Use of Trypsin To Monitor Conformational Changes of Mitochondrial Adenosinetriphosphatase Induced by Nucleotides and Phosphate†

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ABSTRACT: Upon incubation with trypsin, the adenosine-5′-triphosphatase (ATPase) activity of the nucleotide-depleted F_1 is first rapidly and slightly activated and then slowly inactivated. The first phase is simultaneous with the conversion of the α subunit into an α' fragment which migrates between α and β on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The second phase is related to the proteolysis of the three main subunits, α', β, and γ. Preincubation of the enzyme with low concentrations of adenosine 5′-diphosphate (ADP) or adenosine 5′-triphosphate (ATP) does not modify the slight increase of activity but efficiently prevents the inactivation induced by trypsin. The $\alpha \rightarrow \alpha'$ conversion is not affected whereas the further proteolysis of α', β, and γ does not occur. On the contrary, even high concentrations of GDP only slightly lower the trypsin-induced inactivation. The presence of endogenous tightly bound nucleotides also partially lowers the sensitivity to trypsin since F_1 is less rapidly inactivated and proteolyzed than the nucleotide-depleted F_1. Phosphate, at high concentrations, both slows down the first phase of activation and simultaneous $\alpha \rightarrow \alpha'$ conversion and prevents the second phase of inactivation and proteolysis of the main subunits. Pretreatment of the nucleotide-depleted F_1 with trypsin under conditions where the ATPase activity is largely inhibited only slightly modifies, however, the hysteretic behavior of the enzyme: the ADP binding and the concomitant hysteretic inhibition of the residual activity are not markedly diminished. The purified ATPase–ATP synthase complex binds very few ADP's and is not hysteretically inhibited. Its ATPase activity is rapidly activated but not further inhibited by trypsin. Preincubation of the complex with ADP does not modify the effects of trypsin.

It is now generally recognized that the terminal enzyme of oxidative phosphorylation is an anisotropic and reversible adenosine-5′-triphosphatase (ATPase)[1]–ATP synthase which catalyzes the synthesis of ATP from ADP and phosphate. The complex contains an F_0 sector allowing proton translocation across the membrane and an F_1 sector bearing the binding sites of nucleotides and phosphate [see the reviews of Penefsky (1979) and Senior (1979)]. The F_1 sector or F_1-ATPase is an oligomer composed of several types of subunits, which contain various classes of nucleotide or phosphate binding sites.

We have recently shown (Di Pietro et al., 1980) that preincubation of F_1-ATPase with micromolar concentrations of ADP leads to a binding of ADP that induces a progressive, hysteretic, inhibition of MgATP hydrolysis; the inhibition and the ADP binding can be reversed by excess ATP, in the absence of Mg^{2+}. The binding of ADP occurs at regulatory site(s), specific for adenine nucleotides (Harris et al., 1978; Baubichon et al., 1981; Di Pietro et al., 1981), but hydrolysis of MgATP at the catalytic site(s) is necessary to observe the inhibition which follows biphasic kinetics. It has been proposed that the addition of the substrate MgATP could induce a conformational change of the enzyme from a highly active state to a partially active state (Di Pietro et al., 1980).

Several classes of phosphate binding sites have been found in F_1 (Penefsky, 1977; Kasahara & Penefsky, 1978). The reactivity toward inhibitors of ATPase activity was modified in phosphate buffer (Di Pietro et al., 1979), which suggested a phosphate dependency of the enzyme conformation.

The purpose of this study was to monitor eventual nucleotide- or phosphate-dependent conformational changes of F_1-ATPase by studying the effects of trypsin on the ATPase activity and the subunit proteolysis. Trypsin has been shown to induce subtle modifications of F_1-like ATPases obtained from chloroplasts (Deters et al., 1975), bacteria (Bragg & Hou, 1975, 1978; Hockel et al., 1976; Ritz & Brodie, 1977; Dunn et al., 1980; Mollinedo et al., 1980), or mitochondria (Leimgruber & Senior, 1976; Maïrouch & Godinot, 1977; Hundal & Ernster, 1981; Pedersen et al., 1981; Skerrett et al., 1981; Todd & Douglas, 1981). Since controversial effects of trypsin on the release of tightly bound nucleotides were reported for the beef heart (Leimgruber & Senior, 1976) or the rat liver (Pedersen et al., 1981) mitochondrial enzyme, we used as a starting material either the nucleotide-depleted F_1 (Penin et al., 1979) or F_1 containing tightly bound nucleotides. The results were then compared to those obtained with a highly coupled ATPase–ATP synthase complex (Penin et al., 1982).

The present paper shows that important conformational changes of soluble F_1-ATPase are induced by adenine nucleotides or phosphate, leading to trypsin-resistant states of the enzyme. The presence of tightly bound nucleotides also partially protects against inactivation and proteolysis of the enzyme by trypsin. The results obtained with the isolated ATPase–ATP synthase complex suggest that its regulatory ADP site(s) is (are) already occupied by ADP and that the conformation of F_1-ATPase after ADP binding is close to that of F_1 inside the complex.

Experimental Procedures

Materials. Nucleotides were purchased from Boehringer Mannheim; [U-^{14}C]ADP (510 mCi/mmol) came from the Radiochemical Centre, Amersham, England. Their purity was

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[1] Abbreviations: ATPase, adenosine-5′-triphosphatase; F_1, pig heart mitochondrial F_1-ATPase prepared according to the procedure of Penin et al. (1979), omitting the last step (gel filtration in the presence of 50% glycerol); nucleotide-depleted F_1, pig heart mitochondrial F_1-ATPase prepared according to Penin et al. (1979); ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; AMP-P(NH)P, adenosine 5′-(β,γ-imidotriphosphate); NADH, reduced nicotinamide adenine dinucleotide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; PEI, poly(ethylenimine); BAEE, N^{α}-benzoyl-L-arginine ethyl ester; FSBA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine.

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checked by thin-layer ascending chromatography on poly(ethylenimine)–cellulose. The trypsin, from bovine pancreas, was from Sigma as type XI, i.e., freed from chymotrypsin activity. The trypsin inhibitor, from soybean, was also from Sigma as type I.S. All other reagents were of the highest purity commercially available.

Enzyme Preparations. Pig heart mitochondria were obtained at 0–4 °C as previously described (Gautheron et al., 1964). The mitochondrial F_1-ATPase was purified by the method of Penin et al. (1979). The last step of gel filtration on Ultrogel ACA 34 in the presence of 50% glycerol which removed tightly bound nucleotides led to nucleotide-depleted F_1 while omitting this step yielded F_1. F_1 contained 1.8 ± 0.2 mol of ADP/mol and 0.3 ± 0.03 mol of ATP/mol; it was stored as an ammonium sulfate suspension at 0–4 °C. The nucleotide-depleted F_1 was virtually devoid of tightly bound nucleotides (0.17 ± 0.18 mol of ADP/mol and 0.27 ± 0.04 mol of ATP/mol); it was stored frozen at −80 °C in 100 mM Tris–H_2SO_4–5 mM EDTA–50% glycerol, pH 8.0. The ATPase–ATP synthase complex was purified as described (Penin et al., 1982). The complex was obtained in 0.25 M sucrose–10 mM Tris–H_2SO_4–10 mM $MgSO_4$, pH 7.4, and stored frozen in liquid nitrogen. Just before use, an aliquot was centrifuged at 9000*g* for 5 min to sediment and eliminate eventual inactive aggregates.

The protein content of enzyme solutions was estimated by the procedure of Lowry et al. (1951) with bovine serum albumin as the standard. The molecular weight of F_1-ATPase was taken as 380 000 (Di Pietro et al., 1975).

The active ATPase inhibitor protein was a generous gift from Drs. Y. Galante and Y. Hatefi. It was purified according to Horstman & Racker (1970) as modified (Galante et al., 1981).

Assay of ATPase Activity. The measurements were performed on 0.5–4-μL aliquots at 30 °C in 0.62 mL of 50 mM Tris–H_2SO_4 buffer, pH 8.0, 3.3 mM ATP, and 3.3 mM $MgSO_4$, by using an ATP regenerating system (phosphoenolpyruvate, pyruvate kinase), lactic dehydrogenase, and NADH and following with a spectrophotometer the rate of NADH disappearance at 340 nm [Pullman et al. (1960) as modified by Baubichon et al. (1982)]. The specific ATPase activity measured in the presence or the absence of 10 mM sodium bicarbonate as an activating anion was respectively 90–100 or 35–40 units/mg of protein for nucleotide-depleted F_1 and 70–80 or 35–40 units/mg of protein for F_1 (1 unit = 1 μmol of ATP hydrolyzed/min). The activity of the ATPase–ATP synthase complex (2.7–3.0 units/mg) was insensitive to anion activation. The values obtained were not affected by 4 mM Na_2S, indicating the absence of NADH oxidase activity in the complex.

Assay of Trypsin Activity. A stock solution of trypsin (2 mg/mL of distilled water) was divided in fractions and stored frozen at −80 °C without loss of activity. The latter was measured according to Rick (1974) in 50 mM Tris-HCl–20 mM $CaCl_2$, pH 8.0, containing 0.9 mM N^{α}-benzoyl-L-arginine ethyl ester (BAEE) as the substrate and following the increase in absorbance at 255 nm. A specific activity of 18–22 μmol of BAEE hydrolyzed min^{-1} mg^{-1} was obtained at 30 °C. The trypsin activity was 99% inhibited in less than 0.5 min by a 5-fold excess (w/w) of trypsin inhibitor.

Incubation of Trypsin with the ATPase Preparations. F_1-ATPase (0.2–2 mg of protein/mL) was routinely incubated (unless otherwise indicated) in 50 mM Tris–H_2SO_4–1.5 mM $MgSO_4$–10% glycerol, pH 8.0, at 30 °C in the presence of trypsin (2–100 μg/mg of F_1-ATPase). At intervals, 0.5–5-μL aliquots were used for the assay of ATPase activity. When indicated, the enzyme was first preincubated with nucleotides, phosphate, or KCl for 30 min before trypsin addition. The ATPase–ATP synthase complex (0.6–1.0 mg of protein/mL) was preincubated for 30 min at 30 °C in 0.25 M sucrose–10 mM Tris–H_2SO_4, pH 8.0, containing either 10 mM $MgCl_2$ or 1 mM EDTA. When used, ADP was added at a concentration of 10 μM. Trypsin (30 μg/mg) was added, and the ATPase activity was measured as a function of time on 4-μL aliquots.

Table I: Protection by Glycerol against the Inactivation of F_1-ATPase by Trypsin[a]

% glycerol (w/v)	half-time of inactivation[b] (min)
5	12
10	29
20	66
26.7	100

[a] The nucleotide-depleted F_1 (0.18 mg of protein/mL) was incubated at 30 °C in 40 mM Tris–H_2SO_4–1 mM EDTA, pH 8.0, containing the indicated amounts of glycerol. Trypsin was added (100 μg of trypsin/mg of F_1-ATPase), and the remaining ATPase activity was measured as a function of time in the absence of an activating anion. Controls conducted in parallel experiments in the absence of trypsin were virtually constant. [b] The half-time of inactivation was that obtained when the residual ATPase activity was 50% of the value measured before trypsin addition. The indicated values were in fact overestimated since a slight activation preceded the inactivation (see Figure 1).

F_1-ATPase Proteolysis by Trypsin. F_1-ATPase (2 mg of protein/mL) was preincubated as indicated in the legends of each figure or table with nucleotides, P_i, KCl, or glycerol and submitted to trypsin action as described above. At intervals, 50-μL aliquots were added to Eppendorf tubes containing 30 mg of solid urea, 2.5 μL of pure β-mercaptoethanol, and 10 μL of 10% $NaDodSO_4$. The tubes were vigorously vortexed and heated at 100 °C for 3 min to both depolymerize F_1-ATPase and inactivate the trypsin, according to Bragg & Hou (1975). The samples were frozen until used for analysis by $NaDodSO_4$–polyacrylamide gel electrophoresis. The latter was performed according to Laemmli (1970) by using 14% acrylamide in the separating gel and 4% acrylamide in the stacking gel. The staining with Coomassie blue, destaining, and recording were performed as previously described (Penin et al., 1982).

Measurements of the ADP-Induced Hysteretic Inhibition and Concomitant [^{14}C]ADP Binding. After incubation of F_1-ATPase (1.4 mg of protein/mL) in 50 mM Tris–H_2SO_4–1.5 mM $MgSO_4$–10% glycerol, pH 8.0, with trypsin (7 μg/mL) for the indicated times, 50-μL aliquots were supplemented with trypsin inhibitor (35 μg/mL). [^{14}C]ADP (200 μM) was added and incubated for 20 min. The ADP-induced hysteretic inhibition and the concomitant ADP binding were measured as described previously (Di Pietro et al., 1980, 1981).

Results

Inactivation of F_1-ATPase by Trypsin in Various Conditions. Effects of Glycerol. The rate of inactivation increased with the trypsin/F_1-ATPase ratio or with the concentration of F_1-ATPase at a constant trypsin/F_1-ATPase ratio and glycerol concentration.

Table I shows that increasing the glycerol concentration, the presence of which is necessary to maintain a good stability of the nucleotide-depleted F_1 (Garrett & Penefsky, 1975; Penin et al., 1979), decreased the rate of inactivation as shown by the increase in the half-time of inactivation. However, the

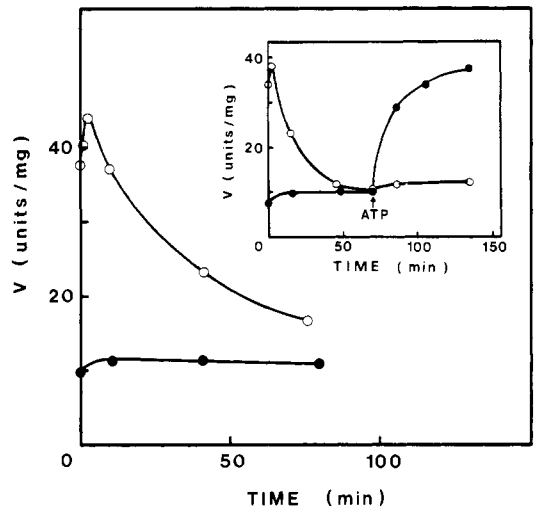

FIGURE 1: Protection of ADP against the trypsin-induced inactivation of F_1-ATPase. The nucleotide-depleted F_1 (9 μg of protein) was preincubated at 30 °C in 50 μL of 44 mM Tris-H_2SO_4–0.6 mM $MgSO_4$–11% glycerol, pH 8.0, both in the presence (●) and in the absence (○) of 10 μM ADP. After 30 min, 5 μL of a trypsin solution (0.09 mg/mL of water) was added, and the activity was followed at intervals by introducing samples of 2–4 μL in the ATPase assay that did not contain an activating anion. For the samples preincubated with ADP, the ADP-induced stable and inhibited rate of ATPase activity was measured after 1 min of ATP hydrolysis as described previously (Di Pietro et al., 1980, 1981). Insert: Effects of ADP in the absence of Mg^{2+} and reversal by ATP. Mg^{2+} was replaced by 1 mM EDTA, and the ADP concentration was 500 μM in the preincubation medium. A ratio of 100 μg of trypsin/mg of F_1-ATPase was used. After 70 min, a 5-fold excess of trypsin inhibitor with respect to trypsin and 6.3 mM ATP were added, and the activity was followed at intervals. Controls performed with water instead of trypsin were virtually constant during the time of the experiments.

activity of trypsin on BAEE was not changed by glycerol (or nucleotides as shown below).

Effects of Preincubation of F_1-ATPase with ADP on Its Inactivation by Trypsin. A careful examination of the pattern of the nucleotide-depleted F_1 activity as a function of the incubation time with trypsin showed that the inactivation was in fact preceded by a slight, but significant and reproducible, activation. In the absence of effectors (Figure 1, open circles), a maximal activation of 14 ± 5% (n = 7) was obtained, but this value was probably underestimated since the activation phase was rapidly followed by an inactivation. The latter was a slow process and led to almost complete loss of activity when the incubation was prolonged or when great amounts of trypsin were used (not shown).

Preincubation of the enzyme with ADP (closed circles) induced a partial hysteretic inhibition of ATPase activity as previously described (Di Pietro et al., 1980, 1981; Baubichon et al., 1981, 1982). The low remaining activity was also enhanced by trypsin (20 ± 6%, n = 7) but did not exhibit the subsequent long-term inactivation. It is known that the F_1-ATPase which has been partially inhibited by preincubation with ADP slowly recovers its original activity upon addition of excess ATP to the preincubation medium, in the presence of EDTA to avoid ATP hydrolysis (Harris et al., 1978; Di Pietro et al., 1980). This reversal of hysteretic ADP inhibition was also observed here when ATP was added after a 70-min action of trypsin on the nucleotide-depleted F_1 inhibited by ADP in the absence of Mg^{2+} (insert of Figure 1). A full reactivation was observed, indicating that the ADP bound at the regulatory site(s) induced a conformation of the enzyme that was insensitive to trypsin attack. This means that the enzyme recovered 112% of its initial activity including the initial phase of trypsin activation. In contrast, when the enzyme was preincubated in the absence of ADP, no reversal of trypsin inactivation was induced by ATP. This indicated strong modifications of the enzyme due to trypsin proteolysis.

Table II: Effects of Nucleotides and Phosphate on the Trypsin-Induced Inactivation of F_1-ATPase[a]

addition	concn (mM)	% initial ATPase activity[b]
none		43
ADP	0.01	112
	0.5	118
ATP[c]	0.01	101
	1	115
GDP	0.2	54
	1.5	76
AMP	10	60
P_i	5	46
	200	103

[a] The nucleotide-depleted F_1 was preincubated as described in Figure 1 in the presence of the indicated concentrations of nucleotide or phosphate. After an 80-min incubation with trypsin (50 μg/mg of F_1-ATPase), the residual ATPase activity was measured in the absence of activating anion and compared to the initial activity. [b] The initial ATPase activity, measured before the addition of trypsin, was always 36 units/mg of protein except in the presence of ADP; values of 13.3 or 9.0 units/mg of protein were obtained respectively for 0.01 or 0.5 mM ADP. [c] Preincubation with ATP was conducted in the presence of 1 mM excess EDTA with respect of $MgSO_4$ to avoid ATP hydrolysis.

F_1 (containing tightly bound nucleotides) was not as sensitive to trypsin as the nucleotide-depleted F_1. Indeed, on the one hand, no initial activation was experimentally observable; on the other hand, an apparent half-time of inactivation of 26 min was obtained with F_1 under conditions where a value of 13 min was obtained with the nucleotide-depleted F_1. The ATPase activity of F_1, previously partially inhibited by preincubation with 200 μM ADP, was slightly activated by trypsin, as it was for the nucleotide-depleted F_1. However, the protection by the pretreatment of the enzyme with ADP against the trypsin-induced inactivation was slightly lower since 99% of the initial activity was observed with F_1, as compared to the 112% obtained with the nucleotide-depleted F_1.

Since the natural ATPase inhibitor has been shown to be very sensitive to trypsin proteolysis (Pullman & Monroy, 1963), inducing an increase in ATPase activity, its presence was investigated in our preparations. F_1, as obtained before the last step of gel filtration in 50% glycerol, effectively contained a protein band between the δ and ϵ subunits in Na-$DodSO_4$–polyacrylamide gel electrophoresis (Penin et al., 1979). This ATPase inhibitor-like fraction was readily proteolyzed upon incubation of F_1 with trypsin (not shown). It could be purified from a solution of F_1 either by heating at 75 °C (Warshaw et al., 1968) or by gel filtration on ACA 34 with 50% glycerol. The purified ATPase inhibitor-like fraction did not have any inhibiting effect however when incubated with F_1 at pH 6.7 in the presence of MgATP according to Horstman & Racker (1970). The same conditions gave a 90–95% inhibition with purified active ATPase inhibitor.

Table II shows that low concentrations of ADP or ATP were able to afford important protection against trypsin whereas GDP at relatively high concentrations only induced a weak protection. As a control, a very high AMP concentration did not have any marked effect. Another important protective effect against the trypsin-induced inactivation was obtained with a high concentration of phosphate. Lower concentrations of phosphate, in the range 0.5–5 mM, did not induce any protective effect even when assayed at pH 6.4, i.e., under

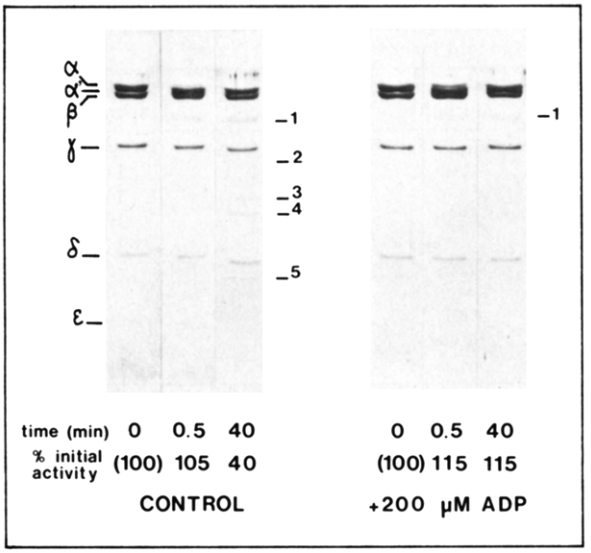

FIGURE 2: Effects of ADP on the $NaDodSO_4$–polyacrylamide gel electrophoresis pattern of F_1-ATPase after proteolysis by trypsin. The nucleotide-depleted F_1 (250 μg) was preincubated in 120 μL of 40 mM Tris–H_2SO_4–1.1 mM $MgSO_4$–10.3% glycerol, pH 8.0, both in the absence and in the presence of 200 μM ADP. After addition of trypsin (5 μg/mg of F_1-ATPase), the remaining ATPase activity was measured at intervals, and fractions of 25 μL (containing 50 μg of protein) were depolymerized and analyzed as described under Experimental Procedures. The electrophoresis on $NaDodSO_4$–polyacrylamide gels was performed on 11-μL fractions (11 μg of protein).

conditions where the specific phosphate binding site was saturated by the monovalent, active, phosphate form according to Penefsky (1977). Such an acidic pH provoked some loss of ATPase activity and slowed down the trypsin activity. However, it did not appreciably modify the enzyme behavior since the ADP-induced hysteretic inhibition and the protection of ADP against the subsequent trypsin-induced inactivation of the nucleotide-depleted F_1 were practically identical at pH 8.0 or 6.4.

A control was made to verify that the effectors studied, i.e., nucleotides and glycerol (cf. Table I), did not significantly modify the trypsin activity with BAEE as the substrate. The effects of 200 mM phosphate buffer could not be checked since it provoked a precipitate when added to the trypsin assay medium. However, the use of an equivalent ionic strength in KCl did not modify the trypsin activity. An activating anion of the ATPase activity, like bicarbonate, accelerated F_1-ATPase inactivation and proteolysis by trypsin. This effect was, however, at least partially due to a direct action of bicarbonate on the trypsin activity even with BAEE as the substrate.

Effects of ADP on the Trypsin-Induced Proteolysis of F_1-ATPase. When analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis by the procedure of Laemmli (1970), the nucleotide-depleted F_1 showed the usual five types of subunits, α, β, γ, δ, and ε (Figure 2). Addition of trypsin produced two types of effects on the electrophoresis pattern. The first and rapid effect was a slight activation and a simultaneous proteolysis of the α subunit, giving a new subunit (α′) of apparent molecular weight between those of original α and β. This α → α′ conversion was more visible when lower amounts of protein (4 μg as in Table III) were analyzed (not shown here). A very faint fragment (fragment 1) was also observed. The second and slower effect, concomitant with a large inactivation, was characterized by the proteolysis of the main subunits (α′, β, and γ) (see also the control experiment in Table III) and by the appearance of the new fragments (2–5).

Table III: Effects of Phosphate and Ionic Strength on Inactivation and Proteolysis of F_1-ATPase by Trypsin[a]

	time (min)	% initial value[b] α	α + α′[c]	β	γ	ATPase activity
control	1	26	100	100	100	113 (98)[d]
	4	0	79			100 (87)
	10		56	67	95	80 (69)
$+P_i$	1	98	100	100	100	103
	4	60	108			109
	10	20	97	97	100	115
+KCl	1	60	60	96	68	100
	4	32	43			100
	10	8	19	86	26	83

[a] The conditions were the same as those in Figure 2 except that 200 mM KH_2PO_4 or 600 mM KCl replaced ADP during preincubation. Samples of 4 μL (4 μg of protein) were analyzed by electrophoresis. The Coomassie blue stained chromatograms were recorded, and the area of each subunit was estimated. [b] The initial values were those obtained before the addition of trypsin. The initial ATPase activity, measured in the absence of activating anion, was 40 units/mg of protein for the control or for the assay preincubated with phosphate. It was reduced to 3 units/mg after preincubation with KCl for 45 min. [c] The initial value of α + α′ area was that of α since α′ was absent at zero time. [d] The percent of residual activity calculated with respect to the maximal ATPase activity (115% of the initial activity, see Figure 1) is in parentheses.

Preincubation of the nucleotide-depleted F_1 with ADP did not modify the rapid proteolysis of α or the appearance of α′ and the minor fragment 1. A careful kinetic study conducted with lower amounts of trypsin (not shown here) indicated that this ADP-insensitive decrease in α subunit paralleled the increase in α′ and the slight increase in activity. On the contrary, ADP efficiently prevented the slow proteolysis of the main subunits α′, β, and γ and the concomitant obtention of the fragments 2–5. The same kind of protective effect against proteolysis was induced by ATP or by the analogue AMP-P(NH)P.

Effects of Phosphate and Ionic Strength. Table III shows that the complete α → α′ conversion of the control was rapid and achieved in less than 4 min.

If we consider that Coomassie blue staining roughly reflects the amount of protein in subunits, the proteolysis of the subunits α + α′ and β appeared to occur approximately at the same rate, in good correlation with the loss of ATPase activity. On the contrary, the γ subunit was hardly modified under the conditions of the experiment. Preincubation of the enzyme with 200 mM phosphate markedly slowed down the α → α′ conversion and the simultaneous increase in activity and prevented subsequent proteolysis of the main subunits. Replacing phosphate by an equivalent ionic strength in KCl resulted in a dramatic loss of ATPase activity with time since a 92% inhibition was produced by a 45-min preincubation with 0.6 M KCl. Upon the addition of trypsin, KCl slowed down the α-subunit proteolysis in the same manner as phosphate although less efficiently. The decrease in α, however, was not directly correlated with an increase in α′, indicating that in this case either α did not convert into α′ or α′ was immediately further proteolyzed into secondary fragments and did not accumulate in the medium. The sum α + α′ rapidly decreased with time in the same manner as the γ subunit. On the contrary, the β-subunit proteolysis was not more rapid than that of the control without KCl. It seemed to decrease in parallel with the residual ATPase activity, even though the ATPase activity remaining after a 45-min incubation with KCl was already very low.

Effects of Trypsin Pretreatment on the ADP-Induced Hysteretic Inhibition of F_1-ATPase and the Concomitant ADP

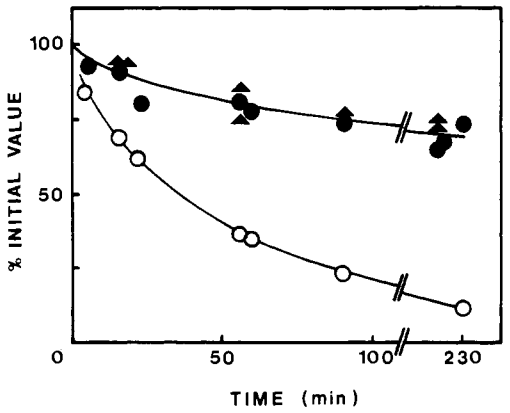

FIGURE 3: Differential trypsin effects on ATPase activity, ADP-induced hysteretic inhibition, and concomitant ADP binding. The nucleotide-depleted F_1 (680 μg of protein) was incubated in 500 μL of 50 mM Tris–H_2SO_4–1.5 mM $MgSO_4$–10% glycerol, pH 8.0, in the presence of 3 μg of trypsin. At intervals, 50-μL aliquots were mixed with 5 μL of trypsin inhibitor (1.5 μg), and the ATPase activity (○) was measured in the presence of 10 mM (sodium) bicarbonate as an activating anion. After addition of 6 μL of 2 mM [^{14}C]ADP (0.2 mM final concentration) and incubation for 20 min, the stable, inhibited rate of ATPase activity was measured, and the percentage of hysteretic inhibition (▲) was calculated. The samples were then submitted to the filtration–centrifugation method (Penefsky, 1977) to measure the binding of [^{14}C]ADP (●). The initial values, taken as 100%, were the following: ATPase activity, 96 units/mg of protein; hysteretic inhibition, 87% (a value of 12.5 units/mg of protein was obtained for the ADP-induced stable, inhibited rate); [^{14}C]ADP binding, 3.2 mol/mol of enzyme.

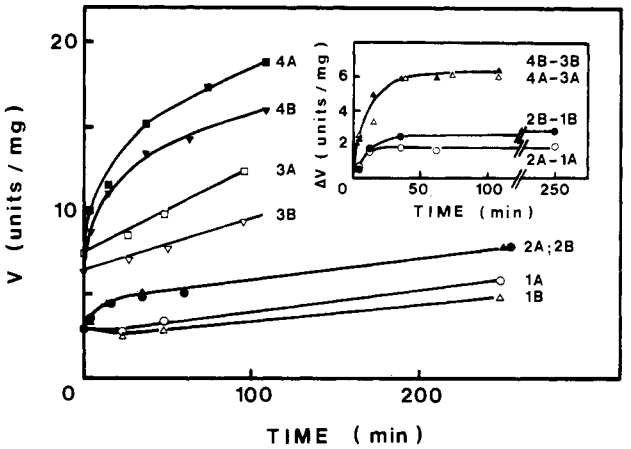

FIGURE 4: Modification by trypsin of the hydrolytic activity of the ATPase–ATP synthase. Effects of ADP and Mg^{2+}. The ATPase–ATP synthase complex was equilibrated with 0.25 mM sucrose–10 mM Tris–H_2SO_4, pH 7.5, containing either 10 mM $MgSO_4$ (curves 1 and 2) or 1 mM EDTA (curves 3 and 4) by filtration–centrifugation through Sephadex G-50 columns (equilibrated with the corresponding buffer). The complex (0.61 mg of protein/mL) was preincubated at 30 °C both in the presence (curves B) and in the absence (curves A) of 10 μM ADP for 30 min. Trypsin (30 μg/mg of complex protein) was added (curves 2 and 4) or omitted (curves 1 and 3), and the ATPase activity as a function of time was followed on 4-μL aliquots. Insert: Stimulation of ATPase activity by trypsin. The difference in the rate (Δv) was obtained by subtracting the values obtained in the absence of trypsin (curves 1 and 3) from those measured in the presence of trypsin (curves 2 and 4).

Binding. Preincubation with trypsin of a relatively high concentration of nucleotide-depleted F_1 (1.4 mg of protein/mL) led to such a rapid inhibition of ATPase activity that the initial slight activation could not be observed. At the same time, the hysteretic inhibition of the residual ATPase activity and the concomitant ADP binding were only slightly modified (Figure 3). Both patterns of ADP-induced hysteretic inhibition and ADP binding as a function of incubation time with trypsin were superimposed. They were only diminished by 15% or 25% under conditions where the ATPase activity was respectively 50% or 75% inhibited.

The same difference in sensitivity toward trypsin was found with F_1 (containing tightly bound nucleotides). The curves were slightly less distant since the ADP binding and the concomitant inhibition were decreased by 18% or 38% when the hydrolytic activity was respectively 50% or 75% inhibited by trypsin.

Modification by Trypsin of ATPase Activity of the ATPase–ATP Synthase Complex. When incubated for 250 min at 30 °C in the presence of Mg^{2+}, the ATPase activity of the complex increased from 2.9 to 5.9 units/mg of protein (Figure 4, curve 1A). This increase in activity was accompanied by an important loss in sensitivity to oligomycin (20 μg/mg of protein), which was reduced from 89% to 26% by an 80-min incubation (not shown). Addition of trypsin rapidly increased the initial ATPase activity, and the pattern was then approximately parallel to that of the control (compare curves 2A and 1A). After gel filtration in the presence of excess EDTA, the ATPase activity of the complex was raised to 7.4 units/mg of protein (curve 3A), and the rapid increase induced by trypsin was even higher (curve 4A). Preincubation of the complex with ADP, at the same concentration that protected the soluble F_1-ATPase against trypsin, hardly inhibited the ATPase activity (curves B). The insert of Figure 4 shows that the trypsin-induced activation of ATPase activity never decreased in the time scale studied or was appreciably modified by preincubation of the complex with 10 μM ADP, whether either Mg^{2+} or EDTA was present. Preincubation, even with a high ADP concentration (200 μM), hardly modified the ATPase activity (not shown). An inhibition of 9 ± 3% (n = 11) was concomitant with a low ADP binding of 0.5 ± 0.1 nmol/0.5 mg of protein (n = 6).

Discussion

The present work has shown that trypsin could be suitably used to monitor conformational changes induced by effectors of the mitochondrial ATPase by measuring both the modification of the activity and the proteolysis of subunits. Important conformational changes of the soluble F_1-ATPase have been observed here in the presence of the physiological effectors nucleotides and phosphate.

Induction by Nucleotides of a Trypsin-Resistant Conformation of F_1. Saturation of the site(s) responsible for the hysteretic behavior of the enzyme (Di Pietro et al., 1980, 1981; Baubichon et al., 1981, 1982) by low concentrations of ADP, ATP, or AMP-P(NH)P proved to induce a large conformational change leading to a trypsin-resistant conformation. The latter was probably more compact since all the main subunits were no longer proteolyzed and the ATPase activity was retained. The fact that ATP could fully reverse the ADP-induced hysteretic inhibition after a 70-min trypsin treatment is an additional argument in favor of a very compact conformation induced by added ADP.

The presence of endogenous tightly bound nucleotides in the enzyme preparation also decreases the sensitivity to trypsin, probably by inducing a less relaxed conformation. These effects of the nucleotides on the enzyme conformation might bring a new light on their possible role in the regulation of the enzyme mechanism. To our knowledge, it is the first time that a nucleotide-induced protection against trypsin effects is reported. Our results are compatible with other studies conducted under variable conditions, showing that trypsin inhibited F_1-ATPase from both bacteria (Hockel et al., 1976; Mollinedo

et al., 1980) and yeast mitochondria (Todd & Douglas, 1981) in the absence of nucleotides whereas no inhibition was obtained with F_1-ATPase from chloroplasts (Deters et al., 1975) or bacteria (Dunn et al., 1980) in the presence of 1 mM ATP. In other conditions, nucleotide-dependent conformational changes of F_1-ATPase have been revealed by variations in aurovertin fluorescence (Chang & Penefsky, 1973), by modifications in thiol accessibility (Godinot et al., 1975, 1977), or by a lag period observed in pre-steady-state kinetics (Recktenwald & Hess, 1977, 1979; Roveri et al., 1980).

High concentrations of GDP hardly modified the effects of trypsin and were not able to induce the hysteretic inhibition of the enzyme. Moreover, GDP binding did not prevent the fixation of ADP at site(s) responsible for the hysteretic inhibition, sites very selective for the adenine nucleotides (Harris et al., 1978; Baubichon et al., 1981; Di Pietro et al., 1981). Therefore, the conformational changes detected here by the trypsin effect were, indeed, due to ADP binding at those selective sites which can be considered as regulatory sites. This conclusion does not exclude the possibility that ADP binding at catalytic sites might interfere in the overall process. Another conformational change can be detected after ADP preincubation of F_1 when its hydrolytic activity is started by the addition of MgATP. Progressively, the hysteretic inhibition develops, and anions can no longer stimulate the activity (Baubichon et al., 1982). This latter conformational change was also prevented by cross-linking of the subunits with dimethyl suberimidate (Chernyak et al., 1981).

Preincubation of F_1-ATPase with ADP efficiently prevented the trypsin-induced inactivation. On the contrary, pretreatment of the enzyme with trypsin only slightly diminished the further ADP binding and concomitant hysteretic inhibition whereas the ATPase activity was largely inhibited. This weak sensitivity of the regulatory site(s) responsible for the hysteretic inhibition constitutes an additional argument to differentiate this (these) site(s) (Di Pietro et al., 1980) from the tightly bound nucleotide binding sites which have been found very sensitive to trypsin (Leimgruber & Senior, 1976).

Limited Proteolysis of the α Subunit of F_1 by Trypsin. The presence of nucleotides did not affect the $\alpha \rightarrow \alpha'$ conversion of F_1 as observed by Hundal & Ernster (1981) with an enzyme preparation containing tightly bound nucleotides. With the latter enzyme, the $\alpha \rightarrow \alpha'$ conversion did not modify the ATPase activity (Leimgruber & Senior, 1976; Skerrett et al., 1981; Hundal & Ernster, 1981) while here with the nucleotide-depleted enzyme an activation was observed during the conversion. Inasmuch as no protein inhibitor could be detected by usual methods in our preparation (Penin et al., 1979), we conclude that this activation is probably not related to modifications of the endogenous protein inhibitor. In contrast, the enzyme preparation is very sensitive to the externally added protein inhibitor.

Many comments have been made on these $\alpha \rightarrow \alpha'$ types of conversion and their consequences (Leimgruber & Senior, 1976; Ritz & Brodie, 1977; Bragg & Hou, 1978; Dunn et al., 1980; Hundal & Ernster, 1981). The fact that we observed an activation with our enzyme preparations in the presence of externally added ADP during the $\alpha \rightarrow \alpha'$ conversion, while no activation was reported in all the experiments conducted in the absence of external ADP with enzymes containing tightly bound nucleotides, brings a further argument in favor of the large conformational change due to ADP binding at regulatory site(s). In our case, the $\alpha \rightarrow \alpha'$ conversion favors the MgATP hydrolysis. The rate of proteolysis of α into α' diminished with the ionic strength, which therefore reduced the reactivity of the α subunit toward trypsin. This agrees with the lack of trypsin-induced $\alpha \rightarrow \alpha'$ conversion of F_1-ATPase from rat liver mitochondria in 200 mM phosphate buffer (Pedersen et al., 1981).

Induction by Phosphate of a Trypsin-Resistant Conformation of F_1. The presence of phosphate also induced a conformational change. Although the phosphate concentration was high, inducing a high ionic strength, such a buffer has been widely used by several authors since it afforded good stability and activity of F_1-ATPase. Our results have shown that phosphate seemed to stabilize the quaternary structure of the enzyme and to prevent the unfavorable effects induced by a high ionic strength. The latter has been found to favor enzyme dissociation into subunits at 0 °C with a concomitant loss of ATPase activity (Penefsky & Warner, 1965), the dissociated subunits being much more sensitive to trypsin proteolysis (Undal & Ernster, 1981). The loss in ATPase activity at 30 °C and the concomitant increase in the subunits' proteolysis in the presence of 0.6 M KCl might therefore be due to more or less partial dissociation into subunits. The presence of phosphate totally prevented these unfavorable effects by inducing a conformational change leading to a trypsin-resistant state. This explains why almost no inhibition was obtained in this buffer even with high amounts of trypsin (Maïrouch & Godinot, 1977; Pedersen et al., 1981). The conformational change was not due to saturation of the high-affinity binding site of phosphate (Penefsky, 1977) located in the β subunit (Lauquin et al., 1980) since low concentrations of the monovalent phosphate form at pH 6.4 did not induce any protection against trypsin inactivation. It might more likely be related to saturation of one of several other phosphate binding sites with millimolar affinities (Kasahara & Penefsky, 1978). Such a phosphate-induced conformational change had previously been characterized by a greater sensitivity to 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine (FSBA), a nucleotide analogue, or to other hydrophobic compounds (Di Pietro et al., 1979).

Extensive Proteolysis of the Various Subunits of F_1 in the Absence of Nucleotides or Phosphate. Other indications concerning the F_1-ATPase molecule arrangement have been provided by the study of the effects of high ionic strength on the subunits' proteolysis. The increased proteolysis of the $\alpha + \alpha'$ and γ subunits would indicate that important subunit–subunit interactions have been lost, making some regions accessible which are normally masked in the active molecule. On the contrary, the β-subunit proteolysis was practically unmodified by the ionic strength; this would indicate that the β subunit occupies a relatively external position in the F_1-ATPase molecule and agrees with conclusions drawn from chemical labeling (Ludwig et al., 1980) or neutron scattering (Satre & Zaccai, 1982) studies of soluble ATPase.

Analogy between the Conformation of F_1 in the ATPase–ATP Synthase Complex and in Isolated F_1 Inhibited by ADP. In contrast to F_1-ATPase, the ATPase–ATP synthase complex showed the same sensitivity to trypsin whether it was preincubated in the presence of ADP or not. The ATPase activity of the complex was rapidly enhanced, which might be due to the rapid proteolysis of the ATPase inhibitor protein (Pullman & Monroy, 1963) or of the oligomycin sensitivity conferring protein (Todd & Douglas, 1981). It has been shown that the trypsin-induced increase in the ATPase activity of the complex became less sensitive to oligomycin inhibition, essentially due to F_1 solubilization (Gautheron et al., 1977). After the activation phase, no inhibition of ATPase activity was observed whether ADP was present or not. This suggested that ADP did not bind to the complex. Direct measurements indicated

a binding of 0.5 nmol of ADP/0.5 mg of protein, i.e., about 0.5 mol of ADP/mol of complex, concomitant with a 9% inhibition. These values are very low as compared to those obtained with F_1-ATPase, i.e., 2–3 mol of ADP/mol and 75–85% inhibition (Di Pietro et al., 1980, 1981; Baubichon et al., 1981, 1982). This indicates that either the ADP binding sites are not accessible to the external medium or they are already occupied by ADP. In the latter, more probable case, the presence of the endogenous ATPase inhibitor protein (Penin et al., 1982) might prevent exchange of the endogenous nucleotides with the medium ADP as has been shown with submitochondrial particles (Harris et al., 1977) or with isolated F_1 (Klein et al., 1981). Such an antagonistic effect of the ATPase inhibitor protein and the binding of external ADP seems supported by the fact that the preparations which are devoid of the inhibitor protein either as submitochondrial particles (Fitin et al., 1979; Vasilyeva et al., 1980) or as F_1-ATPase (Harris et al., 1978; Di Pietro et al., 1980) are sensitive to ADP inhibition whereas the preparations containing inhibitor protein either as submitochondrial particles (Harris et al., 1978) or as F_1-ATPase (Lowe & Beechey, 1982) are not inhibited by added ADP. Our results suggest that the ADP binding sites of the ATPase–ATP synthase are already saturated and that the trypsin-resistant conformation of F_1 induced by ADP would therefore be much more similar to that of F_1 inside the membrane-bound complex than that of F_1 as obtained in solution. Low concentrations of trypsin induced the conversion of α to α' in the isolated ATPase–ATP synthase complex (F. Penin, unpublished experiments) as for isolated F_1.

Due to the ADP-induced conformational change of F_1-ATPase, the "activating" anions, such as bicarbonate, no longer activate the inhibited enzyme. It is interesting to note that F_1 integrated in the ATPase–ATP synthase complex has a low activity and cannot be stimulated by activating anions (Cantley & Hammes, 1973; Ebel & Lardy, 1975). All the above results suggest that ADP-inhibited F_1 and the membrane-bound enzyme have a similar conformation.

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Registry No. ATPase, 9000-83-3; ADP, 58-64-0; ATP, 56-65-5; GDP, 146-91-8; AMP, 61-19-8; phosphate, 14265-44-2; glycerol, 56-81-5; trypsin, 9002-07-7.

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Dynamic Aspects of Insulin Action: Synchronization of Oscillatory Glycolysis in Isolated Perifused Rat Fat Cells by Insulin and Hydrogen Peroxide†

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ABSTRACT: Glucose oxidation to CO_2 was investigated in isolated perifused rat epididymal fat cells. Insulin stimulated rates of oxidation up to 30-fold. Multiple pulses of insulin or prolonged perifusion with the hormone led to a time-dependent desensitization of the cells. The action of insulin could be mimicked by H_2O_2. Reversal of H_2O_2 effects was associated with a damped oscillation of large initial amplitude. Initiation of perifusion with insulin induced rates of glucose oxidation that oscillated around a mean elevated rate with an amplitude of about ±4% of the mean, significantly larger than the measurement error. Basal rates did not show clear oscillations. The oscillations after insulin had a statistically significant period of around 14 min. The results were the same with C1- or C6-labeled glucose and occurred in the presence of both 0.275 and 5.5 mM glucose in the perifusion medium. The oscillations were interpreted as the result of insulin- or H_2O_2-induced synchronization of oscillatory glycolysis by individual fat cells. The similarity of the observed oscillatory period with the period of oscillatory insulin secretion by pancreatic β cells suggests that oscillatory glycolysis may constitute the internal pacemaker for the latter process.

Glycolysis is well-known to oscillate in a variety of systems. The best studied case is yeast (*Saccharomyces carlsbergensis* and *Saccharomyces cerevisiae*) where oscillations could be observed in individual cells, in cell populations, and in cell extracts [for a review, see Hess (1979)]. Evidence for oscillations has also been found in mammalian systems, i.e., cell-free tissue extracts from beef heart (Frenkel, 1965, 1966, 1968a-c) or rat leg muscle (Tornheim & Lowenstein, 1973-1975). Evidence that such oscillations occur in intact mammalian cells is still very limited, however, and consists of observations in cultured mouse fibroblasts (L cells) (Werrlein & Glinos, 1974) and cell suspensions of Ehrlich ascites tumor cells (Ibsen & Schiller, 1967, 1971). Because individual cells may oscillate out of phase, oscillations in cell populations are usually only seen after synchronization. The present report demonstrates that glycolysis in isolated rat epididymal fat cells oscillates and that perifusion with insulin or hydrogen peroxide can synchronize the oscillations.

Materials and Methods

Epididymal fat cells were isolated from 120-150-g Sprague-Dawley rats (Taylor Laboratories, Bellevue, WA) according to Rodbell (1964). Materials were those specified in Muchmore et al. (1981), except that bovine serum albumin (BSA)[1] was a product of Armour Pharmaceutical Co. (CRG-7, lot TX-3). Fat cells were perifused in the apparatus described by Little & de Haën (1980), with the following modifications: the pump was a Technicon Pump 1 equipped with a main buffer line (0.9 mL/min) and a line (0.1 mL/min) for additions of insulin, H_2O_2, or control medium, which joined the main buffer line 1 cm before the entrance to the perifusion chamber. Another line (0.1 mL/min) for addition of NaOH (0.35 M) containing EDTA (26 mM) joined the perifusion

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[1] Abbreviations: BSA, bovine serum albumin; KRB, Krebs-Ringer bicarbonate buffer modified as described in the text; KRP, Krebs-Ringer phosphate buffer modified as described in the text; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid.

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