Steck, T. L., Ramos, B., & Strapazon, E. (1976) *Biochemistry 15*, 1154–1161.
Tyler, J. M., Hargreaves, W. R., & Branton, D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5192–5196.
Ungewickell, E., & Gratzer, W. (1978) *Eur. J. Biochem. 88*, 379–385.
Yoshimura, A., Yamashina, S., & Ohnishi, S. (1985) *Exp. Cell Res. 160*, 126–137.

Partial Proteolysis as a Probe for Ligand-Induced Conformational Changes in the Isolated β Subunit of the H^+-Translocating $F_0 \cdot F_1$ ATP Synthase†

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ABSTRACT: The isolated β subunit of the *Rhodospirillum rubrum* $F_0 \cdot F_1$ ATP synthase contains two nucleotide binding sites, a Mg-independent and a Mg-dependent site [Gromet-Elhanan, Z., & Khananshvili, D. (1984) *Biochemistry 23*, 1022–1028]. Phosphate (P_i) binds only to the second site [Khananshvili, D., & Gromet-Elhanan, Z. (1985) *Biochemistry 24*, 2482–2487]. Binding of these ligands has been found to induce conformational changes in the β subunit that can be followed by their effect on trypsin sensitivity of the subunit. With a ratio of 1 mol of trypsin/100 mol of β, the subunit is digested in the absence of ligands with a half-time of 10 min. $MgCl_2$ has no effect on the trypsin sensitivity of β, but the other ligands show pronounced effects. Binding of either ADP or ATP to the Mg-independent site results in partial protection of the β subunit against its digestion by trypsin, increasing the $t_{1/2}$ to 20 min. A further decrease in the sensitivity to trypsin occurs on binding of MgADP to the second Mg-dependent site, increasing the $t_{1/2}$ to 30 min. Binding of MgATP or MgP_i to this site causes, however, an opposite effect, resulting in a decrease in the $t_{1/2}$ to 3 and 6 min, respectively. These results indicate that ligand binding induces two distinct changes in the conformation of the isolated β subunit. One conformational state is obtained on occupation of the Mg-independent nucleotide binding site and is further stabilized by MgADP. The second conformational state is obtained on binding of MgATP or MgP_i, suggesting that it is induced by occupation of the γ-phosphoryl subsite in the Mg-dependent catalytic site on the β subunit.

Energy-transducing membranes contain a reversible proton-translocating ATP synthase–ATPase complex $F_0 \cdot F_1$. It is comprised of two portions: an intrinsic membrane portion, F_0, that mediates proton movement and an extrinsic membrane portion, F_1, that is the catalytic sector (Kagawa et al., 1979; Penefsky, 1979; Nelson, 1981; Futai & Kanazawa, 1983; Senior & Wise, 1983). F_1 consists of five types of subunits (α, β, γ, δ, ϵ) and functions also as a soluble ATPase. It has been shown to contain several nucleotide binding sites that reside in the two major subunits, α and β (Harris, 1978; Baird & Hammes, 1979; Cross, 1981; Senior & Wise, 1983), and seem to include both catalytic and noncatalytic sites (Cross & Nalin, 1982; Grubmeyer et al., 1982; O'Neal & Boyer, 1984). However, the subunit location of these sites as well as their exact role in the mechanism of ATP synthesis and hydrolysis is still uncertain. One possible approach to the elucidation of the molecular events controlled by the multisubunit F_1 complex is the study of simpler functional systems such as individual subunits of F_1.

Investigations of individual subunits became possible when reconstitutively active α and β subunits have been purified from several bacterial sources (Yoshida et al., 1977; Futai, 1977; Dunn & Futai, 1980; Philosoph et al., 1977; Khananshvili & Gromet-Elhanan, 1982). Direct ligand-binding studies have been carried out until now on the α and β subunits of *Escherichia coli* F_1 (Dunn & Futai, 1980; Issartel & Vignais, 1984) and on the β subunit of *Rhodospirillum rubrum* F_1 (Gromet-Elhanan & Khananshvili, 1984; Khananshvili & Gromet-Elhanan, 1984, 1985a). These studies have established the presence of one Mg-independent nucleotide binding site on Ecα,[1] with K_d values of 0.1 μM for ATP and 0.9 μM for ADP, and one on Ecβ that binds ADP (K_d = 25 μM) and possibly also ATP (K_d = 50–100 μM). On Rrβ two binding sites have been demonstrated: a high-affinity Mg-independent nucleotide binding site with K_d values of 4 μM for ATP and 7 μM for ADP and a low-affinity Mg-dependent site that binds ATP (K_d = 200 μM), ADP (K_d = 80 μM), and P_i (K_d = 270 μM). Ligand-induced conformational changes have also been investigated, mainly with Tα and Tβ (Ohta et al., 1980a,b) and Ecα (Dunn, 1980; Senda et al., 1983). But only in the case of Ecα have the ATP-induced conformational changes been correlated with its high-affinity binding site that has been characterized by direct binding studies. With Ecβ, in contrast to Ecα, addition of ATP has not been found to induce any change in the trypsin sensitivity (Senda et al., 1983), although it has been found to induce some conformational change that could be detected as a quenching of 8-anilinonaphthalene

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[1] Abbreviations: Ecα, Tα, Ecβ, Rrβ, and Tβ, isolated reconstitutively active α and β subunits of the $F_0 \cdot F_1$ complex of *E. coli*, *R. rubrum*, and the thermophilic bacterium PS3; Tricine, *N*-[tris(hydroxymethyl)-methyl]glycine; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate.

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fluorescence (Hirano et al., 1984).

In this investigation we have found ligand-induced changes in trypsin digestion of Rrβ, which indicate that it undergoes large conformational changes on binding of ATP, ADP, and P_i. A clear-cut correlation has been established between the different types of the detected conformational states and the occupation of each of the two binding sites by either ATP, ADP, or P_i.

Materials and Methods

Preparation of the β Subunit. R. rubrum cells were grown and chromatophores prepared as previously described (Gromet-Elhanan, 1970, 1974; Philosoph et al., 1977; Khananshvili & Gromet-Elhanan, 1982). Electrophoretically pure and reconstitutively active Rrβ was isolated, purified, and stored as described by Gromet-Elhanan & Khananshvili (1986). Three different batches of Rrβ were used in this work. Their specific activity for reconstitution (Philosoph et al., 1977) ranged between 45 and 58 units, and they restored 80–90% of the photophosphorylation and Mg^{2+}–ATPase activities of β-less chromatophores. Before each experiment the Rrβ was freed from the ATP and $MgCl_2$ present in the storage buffer as described by Khananshvili & Gromet-Elhanan (1983, 1985a), using three successive elution–centrifugation steps through Sephadex G-50 columns (Penefsky, 1977). In the first two elution–centrifugation steps the columns were preequilibrated with buffer containing 50 mM Tricine–NaOH, pH 8.0, 20% glycerol, 2 mM EDTA, and 50 mM NaCl. In the third step EDTA was omitted in order to avoid interference with $MgCl_2$ during ligand binding.

Ligand Binding and Trypsin Digestion. Rrβ (0.5 mg/mL) was first preincubated at room temperature (23 °C) in TN buffer containing 50 mM Tricine–NaOH, pH 8.0, and 200 mM NaCl with or without various ligands. After 1 h, trypsin was added at a ratio of 1 mol of trypsin/100 mol of Rrβ. Proteolysis with trypsin was terminated by fast mixing with an equal volume of TN buffer containing soybean trypsin inhibitor at a 5-fold excess over trypsin (mol/mol). Samples containing 80–100 μg of trypsinized Rrβ were lyophilized and stored at −20 °C. Before SDS–polyacrylamide gel electrophoresis was performed, the lyophilized samples were dissolved in 120–150 μL of buffer containing 0.1 M sodium phosphate, pH 7.0, 1% SDS, 20% glycerol, and 0.05% bromphenol blue. After 5-min incubation at 80 °C 30–50 μL of each sample was immediately applied on the gel. The remaining aliquot could be stored in liquid nitrogen for a few months without any change in its electrophoretic properties.

Analytical Procedures. Protein was determined according to Lowry et al. (1951). SDS-polyacrylamide gel electrophoresis was carried out according to Weber & Osborn (1969) using an 11% separating gel covered by 2 cm of a 5% stacking gel. Gels were run for about 5 h at 100 V and then fixed in 12% TCA for 1 h and stained overnight at 23 °C in 0.2% Coomassie Brilliant Blue R-250 dissolved in water–ethanol–acetic acid (2:25:10 v/v). Destaining was carried out at 37 °C by continuous shaking in water–ethanol–acetic acid (65:25:8 v/v). The destained gels were stored in 12% TCA at 4 °C.

Gel scanning was carried out at 540 nm in a Beckman DU-8 spectrophotometer equipped with a linear transport module, using a full-scale absorbance of 2.0 o.d. units at a slit width of 0.05 mm. Gels were scanned from top to bottom at a speed of 5 cm/min, and the peak areas were measured with a maximal sensitivity of 1 using the "lowest valley" program. With 5–50 μg of protein a linear relationship was obtained between the peak area corresponding to Rrβ and the amount of Rrβ protein applied on the gel. This peak area, which was used for the quantitative determination of the amount of Rrβ remaining after trypsinization, was independent of the migrating distance in the gel and stayed constant during storage in 12% TCA at 4 °C for at least 4–5 days. The peak area of untrypsinized Rrβ, which was subjected to the same treatment as the trypsinized Rrβ but without trypsin, was taken as the 100% control value.

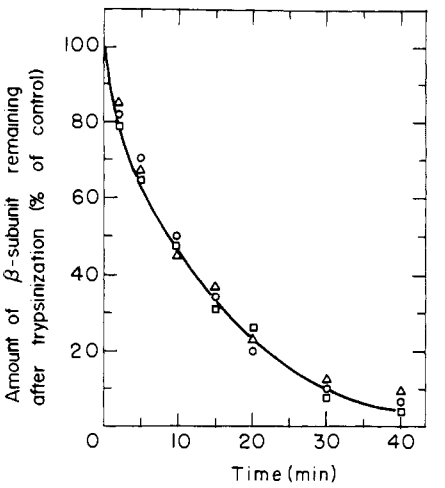

FIGURE 1: Time course of tryptic digestion of Rrβ. Rrβ was preincubated in 50 mM Tricine–NaOH, pH 8.0, without (○) or with (△) 25 mM $MgCl_2$ or 400 mM NaCl (□). After 1 h, trypsin was added, and at the time indicated 50-μL aliquots were removed from each incubation mixture, their proteolytic digestion was stopped, and they were subjected to SDS-polyacrylamide gel electrophoresis as described under Materials and Methods. The amount of Rrβ remaining after trypsinization was determined by measuring the peak area corresponding to Rrβ as outlined under Materials and Methods, using as the control value the peak area of an identical amount of untrypsinized Rrβ.

Materials. ADP, ATP, type XI trypsin (treated with diphenylcarbamoyl chloride), and type 1-S soybean trypsin inhibitor were purchased from Sigma. The electrophoresis LMW calibration kit of Pharmacia was used as molecular weight markers.

Results

Tryptic Digestion of Ligand-Free Rrβ. In all experiments reported below only ligand-free Rrβ was used, so the specific effect of various ligands on the rate and pattern of tryptic digestion could be followed. Trypsinization was carried out at pH 8.0 and 23 °C, since under these conditions Rrβ has been shown to retain all its reconstitutive activity for at least 2 h (Khananshvili & Gromet-Elhanan, 1983) and to bind ATP, ADP, and P_i (Gromet-Elhanan & Khananshvili, 1984; Khananshvili & Gromet-Elhanan, 1984, 1985a). With a molar ratio of trypsin to Rrβ of 1:100, ligand-free Rrβ was completely digested after 1 h, with a half-time of about 10 min (Figure 1). Under the above described conditions tryptic digestion was found to yield a very specific and reproducible pattern of cleaved peptides. During the first few minutes of proteolysis two intermediate peptides (30 000 and 20 000 Da) appear simultaneously (not shown but see Figure 4A), and these are further digested to smaller peptides, so that after 30 min the electrophoretic pattern shown in Figure 2b is obtained.

The kinetics of trypsin digestion as well as the electrophoretic pattern of the resulting peptides were identical for the three preparations of Rrβ used in this study. They were also very similar when Rrβ was preincubated in the presence of up to 400 mM NaCl (Figure 1) or 5–25 mM $MgCl_2$ (Figures 1 and 2c).

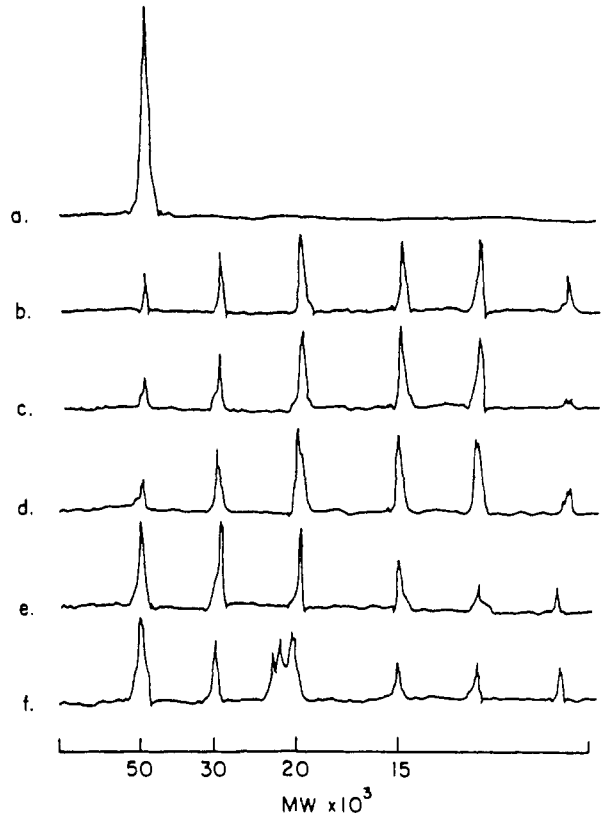

FIGURE 2: Effect of different ligands on the extent and electrophoretic patterns of tryptic digestion of Rrβ. Rrβ was preincubated in the absence (a, b) or presence of either 5 mM $MgCl_2$ (c), 5 mM P_i (d), 5 mM ATP (e), or 5 mM ADP (f). After 1 h trypsin was added to samples b–f, and all samples were further incubated for 30 min. The tryptic digestion was stopped, and the samples were subjected to SDS-polyacrylamide gel electrophoresis and scanned as described under Materials and Methods.

Effect of ATP, ADP, and P_i on the Tryptic Digestion of Rrβ in the Absence of $MgCl_2$. We have earlier reported that in the absence of $MgCl_2$ P_i does not bind to the isolated Rrβ (Khananshvili & Gromet-Elhanan, 1985a), whereas ATP or ADP bind, but only to one Mg-independent, high-affinity site (Gromet-Elhanan & Khananshvili, 1984). In the experiments illustrated in Figure 2d–f Rrβ was preincubated with P_i, ATP, and ADP, respectively, in the absence of $MgCl_2$, under conditions that are optimal for nucleotide binding to their Mg-independent site. It was then subjected for 30 min to our standard conditions of trypsinization by a 1:100 ratio of trypsin to Rrβ at 23 °C. As expected, P_i, which even at high concentrations does not bind to Rrβ in the absence of $MgCl_2$, had no effect on either the rate of Rrβ disappearance or the pattern of the resulting cleaved peptides (compare Figure 2, parts b and d; see also Figures 3C and 6C). On the other hand, binding of either ATP or ADP in the absence of $MgCl_2$ resulted in a marked protection of Rrβ against its digestion by trypsin (Figure 2e,f).

The protective effect of both ATP and ADP was dependent on their concentration during the preincubation and trypsinization stages, reaching saturation at about 100 μM (Figure 3A,B). This concentration dependence is very similar to that recorded for their binding to the high-affinity Mg-independent nucleotide binding site on Rrβ (Gromet-Elhanan & Khananshvili, 1984). But, although the binding of ATP and ADP to their Mg-independent site exerted a similar effect in protecting Rrβ against its digestion by trypsin (Figure 3A,B), they had very different effects on the pattern of the resulting cleaved peptides obtained during proteolysis (compare Figure 2, parts

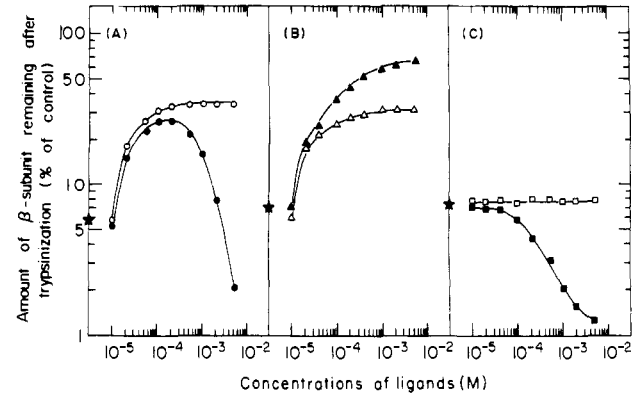

FIGURE 3: Effect of increasing concentrations of ATP, ADP, and P_i on the extent of tryptic digestion of Rrβ. Rrβ was preincubated in the presence of the indicated concentrations of ATP (A), ADP (B), and P_i (C), in the absence of (○, △, □) or presence (●, ▲, ■) of $MgCl_2$. $MgCl_2$ was added at concentrations that maintained a ratio of Mg^{2+} to ATP or ADP of 0.5 and Mg^{2+} to P_i of 2.5 over the whole range of ligand concentrations used. After 1 h of preincubation, trypsin was added to all samples and they were further incubated for 30 min. The tryptic digestion was stopped, and the samples were further treated as described in Figure 1. For each experiment the amount of Rrβ remaining after trypsinization in the absence of any added ligand has also been determined and is indicated by ★.

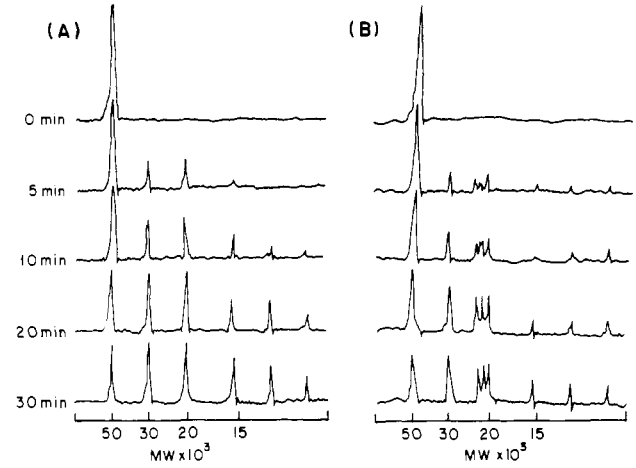

FIGURE 4: Effect of ATP and ADP in the absence of $MgCl_2$ on the extent and electrophoretic pattern of Rrβ tryptic digestion. Rrβ was preincubated with 5 mM ATP (A) or ADP (B) for 1 h followed by the addition of trypsin. The digestion was terminated at the time indicated, and the samples were subjected to gel electrophoresis and scanned as described in Figure 2.

e and f). Whereas binding of ATP did not change the pattern observed in its absence (compare Figure 2, parts b and e), the binding of ADP had a marked influence on the peptide pattern (compare Figure 2, parts b and f).

The differences in the electrophoretic pattern obtained on binding of ATP or ADP to Rrβ in the absence of $MgCl_2$ were not dependent on the concentration of the nucleotides (not shown) and persisted throughout the time course of tryptic digestion of Rrβ (Figure 4A,B). Thus, after 5 min of proteolysis in the presence of ATP two intermediate peptides of 30 000 and 20 000 Da appeared. Longer incubation resulted in a pronounced decrease in the amount of the remaining 50 000-Da Rrβ as well as in the appearance of additional smaller peptides (Figure 4A). On the other hand, 5 min of proteolysis in the presence of ADP led to the appearance of four peptides of 30 000, 23 000, 21 000, and 20 000 Da. Longer incubation resulted here too in a continued decrease in the amount of the remaining Rrβ and in the appearance of additional smaller peptides (Figure 4B). These different effects

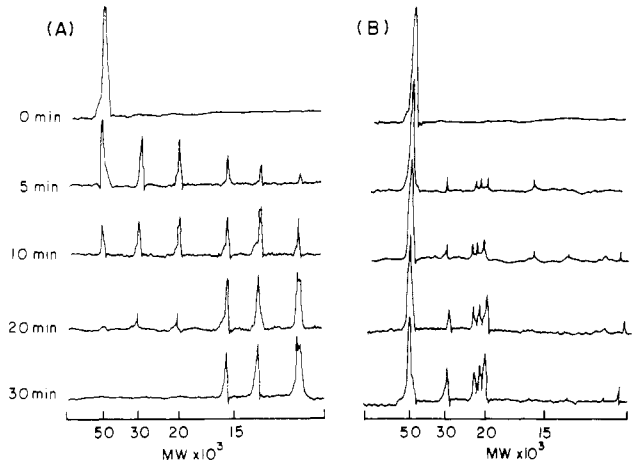

FIGURE 5: Effect of ATP and ADP in the presence of $MgCl_2$ on the extent and electrophoretic pattern of Rrβ tryptic digestion. Rrβ was preincubated with 5 mM ATP (A) or ADP (B) in the presence of 5 mM $MgCl_2$. All other procedures were carried out as described in Figure 4.

of ATP and ADP were observed with all the preparations of Rrβ used in this study.

Effect of ATP, ADP, and P_i on the Tryptic Digestion of Rrβ in the Presence of $MgCl_2$. Our observation that $MgCl_2$ by itself did not change the rate or electrophoretic pattern of Rrβ trypsinization (Figures 1 and 2c) enabled us to check the effect of ATP, ADP, and P_i in the presence of $MgCl_2$. Incubation of Rrβ with up to 50 or 100 μM of ATP, ADP, or P_i in the presence of $MgCl_2$ resulted in effects very similar to those observed in the absence of $MgCl_2$ (Figure 3A–C). With these low ligand concentrations $MgCl_2$ has indeed been shown to have no effect on ligand binding to Rrβ, so ADP and ATP could bind only to their Mg-independent high-affinity site (Gromet-Elhanan & Khananshvili, 1984) and P_i could not bind at all (Khananshvili & Gromet-Elhanan, 1985a). With higher ligand concentrations, under conditions allowing binding of ATP, ADP, and P_i to their Mg-dependent low-affinity site on Rrβ (Gromet-Elhanan & Khananshvili, 1984; Khananshvili & Gromet-Elhanan, 1984, 1985a), the presence of $MgCl_2$ during the preincubation had, however, a very pronounced effect (Figure 3A–C). Thus, increasing the MgATP concentration during the preincubation from 0.1 to 2.0 mM led to a progressive decrease in the protecting effect, until its complete elimination. Higher MgATP concentrations even increased the extent of trypsin digestion of Rrβ above that observed with ligand-free Rrβ (Figure 3A). A similar increase in the extent of trypsin digestion was observed on preincubation of Rrβ with MgP_i at concentrations above 50 μM (Figure 3C). On the other hand, preincubation of Rrβ with MgADP at concentrations above 50 μM yielded an opposite effect, increasing the protective effect above that observed in the absence of $MgCl_2$ (Figure 3B). The increased protection was dependent on the concentration of MgADP, reaching saturation at about 1 mM. This concentration was found to saturate the binding of ADP to its second, low-affinity Mg-dependent site on Rrβ (Khananshvili & Gromet-Elhanan, 1984).

The differences in the electrophoretic pattern obtained during proteolysis after preincubation of Rrβ with ATP or ADP in the absence of $MgCl_2$ (Figure 4A,B) persisted also after their preincubation in the presence of $MgCl_2$ (Figure 5A,B). So under these conditions pronounced differences were observed in both the extent and the electrophoretic pattern of Rrβ trypsin digestion. Thus, 5 min of proteolysis in the presence of MgATP led already to the appearance of the

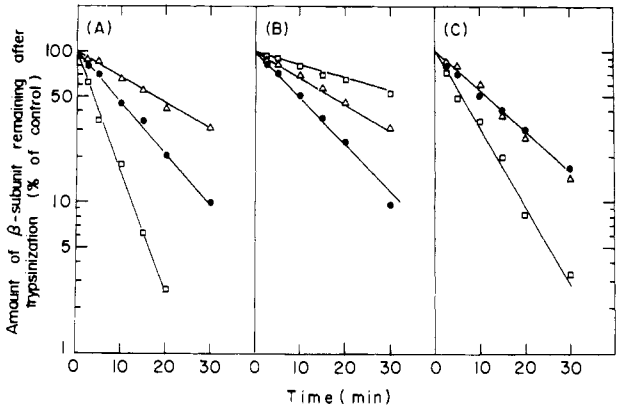

FIGURE 6: Time dependence of Rrβ trypsinization in the presence of ATP, ADP, and P_i. Rrβ was preincubated with 5 mM ATP (A), ADP (B), or P_i (C) in the absence (△) or presence (□) of 5 mM $MgCl_2$. After 1 h trypsin was added, and at the time indicated 70-μL aliquots were removed and subjected to the treatments and assays described in Figure 1. For each experiment the time dependence of ligand-free Rrβ trypsinization (●) has been simultaneously measured.

smaller peptides besides the two intermediate large ones (Figure 5A), and after 30 min only the smaller peptides remained (compare Figures 5A and 4A). With MgADP on the other hand, even after 30 min of trypsinization only the four intermediate peptides could be seen (compare Figure 5, parts A and B).

The effect of saturating concentrations (5 mM) of ATP, ADP, and P_i in the presence of $MgCl_2$ on the time course of Rrβ trypsinization is summarized in Figure 6. The half-time of trypsinization, which is about 10 min for ligand-free Rrβ and increases to 20 min with ATP or ADP in the absence of $MgCl_2$ (Figure 6A,B), decreases to 3 min in the presence of MgATP (Figure 6A) and to 6 min in the presence of MgP_i (Figure 6C). But, in the presence of MgADP it rather increases to 30 min (Figure 6B). These results indicate that binding of either ADP or ATP to their high-affinity Mg-independent site on Rrβ results in a change in its conformation that decreases its sensitivity to trypsin. On the other hand, binding of MgATP or MgP_i to their low-affinity Mg-dependent site on Rrβ induces a different conformational state, which is much more sensitive to trypsin. The data also demonstrate that different conformational states are induced on binding of either ATP or ADP to both sites on Rrβ. This difference becomes especially pronounced when the nucleotides bind to their Mg-dependent site, resulting in a 10-fold difference in the $t_{1/2}$ of trypsin digestion as well as in a different electrophoretic pattern.

Discussion

Binding of ATP to the high-affinity site on Ecα has been shown to lead to a very large conformational change that could be detected by an increase in the sedimentation coefficient of the subunit (Dunn, 1980) or a decrease in its trypsin sensitivity (Senda et al., 1983). Thus, in the absence of any ligands Ecα was cleaved by trypsin to peptides of less than 8000 Da. But in the presence of ATP or ADP, at concentrations sufficient to saturate the high-affinity Mg-independent site (Dunn & Futai, 1980), Ecα was cleaved in the middle of the polypeptide chain, leaving two trypsin resistant domains (Senda et al., 1983). Further evidence for a direct correlation between occupation of the nucleotide binding site and the conformational change detected by protection against trypsin digestion has been obtained in studies with a mutated Ecα, isolated from the F_1 of *E. coli* mutant *Unc* A401 (Stan-Lotter & Bragg, 1984). This Ecα, which has lost its capacity to bind ATP (Bragg et al., 1982), has also lost the protection against pro-

teolysis, being completely digested even in the presence of high concentrations of ATP or ADP.

Senda et al. (1983) have also tested the effect of ATP on the tryptic digestion of Ecβ, but unlike with Ecα, no protective effect has been observed. The results summarized above demonstrate that with Rrβ binding of various ligands caused markedly different effects on the rate as well as the electrophoretic pattern of its digestion by trypsin. Thus, in the absence of any ligands Rrβ was digested by trypsin (at a ratio of 100:1 (mol/mol), respectively), with a $t_{1/2}$ of 10 min (Figure 1). Under these conditions a very reproducible pattern of cleaved peptides was observed, which after 30 min of trypsinization was composed of two intermediate large peptides and a number of smaller ones (Figure 2b). The presence of either $MgCl_2$ (Figures 1 and 2c) or P_i (Figures 2d, 3C, and 6C) had no effect on either the rate or the electrophoretic pattern observed. It is interesting to note that Senda et al. (1983) have obtained a similar pattern of cleaved peptides on incubation of Ecβ with trypsin. But with Rrβ, unlike with Ecβ, preincubation with ATP resulted in partial protection against trypsin digestion, raising the $t_{1/2}$ from 10 to 20 min (Figure 6A,B). The protective effect was obtained with both ATP and ADP and was saturated at nucleotide concentrations of 100 μM in the absence or presence of $MgCl_2$ (Figure 3A,B). Such conditions have been shown to lead to binding of both nucleotides to a high-affinity, Mg-independent site on Rrβ (Gromet-Elhanan & Khananshvili, 1984).

The results therefore suggest that in Rrβ, as in Ecα, the partial protection against tryptic digestion is caused by conformational changes occurring upon binding of the nucleotides to their high-affinity Mg-independent site. There are, however, a number of differences in the nucleotide-induced conformational changes recorded in Ecα and Rrβ. In Ecα binding of either ATP or ADP to its single Mg-independent nucleotide binding site causes an identical large conformational change (Senda et al., 1983; Stan-Lotter & Bragg, 1984), whereas in Rrβ binding of ATP or ADP to their Mg-independent site causes a smaller, but different, conformational change (Figures 2e,f, 4, and 5).

The most pronounced difference between Ecα and Rrβ is, however, exhibited in the capacity of Rrβ to bind ATP and ADP to a second Mg-dependent site (Gromet-Elhanan & Khananshvili, 1984; Khananshvili & Gromet-Elhanan, 1984), and occupation of this second site was found to result in dramatic changes in its conformation (Figures 3, 5, and 6). Thus, binding of MgADP led to an increase in the protective effect, further stabilizing the trypsin resistant conformational state obtained with ADP. On the other hand, binding of MgATP or MgP_i induced a completely different conformational state that is much more sensitive to trypsin. Since Rrβ has not been crystalized yet, it is impossible to determine whether these conformational changes occur locally at the binding site or involve a larger portion of the polypeptide (Steitz et al., 1982).

There are a number of possible explanations for the markedly different conformational changes obtained on binding of MgATP, as compared to MgADP, to the Mg-dependent site on Rrβ. Thus, different types of metal–nucleotide diastereomers could be the true ligands for binding MgADP and MgATP to this site (Cleland & Mildvan, 1979). It has indeed been shown that different metal–nucleotide epimers of ADP and ATP are the substrates for a number of F_1 ATPases (Bossard & Schuster, 1981; Frash & Selman, 1982; Senter et al., 1984; Wieker & Hess, 1985). No such data are as yet available for RrF_1. An additional possible explanation for the difference between MgADP and MgATP is suggested by the identical effects of MgATP and MgP_i on the trypsin sensitivity of Rrβ (Figure 6A,C). A number of earlier observations led us to conclude that MgP_i binds to the γ-phosphoryl subsite in the Mg-dependent nucleotide binding site on Rrβ, which remains empty in the presence of MgADP. So, the conformational state of Rrβ that is much more sensitive to trypsin could be induced by occupation of this subsite in the presence of MgATP and MgP_i. Our earlier observations include (a) the fact that MgATP is 30-fold more effective than MgADP in competitively inhibiting the binding of P_i to Rrβ (Khananshvili & Gromet-Elhanan, 1985a) and (b) the demonstration that chemical modification of Rrβ by diethyl pyrocarbonate and Woodward's reagent K results in elimination of the binding of MgATP and MgP_i, but not MgADP, to their Mg-dependent site on Rrβ (Khananshvili & Gromet-Elhanan, 1985b).

A number of earlier observations have also indicated that the γ-phosphoryl subsite of the Mg-dependent binding site on Rrβ plays an important role in its reconstitutive activity. Thus, the presence of MgATP, but not of MgADP, has been shown to be essential for preservation of the reconstitutive activity of Rrβ during all stages of its isolation, purification, storage, and rebinding to β-less chromatophores (Binder & Gromet-Elhanan, 1974; Philosoph et al., 1977; Khananshvili & Gromet-Elhanan, 1982). Also, Rrβ modified by diethyl pyrocarbonate and Woodward's reagent K has been found to retain its capacity to rebind to β-less chromatophores but lost all its ability to restore their catalytic activity (Khananshvili & Gromet-Elhanan, 1985b). These data, together with the large conformational change observed here, indicate that the γ-phosphoryl subsite of the Mg-dependent nucleotide binding site on Rrβ must be an important part of the catalytic site of the $F_0 \cdot F_1$ ATP synthase, being actively involved in the discrimination between MgADP and MgATP.

Registry No. ATP, 56-65-5; ADP, 58-64-0; MgATP, 1476-84-2; MgADP, 7384-99-8; MgP_i, 13092-66-5; P_i, 14265-44-2; ATP synthase, 37205-63-3.

REFERENCES

Baird, B. A., & Hammes, G. (1979) *Biochim. Biophys. Acta 549*, 31–53.

Binder, A., & Gromet-Elhanan, Z. (1974) in *Proceedings of the 3rd International Congress in Photosynthesis* (Avron, M., Ed.) Vol. 2, pp 1163–1170, Elsevier, Amsterdam.

Bossard, M. J. & Schuster, S. M. (1981) *J. Biol. Chem. 256*, 1518–1521.

Bragg, P. D., Stan-Lotter, H., & Hou, C. (1982) *Arch. Biochem. Biophys. 231*, 669–679.

Cleland, W. W., & Mildvan, A. S. (1979) in *Advances in Inorganic Biochemistry* (Eichorn, G. L., & Marzilli, L. G., Eds.) Vol. 1, pp 163–191, Elsevier/North-Holland, Amsterdam.

Cross, R. L. (1981) *Annu. Rev. Biochem. 50*, 681–714.

Cross, R. L., & Nalin, C. M. (1982) *J. Biol. Chem. 257*, 2874–2881.

Dunn, S. D. (1980) *J. Biol. Chem. 255*, 11857–11860.

Dunn, S. D., & Futai, M. (1980) *J. Biol. Chem. 255*, 113–118.

Frash, W. D., & Selman, B. R. (1982) *Biochemistry 21*, 3636–3643.

Futai, M. (1977) *Biochem. Biophys. Res. Commun. 79*, 1231–1237.

Futai, M., & Kanazawa, H. (1983) *Microbiol. Rev. 47*, 285–312.

Gromet-Elhanan, Z. (1970) *Biochim. Biophys. Acta 223*, 174–182.

Gromet-Elhanan, Z. (1974) *J. Biol. Chem. 249*, 2522–2527.
Gromet-Elhanan, Z., & Khananshvili, D. (1984) *Biochemistry 23*, 1022–1028.
Gromet-Elhanan, Z., & Khananshvili, D. (1986) *Methods Enzymol. 126*, 528–538.
Grubmeyer, C., Cross, R., & Penefsky, H. S. (1982) *J. Biol. Chem. 257*, 12092–12100.
Harris, D. A. (1978) *Biochim. Biophys. Acta 463*, 245–273.
Hirano, M., Takeda, K., Kanazawa, H., & Futai, M. (1984) *Biochemistry 23*, 1652–1656.
Issartel, Y. P., & Vignais, P. V. (1984) *Biochemistry 23*, 6591–6595.
Kagawa, Y., Sone, N., Hirata, M., & Yoshida, M. (1979) *J. Bioenerg. Biomembr. 11*, 39–70.
Khananshvili, D., & Gromet-Elhanan, Z. (1982) *J. Biol. Chem. 257*, 11377–11383.
Khananshvili, D., & Gromet-Elhanan, Z. (1983) *J. Biol. Chem. 258*, 3714–3719.
Khananshvili, D., & Gromet-Elhanan, Z. (1984) *FEBS Lett. 178*, 10–14.
Khananshvili, D., & Gromet-Elhanan, Z. (1985a) *Biochemistry 24*, 2482–2487.
Khananshvili, D., & Gromet-Elhanan, Z. (1985b) *Proc. Natl. Acad. Sci. U.S.A. 82*, 1886–1890.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265–275.
Nelson, N. (1981) *Curr. Top. Bioenerg. 11*, 1–35.
Ohta, S., Tsuboi, M., Oshima, T., Yoshida, M., & Kagawa, Y. (1980a) *J. Biochem. (Tokyo) 87*, 1609–1617.
Ohta, S., Tsuboi, M., Yoshida, M., & Kagawa, Y. (1980b) *Biochemistry 19*, 2160–2165.
O'Neal, C. C., & Boyer, P. D. (1984) *J. Biol. Chem. 259*, 5761–5767.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891–2899.
Penefsky, H. S. (1979) *Adv. Enzymol. Relat. Areas Mol. Biol. 49*, 223–280.
Philosoph, S., Binder, A., & Gromet-Elhanan, Z. (1977) *J. Biol. Chem. 252*, 8747–8752.
Senda, M., Kanazawa, H., Tsuchiya, T., & Futai, M. (1983) *Arch. Biochem. Biophys. 220*, 398–404.
Senior, A. E., & Wise, J. G. (1983) *J. Membr. Biol. 73*, 105–124.
Senter, P., Eckstein, F., & Kagawa, Y. (1983) *Biochemistry 22*, 5514–5518.
Stan-Lotter, H., & Bragg, P. D. (1984) *Biochem. J. 224*, 145–151.
Steitz, T. A., Harrison, R., Weber, I. T., & Leahy, M. (1982) in *Mobility and Function in Proteins and Nucleic Acids*, Ciba Foundation Symposium 93, pp 25–46, Pitman, London.
Weber, M., & Osborn, U. (1969) *J. Biol. Chem. 244*, 4406–4412.
Wieker, H. J., & Hess, B. (1985) *Biochim. Biophys. Acta 806*, 35–41.
Yoshida, M., Sone, N., Hirata, H., & Kagawa, Y. (1977) *J. Biol. Chem. 252*, 3480–3485.

Anomalous Oxygen-18 Exchange during ATP Synthesis in Oxidative Phosphorylation†

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ABSTRACT: The synthesis of ATP from highly enriched [^{18}O]P_i by submitochondrial particles driven by succinate oxidation produces distributions of ^{18}O-labeled ATP species that deviate from the distributions predicted by a simple model for the exchange. Control experiments indicate no change in isotopic distribution when [^{18}O]ATP is synthesized from [^{18}O]ADP by adenylate kinase, which is bound to the submitochondrial particles. The observed deviations are in the opposite direction from that produced by heterogeneity due to multiple pathways for ATP synthesis. Two types of complex models can account for the observed deviations. One model has nonequivalence of the P_i oxygens during the exchange reaction, due to incomplete randomization of the P_i oxygens during the reversible cycles of hydrolysis and synthesis of bound ATP. The other model assumes that, during each turnover, a slow transition must occur between a high-exchange and a low-exchange pathway.

Analysis of the exchange reactions of labeled oxygen atoms between water and phosphate has proven to be an important probe of the mechanisms of a number of enzymes that synthesize or hydrolyze phosphoesters. [See Mitchell (1984) for a recent review.] The simple synthesis model of Scheme I illustrates the probable source of the multiple water oxygens often observed in the products of such reactions. If ATP is synthesized in unenriched water from P_i that has all four

Scheme I

$$E + ADP + P_i \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}ADP{\cdot}P_i \overset{HOH}{\underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}}} E{\cdot}ATP \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} E + ATP$$

oxygens labeled with ^{18}O, then the product will have three ^{18}O-labeled oxygens if $k_{-2} \ll k_3$. However, if $k_{-2} \gg k_3$, then many reversals of step 2 will occur before product release, and most of the ^{18}O label will be lost if the bound phosphate is capable of randomizing its oxygens between reversals. Thus, a model based on nonrestrictive phosphate binding, coupled with a product release rate that is slow in comparison to reaction reversal, predicts extensive oxygen exchange. Most

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