The mechanism lor the ATP-induced uncoupling of respiration in mitochondria of the yeast *Saccharomyces cerevisiae*

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We have recently reported that ATP induces an uncoupling pathway in *Saccharomyces cerevisiae* mitochondria [Prieto, Bouillaud, Ricquier and Rial (1992) Eur. J. Biochem. **208**, 487–491]. The presence of this pathway would explain the reported low efficiency of oxidative phosphorylation in *S. cerevisiae*, and may represent one of the postulated energy-dissipating mechanisms present in these yeasts. In this paper we demonstrate that ATP exerts its action in two steps: first, at low ATP/P, ratios, it increases the respiratory-chain activity, probably by altering the kinetic properties of cytochrome *c* oxidase. Second, at higher ATP/P, ratios, an increase in membrane permeability leads to a collapse in membrane potential. The ATP effect on cytochrome c oxidase corroborates a recent report showing that ATP interacts specifically with yeast cytochrome oxidase, stimulating its activity [Taanman and Capaldi (1993) J. Biol. Chem. **268**, 18754–18761].

INTRODUCTION

Millimolar ATP induces swelling when mitochondria of the yeast *Saccharomyces cerevisiae* are suspended in potassium acetate plus valinomycin and stimulates «-glycerophosphate oxidation [1]. Since ATP triggers potassium acetate-induced swelling only in the presence of valinomycin, a K^+/H^+ exchange was ruled out and involvement of a H^+ uniport was postulated. From these data we concluded that ATP activated a proton-conducting pathway, which we termed 'yeast uncoupling pathway' [1]. Interestingly, a similar effect of ATP has been observed with skeletal-muscle mitochondria [2].

Phosphate has been described to act as a 'coupling factor' in yeast mitochondria [3] and has been found to protect the inner-membrane structure against proton leakage [4]. In our previous paper we showed that phosphate inhibits the ATP-stimulated swelling [1]. Since ATP stimulates both respiration and swelling even in the presence of atractylate, we postulated that the regulatory nucleotide-binding site must be located in the outer face of the mitochondrial inner membrane. Previous reports have also suggested that external ATP regulates respiratory-chain activity in yeasts, probably at the level of the cytochrome c oxidase [5]. In line with this observation, it has been demonstrated that ATP modulates the activity of *S. cerevisiae* cytochrome *c* oxidase [6].

The presence of this uncoupling pathway would correlate with observations made both at the cellular level and with isolated mitochondria. Under a number of growth conditions, and in particular when yeasts are grown on a single carbon source, there is an excess production of reducing equivalents [7]. This has been described to lead to the activation of processes which are non-energy conserving, and thus lower the growth yield (reviewed in [8,9]). Several mechanisms have been proposed to be involved in energy dissipation [9], e.g. deletion of oxidative-phosphorylation sites, wastage of ATP by ATPases, increased proton-conducting capacity at the mitochondrial inner membrane. The induction of an NAD(P)H dehydrogenase which does not translocate protons [10,11] and the activation of a plasma-membrane H^+-ATPase [12] have previously been demonstrated. The newly described yeast uncoupling pathway would be an additional mechanism which would explain previous observations made at the level of the isolated mitochondrion [1]: low P:O ratios [13–15], decrease in P:O ratio in a flux-dependent manner at constant protonmotive force [15,16] and poor stimulation of respiration by ADP [13].

A report has appeared recently [17] where the observed ATP effects on potassium acetate-induced swelling are corroborated. However, the study of the swelling requirements with a number of salts leads to the conclusion that ATP is opening an anion channel which can be inhibited by phosphate. In the present paper we further investigate the effects of ATP on mitochondria and study the mechanism by which ATP stimulates respiration. We conclude that ATP has two distinct effects: first, it increases the activity of the cytochrome *c* oxidase, and second, it alters the membrane permeability.

MATERIALS AND METHODS

Strain, media and isolation of mitochondria

The diploid strain of *S. cerevisiae* W303 was grown aerobically at 28 °C in 1% yeast extract, 2% bactopeptone and 3% lactate as carbon source. Yeast cells were harvested in exponential growth phase, and mitochondria were prepared from protoplasts as previously described [1,18].

Oxygen consumption, membrane potential and pH-gradient measurements

Oxygen consumption was measured at 20 °C in a Hansa-Tech oxygen-electrode chamber in medium containing 0.65 M mannitol, 2 mM $MgCl_2$, 0.5 mM EGTA, 1 mg/ml albumin and 10 mM Tris/maleate, pH 6.8 [19]. Atractylate (10 /zM) and oligomycin (12.5 /zg/ml) were added when indicated. Mitochondrial protein concentration was 0.15 mg/ml. Potassium phosphate concentration was either 1 or 10 mM. K^+ concentration was maintained constant at 15 mM by addition of KCl. As respiratory substrate, 3 mM NADH was used, except for the experiments where the effects of nucleotides on cytochrome *c* oxidase were studied. For those experiments 0.2 mM *NNN'N'*-

Abbreviations used: $DiSC_2(5)$, 3,3'-diethylthiacarbocyanide iodide; DMO, 5,5-dimethyloxazolidine-2,4-dione; TMPD, NNN'N'-tetramethyl-p-phenylenediamine; FCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

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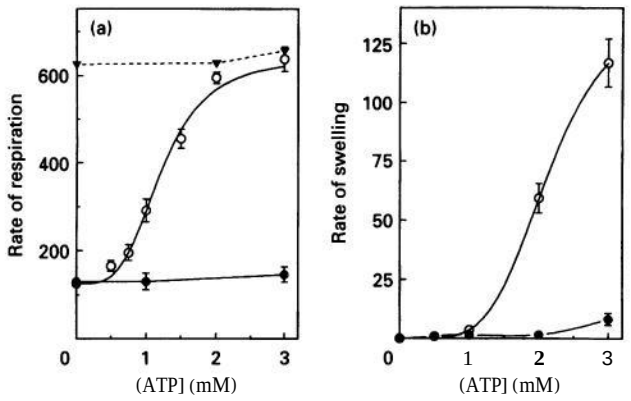


Figure 1 Effect ol increasing ATP concentrations on the rate of respiration (a) and swelling in potassium acetate (b) of *S. cerevisiae* mitochondria

(**a**) Respiration experiments were performed as described in the Materials and methods section: O, 1 mM phosphate; ●, 10 mM phosphate; ▼, 1 mM phosphate plus 10 ytM FCCP. (**b**) Osmotic swelling was determined from the rate of decrease of light-scattering by mitochondria in potassium acetate plus valinomycin in the presence of either 1 mM (O) or 10 mM phosphate (●). Swelling rates are given in arbitrary units, the swelling induced by addition of 1 mM ATP in the absence of phosphate being taken as 100. Results represent means ± S.E.M. of 5 independent experiments.

tetramethyl-p-phenylenediamine (TMPD) plus 5 mM ascorbate were used as substrate. Other additions are described in Figure legends.

Membrane potential was monitored simultaneously under the same conditions, by using the potential-sensitive fluorescent probe 3,3'-diethylthiacarbocyanine iodide [$DiSC_2(5)$; Molecular Probes] (0.16 *fiM*) in a SLM Aminco SPF-500 spectrofluorimeter with excitation at 643 nm and emission at 680 nm [19]. Probe calibration was performed in the presence of valinomycin by addition of increasing concentrations of KCl as described in [20].

The pH gradient was determined from the equilibrium distribution of the weak acid 5,5-dimethyl[2-^{14}C]oxazolidine-2,4-dione ([^{14}C]DMO) essentially as described in [20]. Mitochondria (0.5 mg/ml) were incubated in the above buffer plus [^{14}C]DMO (20 /tM; 0.4 fiCi/ml) and phosphate (1 mM). The mitochondrial suspension was layered on silicone oil AR 200 (Fluka) and mitochondria were separated by rapid centrifugation. Radioactivity was determined in both pellets and supernatants. Matrix volume was determined in parallel experiments using ^{3}HaO and [^{14}C]sucrose.

Osmotic swelling in potassium acetate was determined from the rate of light-scattering decrease exactly as previously described [1]. Protein was determined by the biuret method with albumin as standard. The use of fresh solutions throughout was essential for the reproducibility of the results.

RESULTS AND DISCUSSION

The present paper investigates the changes in membrane potential and rate of respiration brought about by ATP when yeast mitochondria oxidize NADH. The choice of substrate was made by taking into account the ability of *S. cerevisiae* mitochondria

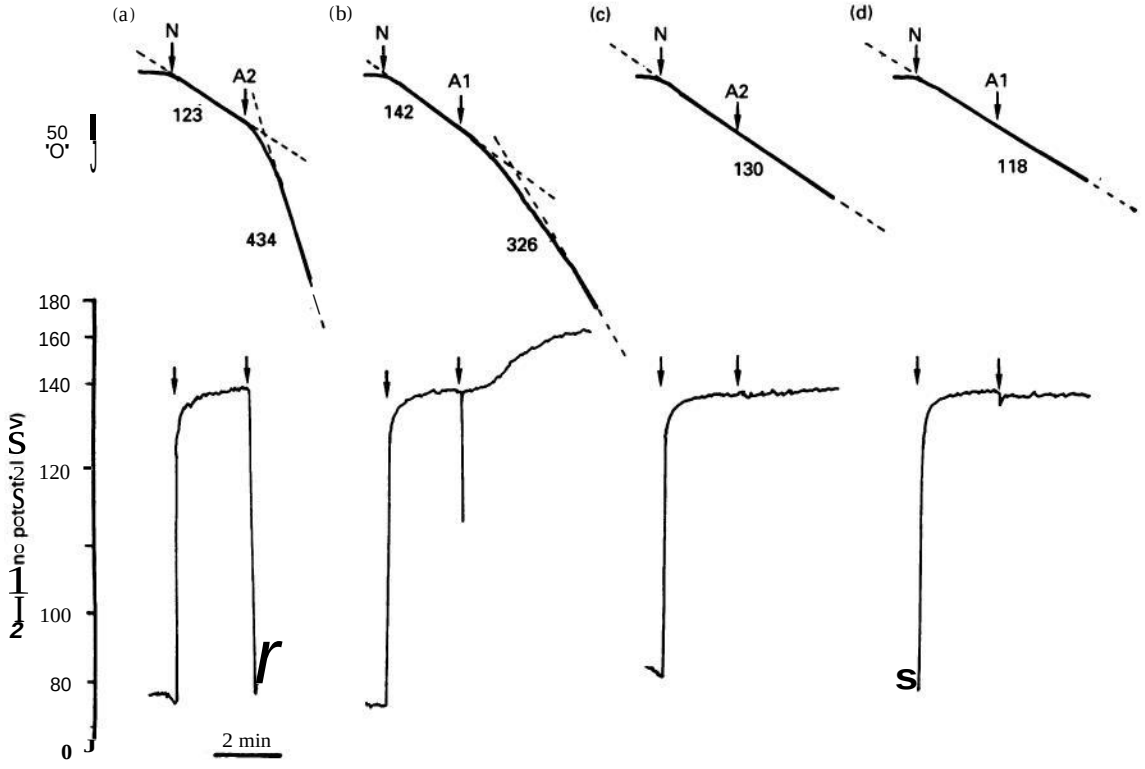


Figure 2 Effect of ATP on oxygen consumption (upper thick traces) and membrane potential (lower thin traces) of yeast mitochondria oxidizing NADH

Effect of 2 mM ATP in the presence of either 1 mM (**a**) or 10 mM (**c**) phosphate. Effect of 1 mM ATP in the presence of either 1 mM (**b**) or 10 mM (**d**) phosphate. Additions: N, NADH (3 mM); A1, ATP (1 mM); A2, ATP (2 mM). Atractylate (10 *fiU*) and oligomycin (12.5 /tg/ml) were always present. Rates ot respiration (numbers beside traces) are expressed in nmol of 'O'/min per mg of protein.

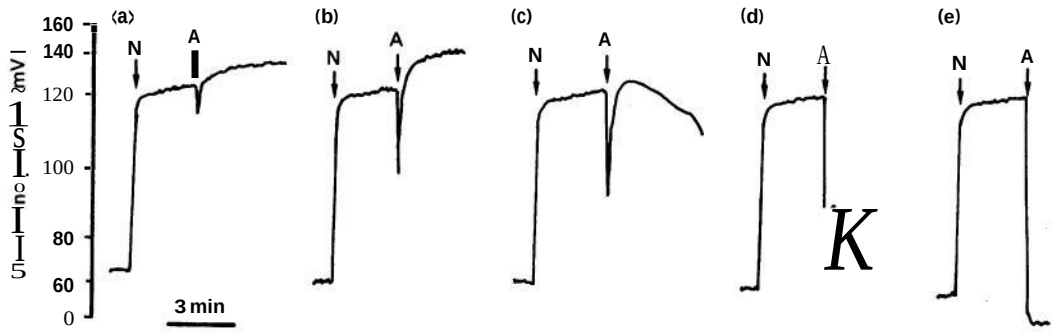


Figure 3 Effect of increasing ATP concentrations on the membrane potential of mitochondria oxidizing NADH

Additions: N, NADH (3 mM); A, ATP, 0.5 mM (a), 1 mM (b), 1.35 mM (c), 1.5 mM (d), 2 mM (e). Other conditions were as in Figure 2.

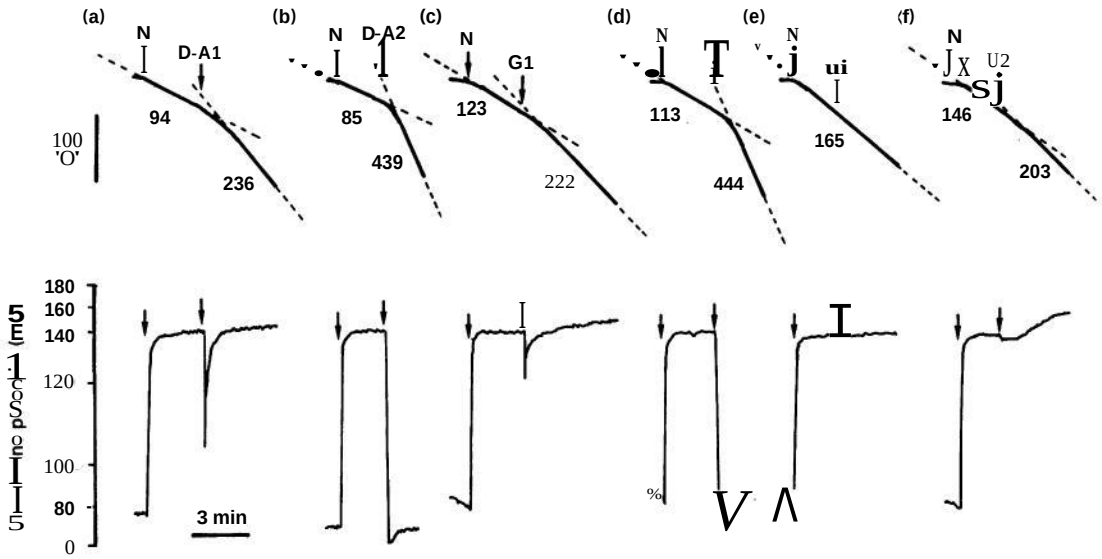


Figure 4 Effect of dATP, GTP and UTP on oxygen consumption (upper traces) and membrane potential (lower traces) of yeast mitochondria oxidizing NADH

(a) dATP (1 mM; D-A1); (b) dATP (2 mM; D-A2); (c) GTP (1 mM; G1); (d) GTP (2 mM; G2); (e) UTP (1 mM; U1); (f) UTP (2 mM; U2). Rates of respiration (numbers beside traces) are expressed in nmol of ^{1}O/min per mg of protein. Other conditions were as in Figure 2.

to oxidize external NADH and the report that with this substrate mitochondria display high respiratory control [4]. Figure 1(a) shows the effect of increasing ATP concentrations on the rate of NADH oxidation, and it is observed that ATP does activate respiration and that the kinetics are sigmoidal, hence similar to those found for the induction of swelling in potassium acetate (Figure 1b and [1]). The incubation mixture contained 1 mM phosphate, since in its absence it was not possible to obtain a stable State-4 rate (results not shown, and [3]). Two observations should be pointed out: first, the highest respiratory rate attained with ATP is equal to that obtained with a saturating concentration of carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) and second, the ATP effects are blocked with high phosphate (10 mM).

The effects of ATP on mitochondrial membrane potential and respiration are presented in Figure 2. Membrane potential was monitored by using the membrane-potential probe $DiSC_2(5)$, which was calibrated with K^+ diffusion potentials in the presence of valinomycin (results not shown). Control experiments demonstrate that, under our standard conditions (1 mM phosphate), addition of NADH causes a fast generation of a membrane potential which stabilizes at a value around 130–140 mV. Parallel experiments performed with the pH-gradient probe [^{14}C]DMO show that the value of the pH gradient in State 4 is 0.23 ± 0.08 unit ($n = 5$). Under these conditions, the respiratory control ratio observed with 0.2 mM ADP is 2.80 ± 0.096 ($n = 16$).

Figure 2(a) shows that when 2 mM ATP is added, in the presence of 1 mM P_i, a collapse in membrane potential and pH gradient is observed. This observation would indicate that either the membrane permeability to protons has increased or, according to Guérin et al. [17], ATP has opened an anion channel which causes the dissipation of the membrane potential. The ATP effects are blocked by 10 mM phosphate (Figures 1 and 2c). When 1 mM ATP is added, there is a progressive increase in membrane potential which stabilizes at values some 10–15 mV higher than in the original State 4 (Figure 2b). The value of the pH gradient under these conditions rises to 0.6 pH unit. The

observed changes are not due to an increased matrix volume, since ATP does not cause mitochondrial swelling in respiration medium (results not shown). The ATP effects on the mitochondrial membrane potential are progressive (Figure 3): low nucleotide concentrations cause a hyperpolarization, whereas high concentrations lead to the collapse in membrane potential. The striking increase in protonmotive force, paralleled with an acceleration of respiration, would agree with data from Figure 1. There it was observed that the ATP concentrations required to stimulate respiration are lower than those required to stimulate swelling in potassium acetate, i.e. channel opening [17]. It should be pointed out, however, that the media used for the two types of experiments (swelling and respiration) are different, and therefore the two ATP titrations may not be comparable.

Since these effects are caused by external ATP [1], experiments were performed in the presence of 10 /tM atractylate and 12.5 /rg/ml oligomycin to avoid interferences caused by the phosphorylation of ADP present in the ATP preparation or produced by non-mitochondrial ATPases. Control experiments have shown that these concentrations of inhibitors prevented the phosphorylation of added ADP (results not shown). The ATP action is corroborated with two nucleoside triphosphates which are not substrates for the mitochondrial ATP synthase: dATP and GTP. Figure 4 shows that dATP and GTP reproduce the effects reported for ATP; thus low nucleotide concentrations stimulate respiration without decreasing the membrane potential, whereas higher concentrations cause its collapse. High phosphate concentrations also block the effects of these nucleotides (results not shown). When the pyrimidine nucleotide UTP was tested, 1 mM nucleotide had no effect on either respiration or membrane potential, whereas at 2 mM it caused a slight stimulation of respiration and a modest increase in membrane potential. This rules out the possibility that the nucleoside triphosphates are exerting their action by lowering the free concentration of bivalent cations. It is interesting that Guérin et al. [17] have also described that GTP causes anion-channel opening, while UTP does not.

The simultaneous rise in respiration and protonmotive force suggests an ATP effect on a respiratory-chain complex. Several observations would point to cytochrome ***c*** oxidase as the putative site of action. First, it has been described that externally added ATP (i.e. in the presence of carboxyatractylate and oligomycin) lowers the level of reduction of cytochrome $a + a_3$ in ***S. cerevisiae*** mitochondria, and thus it has been suggested that cytosolic ATP is a kinetic effector of cytochrome ***c*** oxidase [5]. Interestingly, Mazat et al. [21] have shown that cytochrome oxidase is the main controlling step for both ATP synthesis and respiration in ***S. cerevisiae***. More recently, Taanman and Capaldi [6] have demonstrated that ATP and GTP interact specifically with solubilized ***S. cerevisiae*** cytochrome oxidase and stimulate its activity. The ATP effect on yeast mitochondria differs from that reported for bovine heart cytochrome oxidase, where cytosolic ATP increases the K_m for cytochrome ***c*** [22–24].

Figure 5 shows the effect of ATP on respiration and membrane potential when TMPD and ascorbate are used as substrates, and hence only cytochrome oxidase contributes to the generation of protonmotive force. Experiments clearly show that all the observations made with NADH as substrate (Figure 2) are reproduced when the electron flux is restricted to cytochrome ***c*** oxidase.

The data presented here demonstrate that ATP lowers the phosphorylation efficiency in ***S. cerevisiae*** mitochondria by acting at two levels: first, on the respiratory chain, increasing its activity at the level of the cytochrome c oxidase, and thus in agreement with previous reports [5,6]. Second, it causes the opening of an anion channel that increases membrane permeability [17]. The

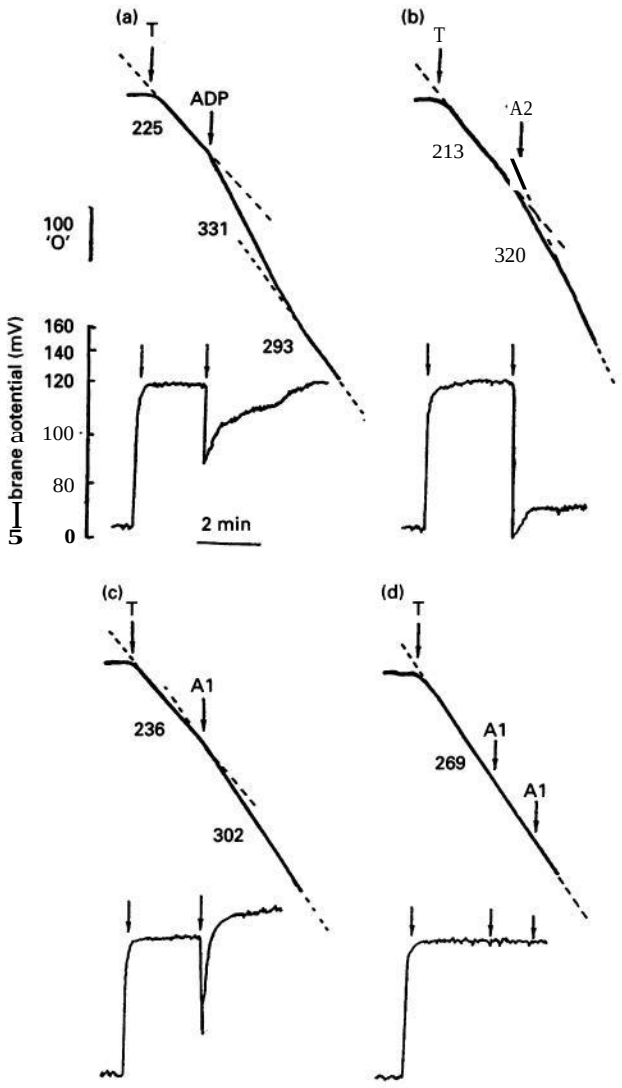


Figure 5 Effect of ADP and ATP on oxygen consumption (upper thick traces) and membrane potential (lower thin traces) of yeast mitochondria oxidizing TMPD/ascorbate

(a) Phosphorylation of ADP (0.2 mM) in the presence of 1 mM phosphate. (b) Effect of 2 mM ATP in the presence of 1 mM phosphate. (c) Effect of 1 mM ATP in the presence of 1 mM phosphate. (d) Effect of ATP in the presence of 10 mM phosphate and 12.5 /rg/ml oligomycin. Additions: T, ascorbate (5 mM) and TMPD (0.2 mM); A1, ATP (1 mM); A2, ATP (2 mM). Rates of respiration (numbers beside traces) are expressed in nmol of 'O'/min per mg of protein.

postulated 'yeast uncoupling pathway' [1] must be composed of two components, although it remains to be established if both are associated with cytochrome ***c*** oxidase. It should be noted that the ATP/P, ratio is the relevant parameter which determines both the rate of respiration and the membrane potential. Therefore, at high P, concentrations, the uncoupling abilities of ATP are inhibited, and yeast mitochondria appear as those from other sources. The cytosolic concentrations of ATP and P, oscillate in a region where, in view of these results, they should modify the phosphorylation efficiency [25,26]. Indeed, it has been described that in ***S. cerevisiae*** the energetic efficiency correlates inversely with the cytosolic ATP concentration [27].

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