in mind the central role of ΔpH in photosynthetic energy transduction, we investigated possible reasons for the inability to demonstrate ATP-dependent ΔpH formation in CF_0CF_1 liposomes. Among others, we considered the possibility that residues of ammonium, carried over with the enzyme from the $(NH_4)_2SO_4$ precipitation step (Pick & Racker, 1979), were responsible for inhibiting the formation of a ΔpH by buffering the protons transported into the liposomes. This would inhibit the formation of a ΔpH while facilitating the formation of $\Delta\psi$. Indeed, the removal of the ammonium by passing the proteoliposomes through a Sephadex column pre-equilibrated with valinomycin and K^+, enabled us to measure ATP-dependent ΔpH formation in CF_0CF_1 proteoliposomes.

Materials and Methods

Preparation of CF_0CF_1

CF_0CF_1 complex was isolated from spinach leaves according to Pick and Racker (1979), with the following modifications: The leaves were ground in 200 mM sucrose, 100 mM NaCl and 50 mM Na-Tricine, pH 8.0. The homogenate was filtered through six layers of cheesecloth and one layer of miracloth, centrifuged for 5 min at 8000 × *g*, the pellet resuspended in 10 mM Na-pyrophosphate, pH 8.0, centrifuged for 7 min at 12,000 × *g* and resuspended in the same solution. The latter step was repeated four times. Solubilization of the membranes was with 0.33% Na-cholate and 16 mM octyl-glucoside. The enzyme precipitated between 35 and 45% $(NH_4)_2SO_4$. About 2 mg protein were mixed with 1 ml of 40 mg/ml soybean phospholipids containing 1.4% Na-cholate (wt/vol), incubated for 20 min at 4°C and stored in liquid nitrogen.

Reconstitution of Proteoliposomes

Liposomes were formed by two methods: 1) "Cholate dilution liposomes" were formed by addition of 50 μl of the protein-lipid-detergent mixture to 2 ml reaction medium containing 30 mM KCl, 3 mM $MgCl_2$, 1 mM KH_2PO_4, 20 mM Na-Tricine, pH 8.0, in

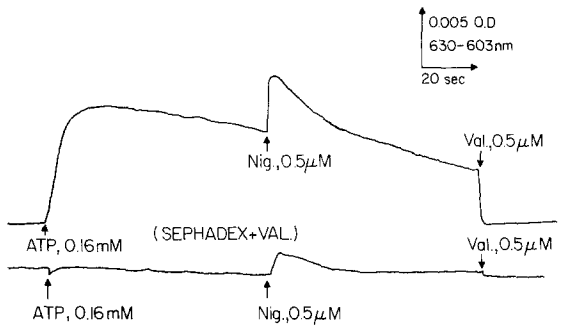

Fig. 1. ATP-dependent $\Delta\psi$ formation in CF_0CF_1 proteoliposomes. Reaction conditions as described under Materials and Methods. The liposomes were prepared by cholate dilution (upper trace), or by Sephadex + valinomycin purification (lower trace). The oxonol VI concentration was 1 μM. The measurement was at room temperature

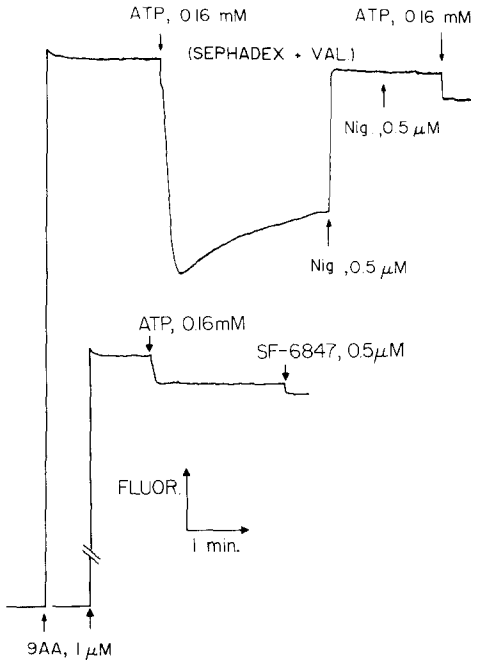

Fig. 2. ATP-induced ΔpH formation in CF_0CF_1 proteoliposomes. Reaction conditions as described under Materials and Methods. The liposomes were prepared by Sephadex + valinomycin (upper trace) or by cholate dilution (lower trace). The measurement was at room temperature

the measuring cuvette. 2) "Sephadex purified liposomes" were formed by placing 50 μl of the protein-lipid-detergent mixture on a Sephadex column (G-50 Coarse, 25 × 0.6 cm) and eluting with 30 mM KCl, 3 mM $MgCl_2$, 1 mM KH_2PO_4, 10 nM valinomycin and 20 mM Na-Tricine, pH 8.0, at room temperature. The flow rate was adjusted to 2 ml/min. The liposomes were collected into the measuring cuvette and diluted with the same reaction mixture to a final volume of 2 ml.

ΔpH and Δψ Measurements

ΔpH was measured by following the fluorescence changes of 9-aminoacridine (final concentration, 1 μM) in a Perkin-Elmer spectrofluorimeter (model MPF-44A) fitted with a magnetic stirrer and temperature control. Excitation was at 420 nm and emission at 465 nm with a 10-nm slit for both. The reaction medium contained in 2 ml about 100 μg protein, 30 mM KCl, 3 mM $MgCl_2$, 1 mM KH_2PO_4, 20 mM Na-Tricine, pH 8.0, and 0.25 to 10 nM valinomycin.

Calibration of the signal was performed by creating an artificial ΔpH across the proteoliposomes (Deamer et al., 1972) as follows: the pH of the liposome suspension was lowered (usually to 4.5 to 6.5) by addition of 10 mM sodium succinate of a preadjusted pH followed by 10-min equilibration at room temperature. The temperature was adjusted to the desired value (usually 0 to 10°C) and the measurement of fluorescence initiated. Sodium hydroxide (1 N) was injected in predetermined amounts to raise the pH to the desired value (usually 8.0), thus establishing a transient ΔpH. Since the total fluorescence of 9-aminoacridine was lower at the higher pH than at the lower pH, the total fluorescence was taken as that observed after addition of excess uncoupler at the higher pH. The calibration was performed at lower temperatures in order to slow down the decay of the ΔpH following NaOH addition.

Alternatively, intraliposomal pH was measured by following fluorescence changes of trapped pyranine, a membrane-impermeable pH indicator (Gould & Bell, 1981). Pyranine trapping was performed by preincubation of the protein-lipid-detergent mixture in the presence of 50 μM pyranine. One ml of the reaction medium containing 50 μM pyranine was added to the Sephadex column and allowed to settle into the Sephadex, followed by 50 to 200 μl of the protein-lipid-detergent-pyranine mixture. The liposomes were eluted with the reaction medium without pyranine. The proteoliposomes thus obtained, contained intraliposomal pyranine, but were free from most of the medium pyranine. The fluorescence of pyranine was measured by excitation at 465 nm and emission at 510 nm with a 10-nm slit for both.

$\Delta\psi$ measurements were performed by following the absorbance changes of oxonol VI as previously described (Shahak et al., 1982).

Assays

Pi-ATP exchange and ATP hydrolysis were measured as previously described (Pick & Racker, 1979).

Chemicals

Octyl-glucoside was obtained from Calbiochem; other chemicals were obtained from Sigma Chemical Co. Sephadex G-50 (coarse) was obtained from Pharmacia.

Results

As was previously shown (Shahak et al., 1982), ATP induced a transmembrane electrical gradient ($\Delta\psi$) in CF_0CF_1 proteoliposomes prepared by cholate dilution as indicated by oxonol VI absorbance changes (Fig. 1, top). The same proteoliposomes

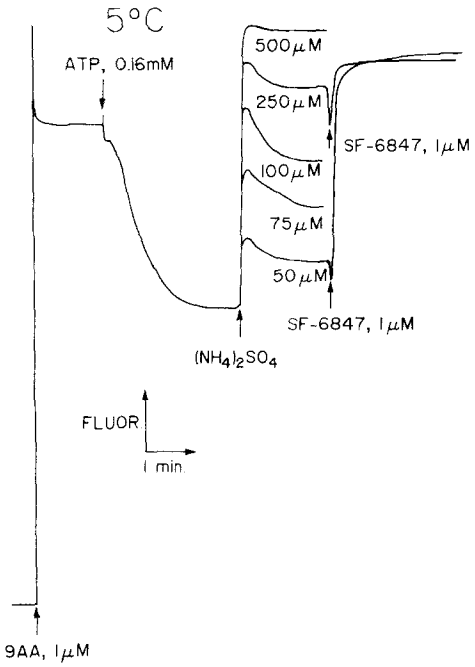

Fig. 3. Effect of ammonium-sulfate on the ATP-dependent ΔpH in Sephadex-purified proteoliposomes. Reaction conditions as in Fig. 2

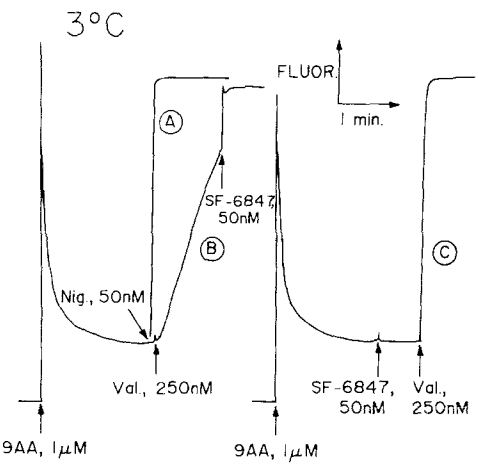

Fig. 4. Spontaneous ΔpH formation in proteoliposomes passed through Sephadex without valinomycin. Reaction conditions as in Fig. 2, except for the omission of valinomycin during the preparation of the proteoliposomes on the Sephadex column, and the temperature which was 3°C

showed no ATP-dependent ΔpH formation as determined by 9-aminoacridine fluorescence changes (Fig. 2, bottom). However, after passing such liposomes through a Sephadex column pre-equilibrated with K^+ and low concentration of valinomycin, ATP-dependent $\Delta\psi$ was no longer observed (Fig. 1, bottom), but a rather large ATP-dependent ΔpH was formed (Fig. 2, top). When the liposomes were passed through Sephadex without valinomycin, no ATP-dependent ΔpH formation was observed (*see* Fig. 5). Valinomycin added to cholate dilution proteoliposomes abolished the ATP-dependent $\Delta\psi$ (Fig. 1) but did not induce the appearance of an ATP-dependent ΔpH (*not shown*).

At lower temperatures (3 to 10°C) the ATP-induced ΔpH was larger in magnitude and slower in development (*not shown*). Even though ATP hydrolysis was slower at low temperatures, which accounts for the slower kinetics, the steady-state level of the ΔpH was larger, probably due to the reduced permeability of proteoliposomes to protons. These results are similar to our previous observations on the temperature dependence of $\Delta\psi$ formation in proteoliposomes as measured with oxonol VI (Shahak et al., 1982).

Uncouplers like nigericin, S-13 and SF-6847 abolished the ATP-induced ΔpH (Fig. 2). High concentration of ATP, S-13 or SF-6847 caused some chemical quenching of 9-aminoacridine fluorescence. This quenching was corrected for in the ΔpH calculations. Preincubation of the proteoliposomes with the energy transfer inhibitor DCCD for 10 min at room temperature completely inhibited the ATP-induced ΔpH (*not shown*). Calibration of the ATP-dependent 9-aminoacridine signal indicated that the quenching observed corresponded to a ΔpH of 2.5 to 3.5 units depending on the temperature and the concentration of ATP.

In order to check if the ammonium which was carried over with the enzyme could be responsible for the difficulties in observing ATP-induced ΔpH, we added ammonium sulfate to proteoliposomes purified by Sephadex + valinomycin. Figure 3 demonstrates that 0.25 mM ammonium sulfate indeed abolished the ATP-induced ΔpH.

When 9-aminoacridine was added to proteoliposomes that were passed through Sephadex without valinomycin, a large spontaneous quenching of the 9-aminoacridine fluorescence was observed (Fig. 4). This fluorescence quenching results most probably, from a ΔpH formed across the liposomes themselves due to efflux of NH_3, which decreases the intraliposomal pH. Indeed the fluorescence quenching was rapidly abolished by nigericin but only poorly by protonophores or valinomycin alone. Combination of valinomycin and a protonophore were very effective (Fig. 4). The observation that ATP did not induce fluorescence quenching of 9-aminoacridine in liposomes which were passed through Sephadex without valinomycin (Fig. 5 right) was most probably due to this large ΔpH which pre-exists in these liposomes. Thus, when low concentrations of valinomycin were added to such liposomes, followed by sufficient time to col-

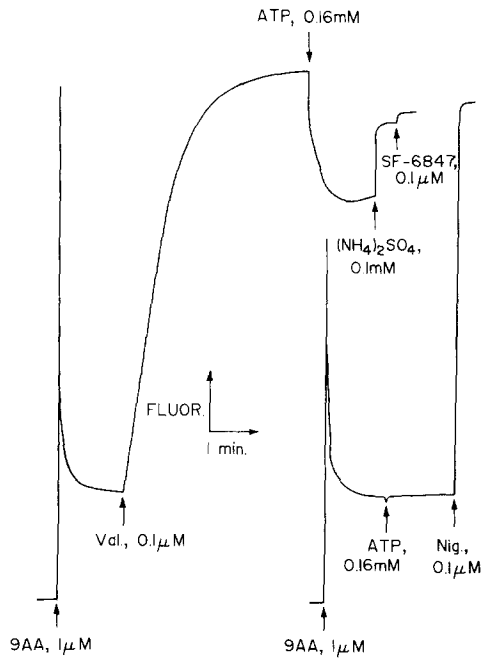

Fig. 5. ATP-induced ΔpH formation in proteoliposomes passed through Sephadex without valinomycin. Reaction conditions as in Fig. 4

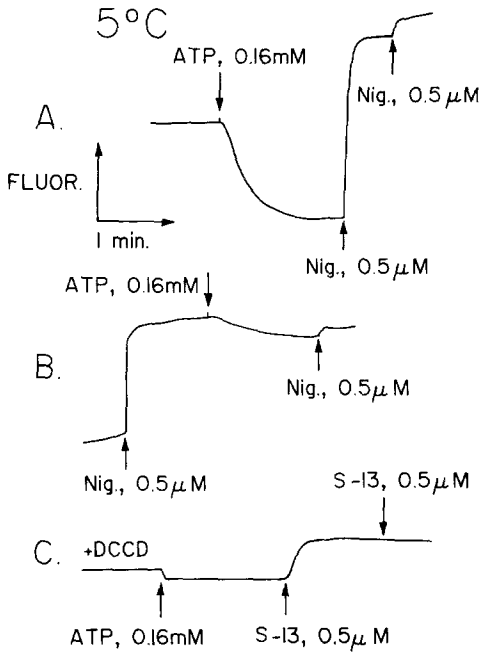

Fig. 6. Measurement of ATP-induced ΔpH in Sephadex-purified proteoliposomes with pyranine. Reaction conditions as in Fig. 2, except for the preparation of the liposomes with pyranine as described under Materials and Methods, and the temperature which was 5°C. In *C* the liposomes were preincubated for 10 min with 100 μM DCCD at room temperature

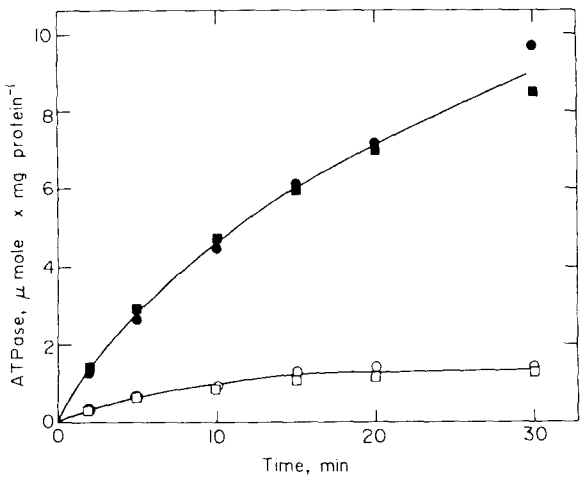

Fig. 7. ATP hydrolysis catalyzed by CF_0CF_1 proteoliposomes. Reaction conditions as in Fig. 1, except that no oxonol was added, and $AT^{32}P$ replaced ATP. The liposomes were either passed through Sephadex + valinomycin (○,●) or prepared by cholate dilution (□,■). ATP concentration was 3 mM (●,■) or 0.15 mM (○,□)

lapse the pre-existing ΔpH, ATP did lead to uncoupler-sensitive ΔpH formation (Fig. 5, left).

ATP-dependent ΔpH formation in CF_0CF_1 proteoliposomes was also observed by following fluorescence changes of trapped pyranine (Fig. 6). The ATP-induced pyranine fluorescence quenching was abolished by uncouplers like nigericin (Fig. 6) or S-13 (*not shown*). It was also inhibited by preincubation of the proteoliposomes with DCCD (Fig. 6). Temperature affected ΔpH formation similarly when followed by either pyranine or 9-aminoacridine. Calibration of the ATP-induced pyranine signal was not possible probably due to interference by left-over amounts of untrapped pyranine.

To find out if the Sephadex reconstitution in the presence of valinomycin does not affect the overall coupling between ATP synthesis or hydrolysis and $\Delta\mu_{H^+}$, we compared the rate of ATP hydrolysis and of Pi-ATP exchange catalyzed by cholate dilution liposomes and by Sephadex-purified liposomes. The results are summarized in Figs. 7 and 8. Since we used a relatively low concentration of ATP for the fluorescence studies (to minimize chemical quenching), we compared these reactions with low (0.15 mM) and high (3 mM) ATP. The rate of ATP hydrolysis was the same for both types of liposomes at each ATP concentration (Fig. 7), and the Sephadex-purified liposomes had a somewhat higher rate of Pi-ATP exchange than the cholate dilution liposomes (Fig. 8). As expected, both reactions were faster with 3 mM ATP, than with 0.15 mM ATP. The

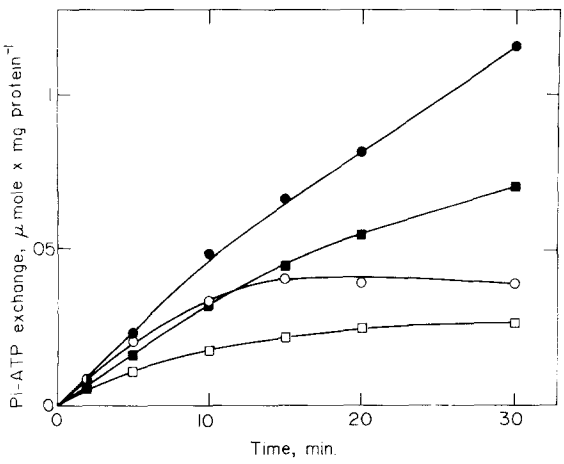

Fig. 8. Pi-ATP exchange catalyzed by proteoliposomes. Reaction conditions as in Fig. 1, except that no oxonol was added, and ^{32}Pi replaced Pi. The liposomes were either passed through Sephadex + valinomycin (○,●) or prepared by cholate dilution (□,■). ATP concentration was 3 mM (●,■) or 0.15 mM (○,□)

improvement in Pi-ATP exchange activity in Sephadex-purified proteoliposomes may be due to the removal of ammonium or cholate, or of both.

Discussion

The data shown indicate that previous difficulties (Dewey & Hammes, 1981; Shahak & Pick, 1983) in demonstrating an ATP-induced ΔpH in CF_0CF_1 proteoliposomes were most probably due to residual $(NH_4)_2SO_4$ introduced into the proteoliposomes with the CF_0CF_1 preparation during their formation. Passing such proteoliposomes through a Sephadex column pre-equilibrated with 10 nM valinomycin, an efficient ammonium ionophore, apparently removes their trapped ammonium by catalyzing a K^+-NH_4^+ exchange. This conclusion is consistent with the following observations: (1) In cholate dilution proteoliposomes, ATP-induced the formation of $\Delta\psi$ but not of ΔpH. (2) In proteoliposomes passed through Sephadex + valinomycin, the converse was true. (3) The addition of ammonium sulfate to Sephadex + valinomycin-purified proteoliposomes, abolished the ATP-induced ΔpH.

Ammonia acted in these proteoliposomes essentially as an internal buffer, rather than an uncoupler. Thus, additions of increasing amounts of $(NH_4)_2SO_4$ to the proteoliposome preparations (Fig. 3) decreased the magnitude of the steady-state ATP-induced ΔpH, rather than temporally decreasing the magnitude of the created ΔpH, as uncouplers do. Furthermore, even 10 mM NH_4Cl was without effect in uncoupling Pi-ATP exchange in cholate dilution proteoliposomes.

The $\Delta\psi$ observed in the cholate dilution proteoliposomes is due to the protons pumped into the liposomes coupled to ATP hydrolysis. Intravesicularly they combine with NH_3 to form NH_4^+ which does not permeate the vesicle. The $\Delta\psi$ so produced prevents further proton pumping, and thus one observes an ATP-dependent $\Delta\psi$ formation without an observable ΔpH.

In proteoliposomes which were passed through Sephadex without valinomycin, only the external $(NH_4)_2SO_4$ was removed. This induces the diffusion of the intraliposomal ammonia (NH_3) out to the medium. The protons remaining inside are then responsible for the observed spontaneous ΔpH (Figs. 4 and 5).

The observation that both ATP-dependent ΔpH and $\Delta\psi$ increase in magnitude at low temperatures seems to indicate a steeper temperature dependence for passive proton and counter-ions permeability than for ATP hydrolysis in the proteoliposome system. Thus, even though the rate of ATP-driven proton uptake decreases at lower temperatures the rate of proton leak across the membrane decreases to an even greater extent indicating a higher activation energy for proton equilibration across the proteoliposome membrane.

Passing the liposomes through Sephadex + valinomycin did not affect the rate of ATP hydrolysis, and somewhat increased the rate of Pi-ATP exchange (Figs. 7 and 8). The latter may again be a consequence of increased tightness relative to simple cholate dilution liposomes, due to removal of ammonium, and possibly cholate.

The reconstitution procedure described in this work provides a new valuable tool for assessing the kinetic and thermodynamic relations between ATP synthesis or hydrolysis and proton translocation in CF_0CF_1 proteoliposomes.

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