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Original Article

Unisite Hydrolysis of [γ^{32}P]ATP by Soluble Mitochondrial F_1-ATPase and Its Release by Excess ADP and ATP. Effect of Trifluoperazine

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Some of the characteristics of unisite hydrolysis of [γ^{32}P]ATP as well as the changes that occur on the transition to multisite catalysis were further studied. It was found that a fraction of [γ^{32}P]ATP bound at the catalytic sites of F_1 under unisite conditions undergoes both hydrolysis and release induced by medium nucleotides upon addition of millimolar concentrations of ADP or ATP. The fraction of [γ^{32}P]ATP that undergoes release is similar to the fraction that undergoes hydrolytic cleavage, indicating that the rates of the release and hydrolytic reactions of bound [γ^{32}P]ATP are in the same range. As part of studies on the mechanisms through which trifluoperazine inhibits ATP hydrolysis, its effect on unisite hydrolysis of [γ^{32}P]ATP was also studied. Trifluoperazine diminishes the rate of unisite hydrolysis by 30–40%. The inhibition is accompanied by a nearly tenfold increase in the ratio of [γ^{32}P]ATP/^{32}Pi bound at the catalytic site and a 50% diminution in the rate of ^{32}Pi release from the enzyme into the media. Trifluoperazine also induces heterogeneity of the three catalytic sites of F_1 in the sense that in a fraction of F_1 molecules, the high-affinity catalytic site has a turnover rate lower than the other two. Trifluoperazine does not modify the release of previously bound [γ^{32}P]ATP induced by medium nucleotides. The latter indicates that hindrances in the release of Pi do not necesarily accompany alterations in the release of ATP even though both species lie in the same site.

KEY WORDS: ATP release; mitochondrial F_1; trifluoperazine; unisite ATP hydrolysis.

INTRODUCTION

The mitochondrial ATP synthase is formed by a membrane moiety (F_0) that allows the coupling of electrochemical H^+ gradients to the synthesis of ATP by the catalytic portion, F_1. The latter may be obtained as a soluble protein that catalyzes the hydrolysis of ATP (Penefsky *et al.*, 1960). It is formed by five different subunits in a stoichiometry of 3α, 3β, 1γ, 1δ, 1ε, in order of decreasing molecular weight (Knowles and Penefsky, 1972a,b; Foster and Fillingame, 1982; Moradi-Améli and Godinot, 1983, Pedersen and Amzel, 1993). F_1 has six binding sites for adenine nucleotides, three of which possess catalytic properties (Garret and Penefsky, 1975; Cross and Nalin, 1982; Xue *et al.*, 1987; Cross, 1988). Some years ago, the important observation was made (Grubmeyer and Penefsky, 1981; Grubmeyer *et al.*, 1982) that at concentrations of ATP lower than those of the enzyme, F_1 catalyzed ATP hydrolysis through the function of only one catalytic site. The kinetics and thermodynamics of the various steps of unisite hydrolysis have been thoroughly studied (Grubmeyer *et al.*, 1982; Cross *et al.*, 1982; Al-Shawi and Senior, 1988; Al-Shawi *et al.*, 1990; Cunningham and Cross, 1988). Also, site-directed mutagenes in F_1 from *E. coli* (Duncan and Senior, 1985; Parsonage *et al.*, 1987; Al-Shawi *et al.*, 1989; Wood *et al.*, 1987; Weber *et al.*, 1994; Omote

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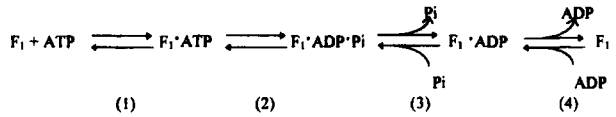
et al., 1995) and inhibitors of F_1 have been used to gain insight into the steps of the catalytic cycle (Kandpal *et al.*, 1985; Tommasino and Capaldi, 1985; Noumi *et al.*, 1987a,b). The reaction sequence of unisite catalysis is



Scheme 1

Although F_1 can function with only one catalytic center, it has been extensively documented (Grubmeyer and Penefsky, 1981; Cross *et al.*, 1982; Hackney and Boyer, 1978; Hutton and Boyer, 1979; O'Neal and Boyer, 1984) that the rate of unisite hydrolysis is accelerated approximately 10^5 times when excess ATP is added. This is due to an increase in the rates of substrate hydrolysis and product release from the catalytic site upon filling of the other catalytic sites, i.e., the alternating site mechanism of Boyer *et al.* (Hackney and Boyer, 1978; Hutton and Boyer, 1979; Kayalar *et al.*, 1977; Boyer, 1993). In confirmation of previous data (Grubmeyer and Penefsky, 1981; Cross *et al.*, 1982; Hackney and Boyer, 1978; Hutton and Boyer, 1979; O'Neal and Boyer, 1984), it was found that under unisite hydrolysis of [γ^{32}P]ATP, the addition of excess ATP or ADP enhanced the rate of hydrolysis of [γ^{32}P]ATP bound to the catalytic site; however, it was now observed that the addition of excess ATP (or ADP) caused the release of a fraction of the bound [γ^{32}P]ATP.

The effect of trifluoperazine (TFP)[2] on unisite ATP hydrolysis was also determined. This was because at saturating ATP concentrations, the maximal inhibition attained with TFP is approximately 70% (García *et al.*, 1995), and thus it was considered of interest to explore the mechanisms that lead to an enzyme that works at 30% of its maximal velocity. It was found that TFP inhibited by about 30% unisite hydrolysis; the inhibition was accompanied by an increase in the ratio of bound ATP/Pi at the catalytic site and a diminution in the rate of Pi release.

In the light of the data observed with and without TFP, it was studied if the alterations of steps 2 and 3 induced by TFP reflect on ATP release from the catalytic site induced by excess nucleotides. The results show that although TFP affects the rates of steps 2 and 3, the release of bound [γ^{32}P]ATP induced by medium nucleotides was not affected. This indicates that impairements in the release of one of the species (Pi) do not necessarily accompany modifications in the release of ATP, even though both share the same site.

[2] Abbreviations: EDTA, ethylenediaminetetraacetic acid; MES, 2[N-morpholino]ethanesulfonic acid; TFP, trifluoperazine; Tris, tris[hydroxymethyl]aminomethane.

MATERIALS AND METHODS

All nonradioactive chemicals were from Sigma; ^{32}Pi was purchased from NEN and used without further purification for the preparation of [γ^{32}P]ATP according to Glynn and Chappell (1964). Soluble F_1 was prepared from bovine heart mitochondria as described elsewhere (Tuena de Gómez-Puyou and Gómez-Puyou, 1977); its ATPase activity at 24°C was 60–80 μmol per min per mg as measured spectrophotometrically at pH 8.0 (García *et al.*, 1995). F_1 was stored at 4°C in a 50% ammonium sulfate suspension that contained 4 mM ATP and 2 mM EDTA, pH 7.4. Before use, the suspension was centrifuged and the pellet dissolved at a concentration of 5 mg per ml in 40 mM MES/Tris, 3 mM Mg-acetate, and 1 mM KH_2PO_4, pH 8.0; the dissolved enzyme was passed by centrifugation through Sephadex G-50 columns (Grubmeyer and Penefsky, 1981) equilibrated with the same buffer. Protein in the eluate was determined according to Lowry *et al.*, (1951) using bovine serum albumin as standard. TFP was freshly prepared for each experiment; its concentration was calculated using a molecular extinction coefficient of log ϵ = 4.5 at 258 and nm (Post *et al.*, 1980).

Unisite ATP Hydrolysis

The standard buffer used in all unisite reactions contained 40 mM MES/Tris, 3 mM magnesium acetate, and 1 mM KH_2PO_4 adjusted to pH 8.0 with KOH. F_1, [γ^{32}P]ATP (1–2 × 10^6 cpm/nmol), TFP, and other reactants added during unisite hydrolysis were dissolved in this standard buffer. Unisite hydrolysis was started by mixing 100 μl of 2 μM F_1 with 100 μl of 0.6 μM [γ^{32}P]ATP. At a ratio of 0.3 ATP/F_1 only the high-affinity hydrolytic site is filled (Grubmeyer *et al.*, 1982; Penefsky, 1988). In studies with TFP, this was added at the desired concentration in both the buffer and enzyme solutions. At various times, the reaction was arrested by mixing with 0.3 ml 8.3% trichloroacetic acid. When the transition of unisite to multisite hydrolysis was studied, Mg-ATP or Mg-ADP (5 mM

final concentration) was added to the unisite reaction mixture (cold chase), and at various times, the reaction was arrested with trichloroacetic acid (5% final concentration). The final volume of the arrested samples was 0.5 or 1.0 ml; to these 0.5 ml of 3.3% ammonium molybdate dissolved in 3.75 N H_2SO_4 and 0.2 ml of acetone were added. The resulting phosphomolybdate complex was extracted with 1 ml of butyl acetate; the organic phase was discarded and the extraction repeated two more times. From the radioactivity of the aqueous phase the amount of [γ^{32}P]ATP that remained was calculated.

Binding of [γ^{32}P]ATP and ^{32}Pi to F_1 during Unisite Hydrolysis

To this purpose, 55 or 110 μl of soluble F_1 (2–4 μM) was mixed with equal volumes of [γ^{32}P]ATP (0.6–1.2 μM, respectively). At the desired times, the mixture was passed by centrifugation (1.5 min) through Sephadex G-50 columns equilibrated with the unisite buffer without ATP; when the effect of TFP was explored, the equilibrating buffer also contained TFP. The eluates were received in 100 μl of 10% SDS. An aliquot was used to measure protein, and the rest divided into two portions. One was used to determine total radioactivity (^{32}Pi + [γ^{32}P]ATP) bound to F_1; the other was extracted three times with butyl acetate after formation of the phosphomolybdate complex as described above. The radioactivity of the remaining aqueous phase was used to calculate the amount of bound [γ^{32}P]ATP; from the difference of the total radioactivity and that of [γ^{32}P]ATP, the amount of ^{32}Pi bound to F_1 was calculated. The eluate of buffer without F_1 contained 1–2% of the total radioactivity introduced.

Rate Constant of ^{32}Pi Release

This was determined by incubation of 100 μl mixtures of [γ^{32}P]ATP with F_1 under unisite conditions with and without TFP. After 20 s the mixtures were passed through Sephadex centrifugation columns and the eluate received in 0.1 ml of 10% SDS (time zero in the experiments). Identical samples of the unisite mixtures were also passed through the centrifuge columns; these eluates were received in empty tubes. At the indicated times (see Results section), the latter eluates were again passed by centrifugation through Sephadex columns and received in SDS for determination of protein, total radioactivity, and its distribution into ^{32}P and [γ^{32}P]ATP as described above. The decrease in the amount of ^{32}Pi bound to F_1 in the second eluates was used to calculate the rate of ^{32}Pi release.

Release of Bound [γ^{32}P]ATP after a Cold Chase

This was determined by incubating F_1 with [γ^{32}P]ATP under unisite conditions, followed by the addition of 5 mM ADP, 10 mM glucose, and 1 mg/ml of hexokinase (360 units). After 5 min, the reaction was arrested with HCl 1.3 N final concentration. The samples were placed in boiling water at 90–93°C for 30 min in order to hydrolyze the existing [γ^{32}P]ATP. Thereafter, the samples were extracted with butyl acetate after formation of the phosphomolybdate complex (see above). The radioactivity that remained in the aqueous phase after the heat treatment ([^{32}P]glucose-6-phosphate) was considered to correspond to [γ^{32}P]ATP that became accessible to hexokinase in the cold chase. Control experiments showed that after heat treatment, less than 1% of [γ^{32}P]ATP remained and that at least 98% [^{32}P]glucose-6-phosphate was heat resistant. Other control experiments showed that all [γ^{32}P]ATP added to a mixture that contained hexokinase and glucose (with and without F_1) was trapped as [^{32}P]glucose-6-phosphate and that 5 mM MgADP did not interfere with hexokinase.

RESULTS

At saturating concentrations of ATP, TFP modifies the kinetics of the ATPase activity of F_1. It diminishes the V_{max} by approximately 70%, whereas the K_m for Mg-ATP is increased from 0.1 to 0.2 mM (García *et al.*, 1995). In consequence, TFP diminishes V_{max}/K_m by about twofold. To probe into the mechanisms that lead to an enzyme that works with 50% efficiency, the effect of TFP on unisite ATP hydrolysis was determined. Figure 1 shows the hydrolysis of 0.3 μM [γ^{32}P]ATP by 1 μM F_1, with and without TFP. In a time course, an initial rapid burst of hydrolysis was followed by a slower rate of ATP breakdown. The initial burst reflects the velocity of binding of ATP to the catalytic site and the establishment of an equilibrium between hydrolysis and synthesis of bound [γ^{32}P]ATP. The slower phase is a state in which ^{32}Pi is produced and slowly released into the media (Grub-

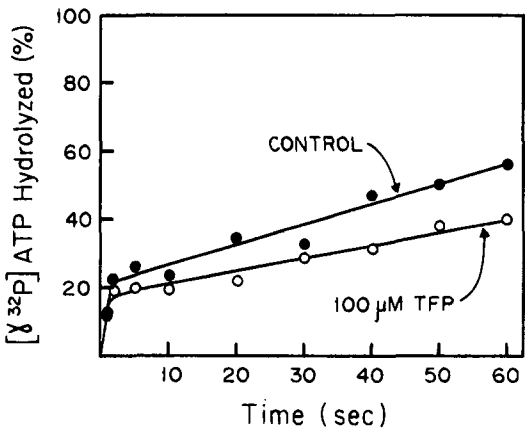


Fig. 1. Unisite hydrolysis of [γ^{32}P]ATP by F_1. Effect of TFP. Unisite hydrolysis was measured as described under Materials and Methods. At zero time, 100 μl of 2 μM F_1 was mixed with 100 μl of 0.6 μM [γ^{32}P]ATP. Where indicated, the mixtures contained 100 μM TFP. At the times shown, the reaction was stopped with 5% trichloroacetic acid final concentration and diluted to a volume of 0.5 ml. After extracting ^{32}Pi, the amount of remaining [γ^{32}P]ATP was determined.

meyer *et al.*, 1982); this was inhibited by TFP by 38% (average of five identical experiments ± 8.3).

Distribution of bound ATP and Pi at the Catalytic Site

The decrease in the rate of unisite [γ^{32}P]ATP hydrolysis induced by TFP suggested that in its presence the amount of ATP that bound to F_1 was lower, or that TFP hindered the breakdown of bound [γ^{32}P]ATP. Therefore, F_1 was incubated under conditions for unisite hydrolysis with and without TFP, and at different times, the mixtures were passed by centrifugation through Sephadex columns. Analysis of the eluate showed that in both cases, most of the radioactivity introduced (0.3 mol/mol F_1) had bound to the enzyme (Figs. 2 and 3). Hence, TFP did not interfere with ATP binding. However, when the distribution of the radioactive label into ^{32}Pi and [γ^{32}P]ATP was determined, a significant difference between the two samples became apparent. TFP shifted the distribution of ^{32}P between [γ^{32}P]ATP and [^{32}P]Pi bound towards [γ^{32}P]ATP. The shift to a higher ATP/Pi ratio at the catalytic site could be observed at concentrations of TFP as low as 5 μM (Fig. 3). This shift was likely due to the binding of TFP to a high-affinity site of F_1, since with 5 μM TFP, the ratio of TFP/F_1 was 2.5.

Release of ^{32}Pi From the Catalytic Site

Under unisite conditions, there is continuous synthesis and hydrolysis of ATP at the catalytic site (Hackney and Boyer, 1978; Kayalar *et al.*, 1977). Thus, it was studied if the change induced by TFP on the ratio of ATP/Pi at the catalytic site was related to the rate at which Pi is released from the enzyme into the media. Accordingly, F_1 was incubated under unisite conditions with and without TFP for 20 s and thereafter passed through Sephadex centrifugation columns; F_1 in the eluate contained [γ^{32}P]ATP and ^{32}Pi (Fig. 4). In order to determine the rate of Pi release, aliquots of identical eluates were filtered again at different times through Sephadex columns. In accordance with the reported value of 3×10^{-3} s^{-1} (Grubmeyer *et al.*, 1982), the rate constant of Pi release in control F_1 was 3.1×10^{-3} s^{-1}; with TFP, this decreased to 1.7×10^{-3} s^{-1} (an inhibition of 45%). In both cases, the data fitted a first-order equation (Fig. 4). The reason why this decrease is slightly (7%) higher than the inhibition of unisite hydrolysis by TFP (see above) is not clear.

Transition of Unisite to Multisite Catalysis

One of the most notable features of F_1-ATPase is its strong positive cooperativity (Grubmeyer and Penefsky, 1981; Cross *et al.*, 1982; Hackney and Boyer, 1978; O'Neal and Boyer, 1984). In fact, the addition of high concentrations of ATP to F_1 catalyzing unisite hydrolysis increases 30 times the rate of hydrolysis at the catalytic site, whereas that of product release increases by a factor of 10^5 (Cross *et al.*, 1982; Cunningham and Cross, 1988; Penefsky and Cross, 1991). In consonance with these data, Fig. 5 shows that the addition of saturating ATP concentrations to F_1 undergoing unisite catalysis produced a rapid breakdown of the bound [γ^{32}P]ATP. However, not all the bound [γ^{32}P]ATP was hydrolyzed after introduction of excess ATP. After the hydrolytic burst (one second after the addition of excess ATP), about 30% of the [γ^{32}P]ATP introduced at the beginning of the experiment was not hydrolyzed. In TFP-treated F_1, the cold chase also produced a burst of hydrolysis; however, in this case, the sum of [γ^{32}P]ATP hydrolyzed during unisite hydrolysis and the hydrolysis attained in the first second after the cold chase was lower than in control F_1. The data of Fig. 5 also show that with or without TFP, the rapid burst of hydrolysis induced by excess ATP was followed by a slower rate of hydrolysis of [γ^{32}P]ATP.

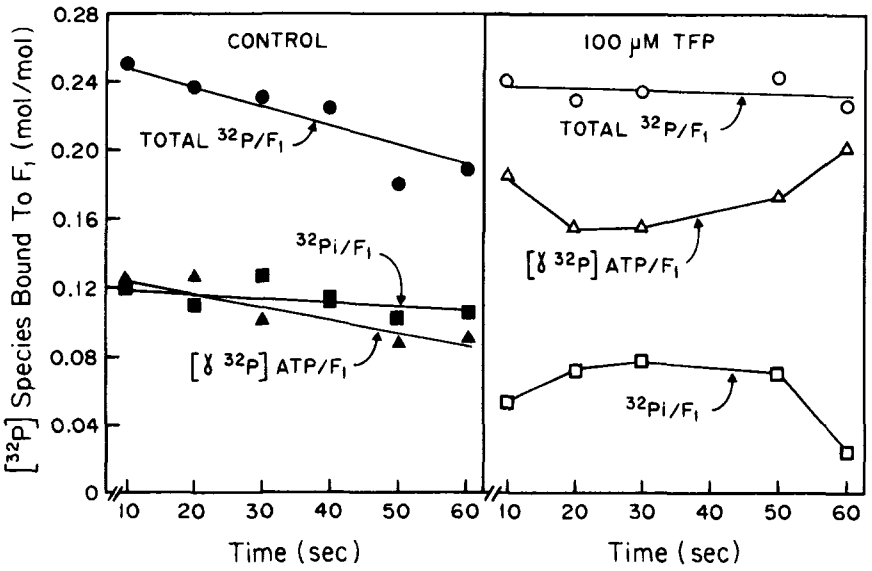


Fig. 2. Amount of [γ^{32}P]ATP and ^{32}Pi bound to F_1 under unisite conditions. Effect of TFP. Unisite hydrolysis was started by mixing 55 μl of 2 μM F_1 with 55 μl of 0.6 μM [γ^{32}P]ATP. At the indicated times, 100 μl of the mixture was passed through centrifuge columns. The eluates were received in 100 μl 10% SDS and the amount of protein, ^{32}Pi, and [γ^{32}P]ATP was determined as described under Materials and Methods. The left and right panels show experiments without and with 100 μM TFP, respectively. (●,○) total ^{32}P ([γ^{32}P]ATP + ^{32}Pi), (■,□) ^{32}Pi, and (▲,△) [γ^{32}P]ATP bound at the catalytic site.

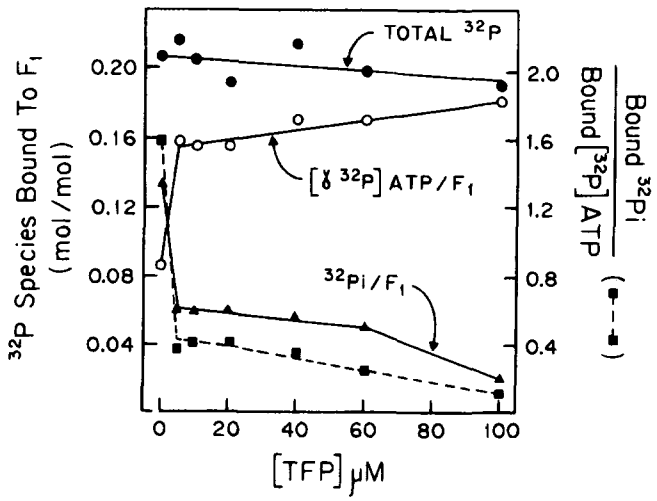


Fig. 3. Effect of different concentrations of TFP on the [γ^{32}P]ATP to ^{32}Pi ratio at the catalytic site of F_1 undergoing unisite hydrolysis. Unisite hydrolysis was started by mixing 110 μl of 4 μM F_1 with 110 μl of 1.2 μM [γ^{32}P]ATP that contained the indicated final concentrations of TFP. Ten seconds later, 100-μl samples were passed through Sephadex columns containing the same concentration of TFP and the eluate was received in 100 μl 10% SDS. In the latter, the amount of protein, total ^{32}P (●), [γ^{32}P]ATP (○), and ^{32}Pi (▲) were determined; (■) ratio of ^{32}Pi/[γ^{32}P]ATP at the catalytic site.

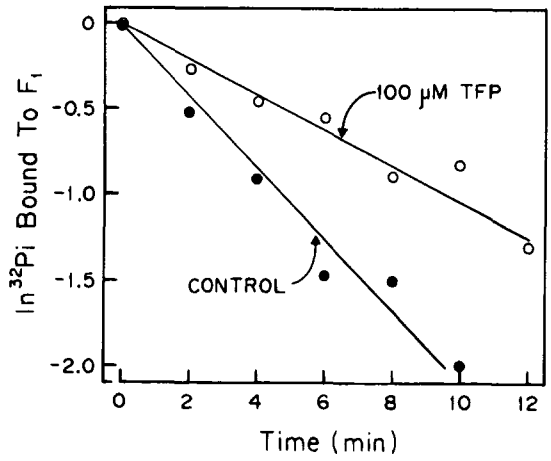


Fig. 4. Rate of Pi release from F_1 catalyzing unisite hydrolysis of [γ^{32}P]ATP. Effect of TFP. Unisite hydrolysis was initiated by mixing 110 μl 4 μM F_1 with 110 μl of 1.2 μM [γ^{32}P]ATP, with and without 100 μM TFP. The experimental details are described under Materials and Methods. At time zero, the samples without and with TFP contained 0.15 and 0.025 nmol of ^{32}Pi per nmol of F_1. The logarithm of the fraction of bound ^{32}Pi remaining at different times is plotted against time. From the slope of the decrease in the amount of ^{32}Pi bound to F_1, the first-order rate constants were calculated. The values obtained were 3.1×10^{-3} s^{-1} and 1.7×10^{-3} s^{-1} for the control and the TFP treated samples, respectively.

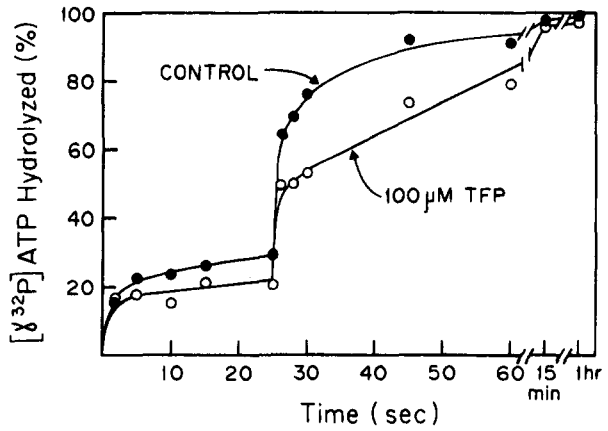


Fig. 5. Transition from unisite to multisite hydrolysis. Effect of TFP. Unisite hydrolysis was measured as in Fig. 1 (2 μM F_1 and 0.6 μM [γ^{32}P]ATP) and was allowed to proceed for 25 s; at this time 100 μl of 15 mM Mg-ATP was added to give a final 5 mM concentration of Mg-ATP in the cold chase. At the times indicated the reaction was arrested with trichloroacetic acid. ^{32}Pi was extracted as described and the amount of [γ^{32}P]ATP remaining was determined. The results show the percent of total [γ^{32}P]ATP hydrolyzed.

These results suggested that the portion of the bound [γ^{32}P]ATP that was not hydrolyzed in 1 s after the cold chase was released and mixed with medium ATP, which thereafter underwent hydrolysis. In the presence of TFP, the rate of [γ^{32}P]ATP hydrolysis that followed the hydrolytic burst was lower than in control F_1, as expected from the inhibiting effect of TFP on ATP hydrolysis at saturating substrate concentrations (de Meis *et al.*, 1988; García *et al.*, 1995).

Hydrolysis and Release of [γ^{32}P]ATP Bound under Unisite Conditions

The possibility that in a cold chase a portion of the [γ^{32}P]ATP bound at the catalytic site could be released into the medium and mixed with cold nucleotides was directly determined. In F_1 loaded with [γ^{32}P]ATP under unisite conditions, the introduction of millimolar concentrations of ADP (similarly to ATP) induces a burst of hydrolysis of previously bound [γ^{32}P]ATP (Grubmeyer and Penefsky, 1981). Hence, excess ADP together with hexokinase plus glucose was added to F_1 undergoing unisite hydrolysis of [γ^{32}P]ATP. As the activity of hexokinase introduced was in a tenfold excess over the hydrolytic activity of F_1, most of the [γ^{32}P]ATP released from the enzyme upon the addition of ADP would be trapped by hexokinase, yielding [^{32}P]glucose-6-phosphate.

Under unisite conditions, only about 2% of the total [γ^{32}P]ATP that had been introduced was accessible to hexokinase (Fig. 6). At this state, the addition of ADP + hexokinase produced a burst of hydrolysis of about 20% of the enzyme bound [γ^{32}P]ATP; the rest was found as [^{32}P]glucose-6-phosphate. Thus, upon the addition of ADP, close to 30% of the bound [γ^{32}P]ATP became accessible to hexokinase, which indicates that this fraction of the previously bound [γ^{32}P]ATP had the capacity to be released into the medium when excess ADP is added. A release of [γ^{32}P]ATP of similar magnitude was observed in the presence of TFP.

In order to further discard the possibility that any free [γ^{32}P]ATP could contribute to the formation of [^{32}P]glucose-6-phosphate induced by ADP (Fig. 6), a control experiment was made in which previous to the addition of excess ADP (± glucose-hexokinase), F_1 (1 μM) was mixed with 0.3 μM [γ^{32}P]ATP and 15 s later the mixture was filtered through Sephadex columns. Ten seconds after filtration, the eluted enzyme retained almost half of added [γ^{32}P]ATP (0.142 mol/mol F_1). Of this bound nucleotide, 70% (0.1 mol/mol F_1) was hydrolyzed in 2 s after adding excess MgADP; 30% (0.042 mol/mol F_1) was released by MgADP and trapped by hexokinase-glucose, and 3% (0.004 mol/mol F_1) was accessible to hexokinase in the absence of MgADP. Thus, as observed with the unfiltered enzyme (Fig. 6), the fraction of [γ^{32}P]ATP not committed to rapid hydrolysis was also trapped by hexokinase in filtered F_1 undergoing unisite hydrolysis.

The overall results of Figure 6 show that the fraction of [γ^{32}P]ATP that underwent hydrolysis nearly equalled the fraction that is released by medium nucleotides. This indicates that notwithstanding the presence of TFP, the rate of [γ^{32}P]ATP release was as fast as that of the splitting reaction (approximately 300 s^{-1}, see Penefsky, 1988). However, it is pointed out that in the presence of TFP, after 2 s of applying a cold chase with either ADP or ATP, about 15% of enzyme bound [γ^{32}P]ATP was not hydrolyzed, and not released into the media (Fig. 7). Nonetheless, this [γ^{32}P]ATP was at a catalytic site as illustrated by the following experiment. To F_1 catalyzing unisite hydrolysis of [γ^{32}P]ATP for 25 s in the presence of TFP, an ATP chase was applied; at 35 s the samples were filtered through Sephadex columns as in Fig. 7. A sample that was received in SDS had 0.025 nmol ATP per nmol F_1. Identical samples were received in empty tubes and quenched with SDS; after 5 min the ATP content decreased to 0.003 nmol per F_1; after 15 min, no ATP was detected. The failure of a fraction of previously

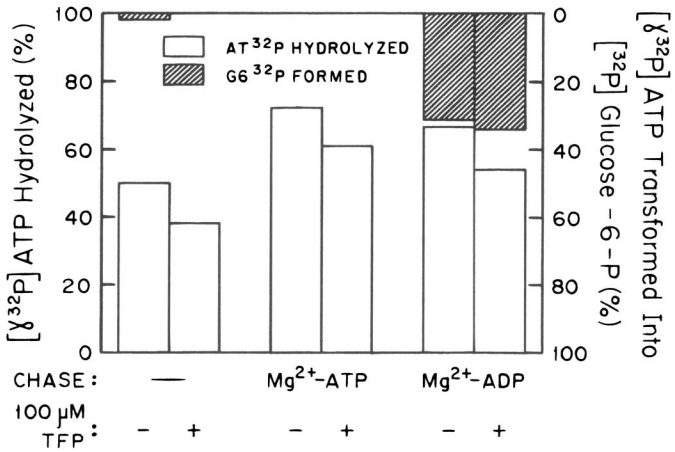


Fig. 6. Hydrolysis and release of [γ^{32}P]ATP bound to F_1 under unisite conditions after a cold chase. Effect of TFP. F_1 was incubated under unisite conditions (6 μM F_1 and 1.8 μM [γ^{32}P]ATP). After 25 s, the reaction was either quenched with HCl (1.3 N, final concentration), or supplemented with 15 mM MgATP or 15 mM MgADP (5 mM final concentrations) as indicated. After 2 s the reaction was stopped with HCl; afterwards, the amount of remaining [γ^{32}P]ATP was determined (open bars). In separate samples, after 25 seconds of hydrolysis, the unisite mixture was supplemented with 5 mM MgADP, 30 mM glucose, and 350 units per ml of hexokinase (HK) (final concentrations); after 5 min the reaction was quenched with HCl. The amount of [γ^{32}P]ATP that remained and [^{32}P]glucose-6-phosphate formed (filled bars) was determined as described under Materials and Methods. The first two bars (left side) show the amount of [γ^{32}P]ATP hydrolyzed at 25 s of incubation. Where indicated, the mixtures contained 100 μM TFP throughout the various treatments. Note that after a cold chase with Mg-ADP + hexokinase, the totality of the radioactivity was accounted for, either as [γ^{32}P]ATP or [^{32}P] glucose-6-phosphate, whereas in the presence of TFP, 15% [γ^{32}P]ATP was not hydrolyzed, nor trapped by hexokinase.

bound ATP to undergo release or hydrolysis after repeated turnovers after a cold chase suggests that TFP produces a heterogeneity of hydrolytic sites. Functional and structural heterogeneity of catalytic sites has been reported (Melese and Boyer, 1985; Beltrán *et al.*, 1988; Bullough *et al.*, 1987; Fromme and Gräber, 1989; Matsuno-Yagi and Hatefi, 1990; Bragg and Hou, 1990; Shapiro and McCarty, 1990; Abrahams *et al.*, 1994). It is also evident that in the presence of TFP, the site that retains and slowly hydrolyzes bound [γ^{32}P]ATP exhibits a high affinity for ATP, otherwise it would have been released into the media.

DISCUSSION

In confirmation of reported data (Grubmeyer and Penefsky, 1981; Grubmeyer *et al.*, 1982; Cross *et al.*, 1982; Penefsky, 1988), it was observed that at concentrations of ATP lower than F_1 concentrations, ATP rapidly binds to the enzyme and thereafter undergoes slow hydrolysis; in this state, excess ATP produces a rapid cleavage of a 70–80% portion of enzyme-bound [γ^{32}P]ATP as the consequence of an enhancement in its rate of hydrolysis and an increase in the rate of product release (Grubmeyer *et al.*, 1982; Cross *et al.*, 1982; Penefsky, 1985, 1988; Souid and Penefsky, 1995 and Fig. 5); Penefsky (1988) suggested that the 20–30% portion of [γ^{32}P]ATP that is not rapidly hydrolyzed was due to enzyme heterogeneity. However, in the present experiments we observed that in the transition of uni- to multisite hydrolysis, a fraction of approximately 30% of the bound [γ^{32}P]ATP is released into the media. This illustrates that upon addition of excess ADP or ATP, [γ^{32}P]ATP bound at the catalytic site under unisite conditions can follow two pathways, i.e., hydrolysis or release. This indicates that the reported unisite rates of ATP binding and

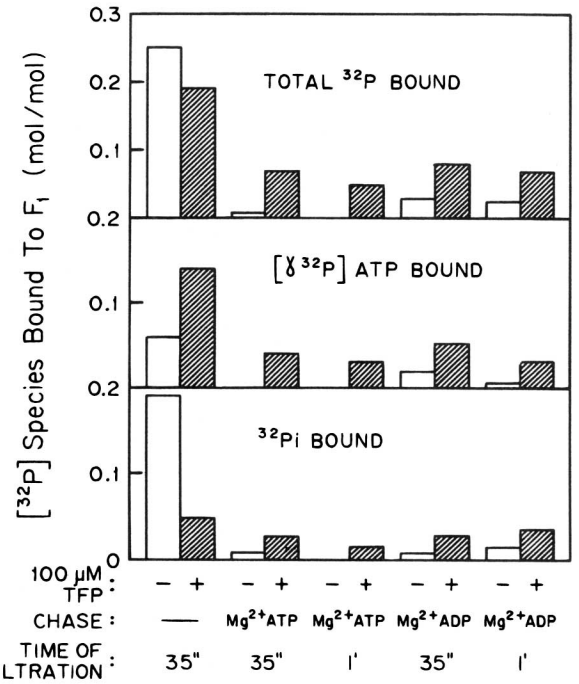


Fig. 7. Amount of [γ^{32}P]ATP bound and ^{32}Pi bound to F_1 after acceleration of unisite hydrolysis by cold chases. F_1 (50 μl, 6 μM) was mixed with [γ^{32}P]ATP (50 μl, 1.8 μM) to start unisite hydrolysis. After 25 s, 100 μl of unisite buffer (no chase), 10 mM MgATP, or 10 mM MgADP were added to give 5 mM final concentrations of cold nucleotides. At the times shown, samples were filtered through centrifuge columns, and the amount of [γ^{32}P]ATP, ^{32}Pi, and protein eluted from the columns were measured as described in Figs. 2–4 and in Materials and Methods. The upper panel shows the total radioactivity bound to F_1, the middle panel shows the amount of [γ^{32}P]ATP, and the lower panel the amount of ^{32}Pi. Control experiments are shown in white bars, and the experiments made in the presence of 100 μM TFP in shaded bars.

hydrolysis in F_1 would be slightly underestimated, since these values have been calculated assuming that none of the bound [γ^{32}P]ATP is released into the media. In fact, in particulate F_1, Suoid and Penefsky (1995) found a value of 2.3×10^5 M^{-1} s^{-1} for ATP binding measured by the cold chase technique which increased to 8×10^5 M^{-1} s^{-1} when measured with a hexokinase trap. It is important to mention that a release of bound [γ^{32}P]ATP under unisite conditions induced by excess ATP has been previously detected by a hexokinase trap; this release was observed in a mutant of F_1 from *E. coli* (β-M209I) in which the unisite hydrolysis and the transition from unisite to multisite catalysis are impaired. This mutant was thought to promote the release of bound [γ^{32}P]ATP by the binding of medium ATP at a second or third catalytic site (Al-Shawi *et al.*, 1989).

This work also probed the mechanisms through which TFP (de Meis *et al.*, 1988; García *et al.*, 1995), similarly to other amphipatic cations (Palatini, 1982; Adade *et al.*, 1984; Chazotte *et al.*, 1982; Laikind and Allison, 1983; Bullough *et al.*, 1989), produces a partial inhibition of ATP hydrolysis at saturating substrate concentrations. It was found that unisite catalysis was partially inhibited by TFP, and that TFP diminished the rate of Pi release during unisite catalysis. As the inhibiting effect of TFP on ATP synthesis, and hydrolysis at saturating ADP or ATP concentrations is prevented by Pi in a competitive form (de Meis *et al.*, 1988; García *et al.*, 1995), it is likely that TFP acts by hindering the movements of Pi to and from the catalytic site. Rosing *et al.* (1977) and Al-Shawi *et al.* (1990) showed that the partition of Pi from the media into the catalytic site requires a fairly large energy input, which implies that the release of Pi from the catalytic site is energetically favorable. Therefore, it is likely that TFP acts by imposing kinetic impairements to the partition of Pi between the media and the catalytic site. This alteration could account for the inhibition of ATP hydrolysis induced by TFP; however, it is noted that TFP also increases the ratio of ATP/Pi bound to the high-affinity catalytic site of F_1, and induces a heterogeneity of the catalytic sites, which may also contribute to its inhibiting effect on ATP hydrolysis. In other conditions, heterogeneity of catalytic sites has also been observed (Bullough *et al.*, 1987; Vázquez-Laslop and Dreyfus, 1990; Murataliev and Boyer, 1994). An increase in the ATP/Pi ratios at the catalytic site during unisite conditions was also observed by Al-Shawi *et al.* (1989) in mutants of the β from *E. coli* in which amino acids located at or near the catalytic site were substituted by less polar residues. Thus, as previously suggested (de Meis *et al.*, 1988; de Meis, 1989), it is possible that TFP increases the hydrophobicity of the catalytic site, thereby shifting the equilibrium between ATP and ADP + Pi.

Taken together, the data of this work show (i) that in the transition of unisite to multisite hydrolysis, a fraction of the previously bound ATP is released by medium nucleotides, and (ii) that in unisite conditions, TFP imposes hindrances in Pi release and increases the ratio of ATP/Pi at the catalytic site. Hence, it was asked if the change in ATP/Pi ratio and the hindrances in Pi release run in parallel to modifications of ATP release from the catalytic site induced by excess adenine nucleotides. The results showed that the amount of bound [γ^{32}P]ATP that is released by medium nucleo-

tides, as well as the initial entrance of [γ^{32}P]ATP into the catalytic site, were nearly of the same extent with and without TFP. However, the rate of Pi release was 50% lower in the presence of TFP. A similar pattern in the effect of TFP is observed in multisite hydrolysis and respiration-driven ATP synthesis; TFP lowers the V_{max} of ATP hydrolysis with only a moderate increase in the K_m for ATP, whereas the inhibition of ATP synthesis driven by electron transport is observed only at relatively low Pi concentrations (García *et al.*, 1995). Therefore, the results in soluble and particulate F_1 indicate that TFP induces alterations in the partition of Pi between the media and the enzyme; however, the results also show that these alterations do not accompany modifications of the partition of ATP, albeit both Pi and ATP occupy the same catalytic site. In this respect, it is pointed out that Al-Shawi and Senior (1992) indicated that the environment of the catalytic site in unisite catalysis is more hydrophobic when it is occupied with ATP than when it has ADP. Therefore, it is possible that these differences in hydrophobicity of the catalytic site are related to different pathways of partition of the two species, ATP and Pi, into and from the catalytic site.

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