Differential Nucleotide Binding to Catalytic and Noncatalytic Sites and Related Conformational Changes Involving α/β-Subunit Interactions As Monitored by Sensitive Intrinsic Fluorescence in *Schizosaccharomyces pombe* Mitochondrial F_1†

Gilles Divita, Attilio Di Pietro,* Bernard Roux, and Danièle C. Gautheron

Laboratoire de Biologie et Technologie des Membranes et des Systèmes Intégrés, UMR 24 du Centre National de la Recherche Scientifique, Université Claude Bernard de Lyon, 43 Boulevard du 11 Novembre 1918, F-69622 Villeurbanne Cedex, France

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ABSTRACT: Mitochondrial F_1 from the yeast *Schizosaccharomyces pombe* exhibits an intrinsic tryptophan fluorescence sensitive to adenine nucleotides and inorganic phosphate [Divita, G., Di Pietro, A., Delêage, G., Roux, B., & Gautheron, D. C. (1991) *Biochemistry 30*, 3256–3262]. The present results indicate that the intrinsic fluorescence is differentially modified by nucleotide binding to either catalytic or noncatalytic sites. Guanine or hypoxanthine nucleotides, which selectively bind to the catalytic site, produce a hyperbolic saturation monitored by fluorescence quenching at 332 nm, the maximal emission wavelength. On the contrary, adenine nucleotides, which bind to both catalytic and noncatalytic sites, exhibit a biphasic saturation. High-affinity ATP binding produces a marked quenching as opposed to the lower-affinity one. In contrast, ADP exhibits a sigmoidal saturation, with high-affinity binding producing no quenching but responsible for positive cooperativity of binding to the lower-affinity site. The catalytic-site affinity for GDP is almost 20-fold higher at pH 5.0 as compared to pH 9.0, and the high sensitivity of the method allows detection of the 10-fold lower-affinity GMP binding. In contrast, high-affinity binding of ADP, or AMP, is not pH-dependent. The selective catalytic-site saturation induces a F_1 conformational change decreasing the Stern–Volmer constant for acrylamide and the tryptophan fraction accessible to iodide. ATP saturation of both catalytic and noncatalytic sites produces an additional reduction of the accessible fraction to acrylamide.

Mitochondrial F_1 is a solubilizable component of the ATPase–ATP synthase (F_o–F_1)[1] complex that catalyzes ATP synthesis from ADP and inorganic phosphate using the proton gradient generated by the respiratory chain inserted in the inner membrane.

Purified F_1 is composed of five types of subunit, with the stoichiometry $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$, and is able to hydrolyze ATP or other nucleoside triphosphates such as GTP or ITP. The two main subunits, α and β, contain the nucleotide and inorganic phosphate binding sites. The catalytic sites also bind the nucleotide analogue GMP-P(NH)P which behaves as a competitive inhibitor (Schuster et al., 1975; Roux et al., 1984). These sites are assumed, on the basis of numerous chemical modifications [for a review, see Vignais and Lunardi (1985)], to be mainly located on the β subunits; however, some authors have proposed that the α subunits might also contribute to the nucleotide binding sites (Williams & Coleman, 1982; Schäfer & Dose, 1984; Tagaya et al., 1988).

Other nucleotide sites are known to be adenine-specific (Harris et al., 1978; Baubichon et al., 1981; Di Pietro et al., 1981). A regulatory function has been put forward on the basis of hysteretic inhibition of ATP hydrolysis (Di Pietro et al., 1980) and on kinetic experiments indicating negative cooperativity (Schuster et al., 1975; Recktenwald & Hess, 1977), which is very sensitive to anions either activating (e.g., bicarbonate) or inhibitory (e.g., azide) (Ebel & Lardy, 1975). Genetic modification in the yeast *Schizosaccharomyces pombe* showed that the α subunit binds the phosphate chain moiety of the nucleotide (Jault et al., 1991) whereas chemical modifications with different nucleotide analogues label either the α subunit (Verburg & Allison, 1990) or the β subunit (Fellous et al., 1984; Boulay et al., 1985; Bullough & Allison, 1986; Van Dongen & Berden, 1986; Cross et al., 1987). These results favor the idea that the adenine binding domain is shared between α and β subunits. In contrast to the β subunit, problems rise with the hardly solubilizable α subunit that contains very few reactive groups: only reactive cysteines have been shown in *S. pombe* F_1 (Falson et al., 1986).

This extreme complexity of nucleotide binding sites, the total number of which is assumed to be six per F_1, is expected to involve large enzyme conformational changes related to selective nucleotide-site saturation and to interactions between catalytic and noncatalytic sites. However, up to now, very limited data on these aspects were available, probably due to the lack of sufficiently sensitive monitoring methods. We recently took advantage, in *S. pombe* F_1, of the presence of two tryptophan residues per α subunit producing an intense intrinsic fluorescence that was modified upon binding of adenine nucleotides or inorganic phosphate (Divita et al., 1991). Gene sequencing (Falson et al., 1991b) indicated that both tryptophans are strategically located with respect to nucleotide-site conserved elements, as follows:

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          190                                   257
DTILNHKRWNN.....APLQYLAPFSGCAMGEWFRDNGKHGLVVYD
element 2          FSBεA                      element 3
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[1] Abbreviations: F_o–F_1, mitochondrial ATPase–ATP synthase; AMP-P(NH)P, adenosine 5′-(β,γ-imidotriphosphate); ATPase, adenosine-5′-triphosphatase (EC 3.6.1.3); EDTA, ethylenediaminetetraacetic acid; F_1, soluble mitochondrial ATPase; FSBεA, 5′-[(*p*-fluorosulfonyl)-benzoyl]-1,N^6-ethenoadenosine; GMP-P(NH)P, guanosine 5′-(β,γ-imidotriphosphate); Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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W^{190} is positioned near element 2 that might interact with the nucleotide adenosine moiety (Walker et al., 1985; Fry et al., 1986) while W^{257} is located between element 3, assumed to react with bound Mg^{2+}, and Y^{245}, equivalent to Y^{244} in beef heart F_1 that is modified by 5'-[(*p*-fluorosulfonyl)benzoyl]-1,N^6-ethenoadenosine (FSBεA) (Verburg & Allison, 1990).

The present paper indicates that the intrinsic tryptophan fluorescence of *S. pombe* F_1 is a very sensitive probe allowing the discrimination between nucleotide binding to catalytic and noncatalytic sites, the monitoring of low-affinity nucleotide binding, and the characterization of related specific conformational changes of the enzyme and critical interactions between α and β subunits.

Experimental Procedures

Materials. Nucleotides were from either Boehringer Mannheim or Sigma. Their purity was controlled by high-pressure liquid chromatography using a Partisil PXS (10-25) SAX column from Whatman, as previously described (Di Pietro et al., 1988). Sodium iodide and sodium thiosulfate were from Merck, and acrylamide came from LKB. *N*-Acetyltryptophanamide was from Sigma. Sodium azide and sodium bicarbonate were from Prolabo. [U-^{14}C]ADP, 18.8 GBq/nmol, was from Amersham. The Micro BCA protein assay reagent kit was from Pierce.

Enzyme Preparation. Purified F_1, from a large-scale glycerol culture of *S. pombe* wild strain (972 h^-), was obtained by a previously described procedure (Falson et al., 1986) including poly(ethylene glycol) precipitation (Falson et al., 1989). The pure enzyme at 4–6 mg of protein/mL of 100 mM Tris-HCl, 50% glycerol, 5 mM EDTA, 10 mM *p*-aminobenzamidine, 10 mM ε-amino-*n*-caproic acid, and 1 mM phenylmethanesulfonyl fluoride, pH 7.5, was stored frozen in liquid nitrogen. Just before use, aliquots were thawed at 30 °C in a water bath and equilibrated in the desired buffer by centrifugation–elution through Sephadex G-50 Fine columns (Penefsky, 1977). As prepared, the enzyme contained a total of 3.6 mol of endogenous nucleotides/mol (half ADP–half ATP) when titrated by high-pressure liquid chromatography on a strong anion-exchange column, as previously described (Di Pietro et al., 1988). According to the criteria defined by Kironde and Cross (1986), all ADP was bound at noncatalytic nonexchangeable sites; 1 mol of ATP/mol of F_1 was readily exchangeable whereas 0.8 mol/mol appeared more difficult to release.

Assay Procedures. Protein concentration was estimated either by a modified Lowry procedure (Bensadoun & Weinstein, 1976) or by the bicinchoninic acid (Micro BCA) method (Smith et al., 1985) using bovine serum albumin as the standard. The molecular mass of F_1 was taken as 380 kDa (Falson et al., 1986).

The ATPase or ITPase activity assay in 50 mM Hepes–KOH, pH 7.5, was measured in the presence of azide or bicarbonate by spectrophotometric recording of NADH oxidation using an ATP- or ITP-regenerating system composed of phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase, as previously described (Falson et al., 1989; Jault et al., 1991).

Measurements of radiolabeled ADP binding were performed as previously described (Di Pietro et al., 1981; Falson et al., 1989). F_1 (50 μg of protein) was incubated for 30 min at 30 °C in 100 μL (final volume) of 20 mM Tris (Mes), 20% glycerol, and 2 mM $MgCl_2$, pH 7.5, in the presence of increasing concentrations of [U-^{14}C]ADP (127 dpm/pmol) with or without 100 μM sodium azide or 20 mM sodium bicarbonate. Nucleotide binding was measured after centrifugation–elution, according to Penefsky (1977), by liquid scintillation counting in Ready Safe (Beckman) medium.

Fluorescence. All measurements were performed at 30.0 ± 0.1 °C with a Bio-Logic spectrofluorometer equipped with a 150-W xenon lamp. The intrinsic fluorescence of native F_1 (0.3–0.4 mg of protein/1.5–1.7 mL) was routinely measured in 20 mM Tris–Mes, 20% glycerol, and 2 mM $MgCl_2$, pH 7.5, after 10-min incubation in the presence or absence of nucleotide or analogue, with or without activating or inhibiting anion. When ATP, GTP, or ITP were used, 1 mM excess EDTA was added to avoid nucleotide hydrolysis. When fluorescence was studied as a function of pH, three different buffers were used: 50 mM glycine (NaOH) from pH 9.0 to 8.0, 50 mM Tris (Mes) from pH 8.5 to 6.0, and 50 mM Tris (acetate) from pH 6.0 to 4.0, in addition to 20% glycerol and either 2 mM $MgCl_2$ or 1 mM EDTA. F_1 was equilibrated in the indicated buffer by centrifugation–elution and incubated for 60 min before fluorescence measurements.

Native F_1 showed a maximal excitation at 291 nm and a maximal emission at 332 nm (Divita et al., 1991). However, excitation was performed at 300 nm when nucleotides were present, in order to minimize their inner-filter effect. The residual effect was estimated in control experiments containing *N*-acetyltryptophanamide and increasing concentrations of nucleotide, up to 300 μM. With hypoxanthine and adenine nucleotides, an inner-filter effect appeared from 90 μM and linearly increased with concentration to reach respectively 4% and 5.5% at 300 μM. The effect was higher with guanine nucleotides: it began from 60 μM and reached 10% at 300 μM. F_1 fluorescence was corrected accordingly. Other controls of nucleotide inner-filter effects were performed with bovine serum albumin (which does not interact with nucleotides either). The obtained values were lower than those obtained with *N*-acetyltryptophanamide, indicating that F_1 corrections determined by reference to *N*-acetyltryptophanamide were not underestimated. Additional corrections were performed for dilution (never exceeding 5%) and for buffer blank (no protein present).

Quenching experiments in the presence of either acrylamide or sodium iodide were performed by sequential addition of aliquots from 6–8 M stock solutions, up to 0.32 M final concentration. The inner-filter effect of acrylamide was very low at 300 nm and corrected according to Calhoun et al. (1983). In the case or iodide, the stock solution contained 0.1 mM sodium thiosulfate to prevent I_3^- formation, and controls with equal concentrations of NaCl indicated that ionic strength did not significantly modify the F_1 fluorescence emission.

The fluorescence quenching data in the presence of either acrylamide or iodide were analyzed according to the Stern–Volmer equation which, when all quenching is collisional (no static quenching), is (Eftink & Ghiron, 1976, 1981)

$$F_0/F = 1 + K_{sv}[Q] \quad (1)$$

where F_0 and F are the fluorescence intensities, respectively, in the absence or the presence of quencher, K_{sv} is the collisional Stern–Volmer constant, and [Q] is the quencher concentration. The plot of F_0/F vs [Q] is linear for a homogeneous population of emitting fluorophores. On the contrary, fluorophore heterogeneity gives a downward curvature, and the modified Stern–Volmer relationship of Lehrer (1971) allows one to get a linear plot:

$$F_0/(F_0 - F) = 1/([Q]f_aK_Q) + 1/f_a \quad (2)$$

where f_a is the fractional number of accessible fluorophores and K_Q their collisional constant. The plot of $F_0/(F_0 - F)$ vs

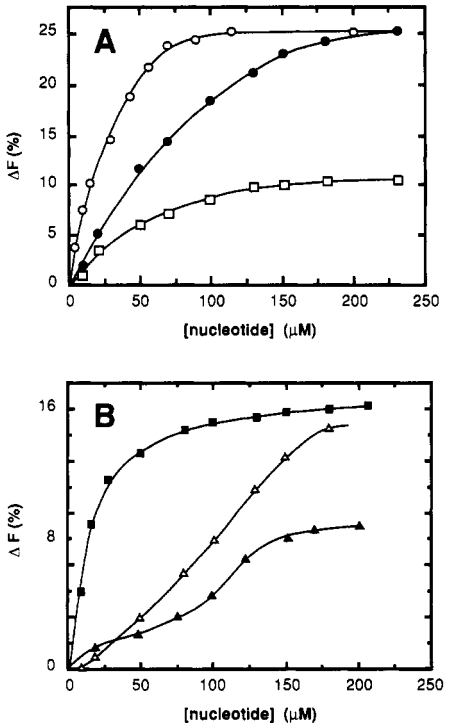

FIGURE 1: Saturation curves of nucleotides or analogues by quenching of the F_1 intrinsic fluorescence. The fluorometer cuvette contained 0.3 mg of F_1 in 1.5 mL of 20% glycerol, 2 mM $MgCl_2$, and 20 mM Tris (Mes), pH 7.5. Incubation for 10 min was conducted at 30.0 ± 0.1 °C in the presence of increasing concentrations of either GDP (●), GMP-P(NH)P (○), or IDP (□) in panel A or of either ADP (△), ATP (■), or AMP-P(NH)P (▲) in panel B. Excitation was performed at 300 nm and emission was recorded at 332 nm. The relative quenching of fluorescence produced by nucleotides, ΔF, was calculated by reference to the control in the absence of nucleotide and after adequate corrections (see Experimental Procedures).

1/[Q] allows graphical determination of f_a.

Results

Nucleotide Binding to F_1 As Monitored by the Intrinsic Tryptophan Fluorescence. Figure 1 shows that the binding of various nucleotides to *S. pombe* F_1 could be characterized and differentiated by quenching of the intrinsic tryptophan fluorescence. A concentration-dependent study indicated that the binding of all tested guanine or hypoxanthine nucleotides or analogues, known to selectively bind to the catalytic site, followed a hyperbolic saturation curve (Figure 1A). A much higher plateau value of quenching was obtained with guanine as compared to hypoxanthine derivatives. Linear double-reciprocal plots (not shown here) allowed extrapolation to maximal quenching values of 33% for guanine nucleotides and of 14% for hypoxanthine c nes and to a nucleotide concentration producing half-maximal c uenching of 31 μM GMP-P(NH)P, 67 μM IDP, or 80 μM GDP. Identical values were obtained when data were analyzed using the Macintosh curve-fitting program Multifit 2.0 (Day Computing, Cambridge). Similar results were obtained for ITP, as compared to IDP, and for GTP, as compared to GDP, in the presence of 1 mM excess EDTA to avoid hydrolysis.

In contrast, a biphasic pattern of quenching versus concentration was obtained with adenine nucleotides or analogues assumed to bind to both catalytic and noncatalytic sites. A positive cooperativity was observed for ADP, with a very low quenching up to about 20 μM and a maximal one above 200 μM ADP (Figure 1B). Very different effects were produced by ATP, with a marked quenching at 20 μM and a lower one at higher concentrations. Another different quenching pattern

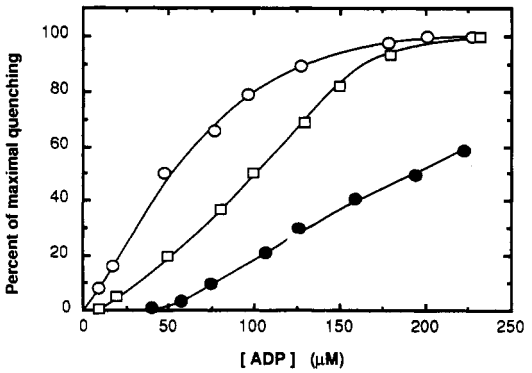

FIGURE 2: Anion effects on the binding of ADP monitored by intrinsic fluorescence quenching. F_1 was first incubated in the absence (□) or the presence of either 100 μM sodium azide (○) or 20 mM sodium bicarbonate (●) and then mixed with increasing concentrations of ADP. Fluorescence measurements were performed as in Figure 1B.

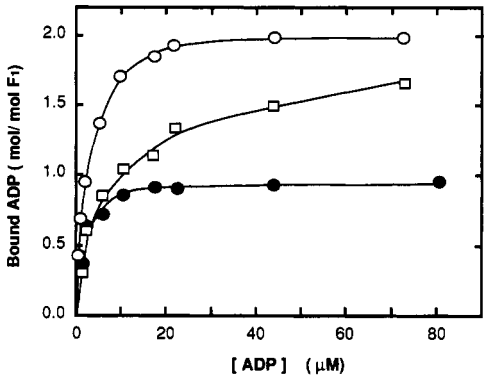

FIGURE 3: Anion effects on the binding of radiolabeled ADP measured by centrifugation–elution. F_1 (0.5 mg of protein/mL) in 20% glycerol, 2 mM $MgCl_2$, and 20 mM Tris (Mes), pH 7.5, was incubated for 30 min at 30 °C with increasing concentrations of [U-^{14}C]ADP at 127 dpm/pmol in the absence (□) or the presence of either 100 μM azide (○) or 20 mM bicarbonate (●). Free or loosely-bound nucleotide was eliminated by centrifugation–elution, and the eluates were counted for the remaining bound radioactivity (see Experimental Procedures).

was observed with adenosine 5'-(β,γ-imidotriphosphate) [AMP-P(NH)P], an ADP and ATP analogue. A biphasic fluorescence quenching was also obtained with the other analogue adenosine 5'-(β,γ-methylenetriphosphate) (not shown here). Double-reciprocal plots evidenced either an upward curvature for ADP and AMP-P(NH)P or a downward curvature for ATP; approximate maximal quenching values were estimated as 17% for ADP, 20% for ATP, and 11% for AMP-P(NH)P.

Anion Effects on Nucleotide Binding. Figure 2 indicates that ADP binding, as monitored by the quenching of intrinsic fluorescence, was very dependent on the presence of anions known to modulate enzyme activity. The addition of 20 mM bicarbonate, which 20–30% activated the hydrolysis of 1 mM ATP at pH 7.5, lowered ADP binding. Significant quenching, which appeared above 10 μM ADP in the control without anion, required more than 40 μM ADP in the presence of bicarbonate, and the half-maximal quenching, normally reached around 100 μM ADP, required as much as 190 μM ADP. On the contrary, the addition of 100 μM azide, which produced 80% inhibition of ATP hydrolysis, increased the apparent affinity for ADP binding. Indeed, a significant quenching was already produced by 10 μM ADP, the lowest concentration tested, and the half-maximal quenching was obtained with only 48 μM ADP. The same maximal quenching was produced by 230 μM ADP whether azide was present or not. From double-reciprocal plots (not shown here),

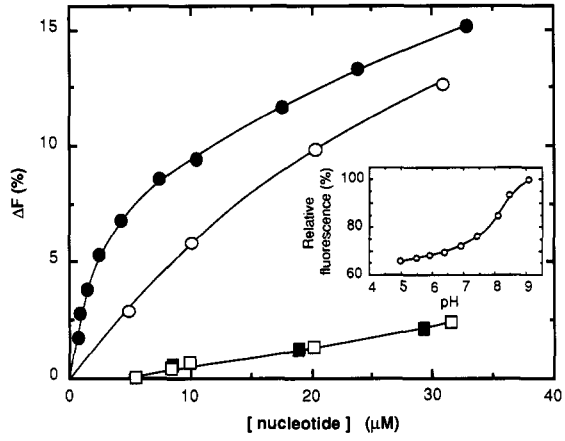

FIGURE 4: pH effects on the F_1 intrinsic fluorescence and quenching by adenine nucleotides. F_1 was incubated at either pH 5.0 (●, ■) or pH 7.5 (○, □) in the presence of increasing concentrations of ATP (●, ○) or (■, □), and fluorescence quenching was measured as in Figure 1B. Insert: F_1 fluorescence intensity as a function of pH. The enzyme was equilibrated, in the absence of any added nucleotide, at the indicated pH value from 9.0 to 5.0; its fluorescence intensity at 332 nm was measured by reference to the value obtained at pH 9.0.

the same maximal value of 17% was estimated even in the presence of bicarbonate. In controls, either bicarbonate or azide alone, in the absence of nucleotides, did not modify the enzyme intrinsic fluorescence.

As opposed to ADP, the binding of IDP, as shown in Figure 1A, was not significantly modified by the presence of any anion; in all cases, quenching values of 5.5% and 11% were obtained at 50 and 230 μM IDP, respectively. Under these conditions, 20 mM bicarbonate did not produce any activation of 1 mM ITP hydrolysis, and 100 μM azide only produced a limited inhibition ($I_{50\%}$ = 460 μM).

Figure 3 shows the effects of bicarbonate and azide on the binding of radiolabeled ADP as measured by centrifugation–elution. A biphasic saturation curve was clearly obtained with the control assay in the absence of anion; Scatchard plots (not shown here) allowed estimation of a total of 1.84 binding sites, with 2 different K_d values of 2.6 and 12 μM. The addition of 100 μM azide increased ADP binding at subsaturating concentrations: 2.08 binding sites were titrated, characterized by the same high affinity (K_d = 2.7 μM) and the loss of biphasicity. On the contrary, only 1.0 site, with high affinity (K_d = 2.2 μM), could be titrated in the presence of 20 mM bicarbonate under the conditions used.

Nucleotide Binding as a Function of pH. It was first necessary to determine the pH effects on enzyme intrinsic fluorescence in the absence of added nucleotides. A constant wavelength for maximal emission of 332 nm was observed when the pH varied in the range 9.0–5.0. Figure 4 (insert) shows that the fluorescence intensity at 332 nm, however, decreased as a function of pH: the decrease was sharp (24%) from pH 9.0 to pH 7.5 and milder (10%) down to pH 5.0.

It is worth mentioning that the enzyme was quite stable in the 9.0–5.0 pH range studied, in the absence or the presence of any nucleotide: less than 5% loss of activity occurred for a 60-min incubation under the conditions used. On the contrary, further lowering of the pH down to 4.0 produced a significant loss of activity (>20%), preventing accurate fluorescence measurements.

ATP binding also appeared to be pH-dependent (Figure 4). Its high-affinity binding increased at pH 5.0 as compared to pH 7.5: a 11 μM ATP concentration at pH 5.0 was sufficient to produce the same 10% quenching as did 21 μM at pH 7.5. In contrast, the high-affinity ADP binding, which produced

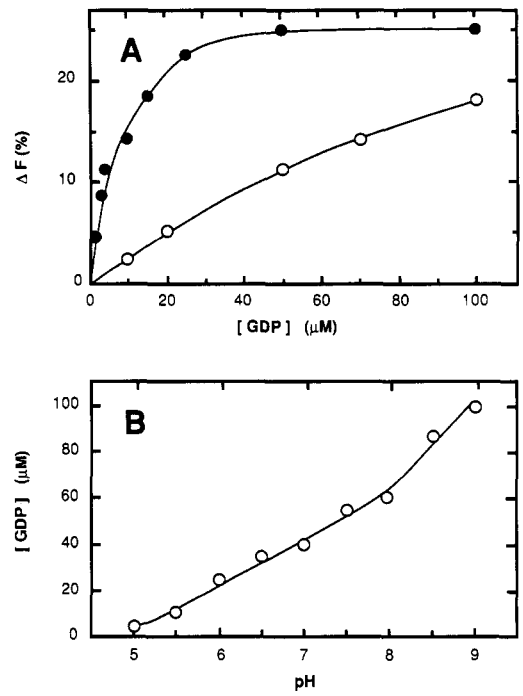

FIGURE 5: pH dependency of GDP binding. (A) The saturation curve of GDP binding at pH 5.0 (●) was measured by fluorescence quenching, in parallel to that obtained at pH 7.5 (○). (B) Similar curves were studied in the wide pH range from 9.0 to 5.0. The GDP concentration producing half-maximal quenching at each indicated pH value was graphically estimated and plotted as as a function of pH.

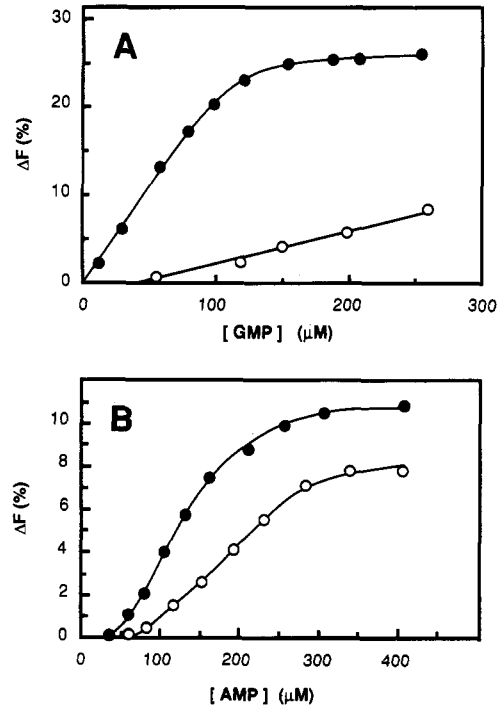

FIGURE 6: Detection of GMP and AMP low-affinity binding: effect of pH. The saturation curves of GMP (A) and AMP (B) were studied at either pH 5.0 (●) or pH 7.5 (○) as described for GDP in Figure 5A.

only a very limited quenching of fluorescence, was not changed, at least up to 30 μM ADP, when the pH was lowered from 7.5 to 5.0.

The binding of GDP was, like that of ATP, very dependent on the pH: a much higher quenching was produced by subsaturating GDP concentrations at pH 5.0 as compared to pH 7.5 (Figure 5A). The half-maximal effect was produced by

Table I: Effects of Nucleotides or Analogues on Tryptophan Accessibility to Acrylamide and Iodide[a]

nucleotide or analogue	concn (μM)	acrylamide K_{sv}[b] (M^{-1})	acrylamide f_a[c]	iodide K_{sv}[b] (M^{-1})	iodide f_a[c]
none		4.0	1.0	1.8	1.0
GDP	300	1.4	1.0	1.3	0.50
GTP	300	1.5	1.0	1.2	0.53
GMP-P(NH)P	120	2.2	1.0	1.25	0.50
IDP	300	3.0	1.0	1.5	0.50
ITP	300	3.1	1.0	1.6	0.55
ADP	10	3.7	1.0	1.7	1.0
	300	2.5	1.0	1.6	0.50
ATP	10	2.8	1.0	1.7	0.55
	300	2.5	0.66	1.7	0.50
AMP-P(NH)P	300	2.0	1.0	1.5	0.50

[a] F_1 was incubated for 10 min at pH 7.5 with either low (10 μM) or saturating (120–300 μM) concentration of nucleotide or analogue and mixed with increasing amounts of either acrylamide or iodide, up to 0.32 M. Excitation was performed at 300 nm, and the intensity of quenched fluorescence was measured at 332 nm. [b] The K_{sv} constant was graphically estimated as the initial slope from the Stern–Volmer plots (see Experimental Procedures). [c] The fractional number of accessible fluorophores (f_a) was extrapolated from the modified Stern–Volmer relationship, according to Lehrer (1971).

a much lower GDP concentration at pH 5.0 (6 μM) than at pH 7.5 (57 μM) whereas the same maximal quenching of 25% was obtained (compare with Figure 1A). When the pH was increased to 9.0, the GDP concentration producing half-maximal quenching increased correspondingly and reached a high value of 100 μM (Figure 5B). Therefore, an almost 20-fold lower GDP concentration was sufficient at pH 5.0 to produce the same effects as those observed at pH 9.0. Titration of endogenous nucleotides by anion-exchange high-pressure liquid chromatography indicated that they were not significantly released or exchanged either when the pH was lowered from 7.5 to 5.0 or after incubation with GDP: 1.8 mol of each ADP and ATP/mol of F_1 remained always present.

Figure 6A shows that GMP binding could also be efficiently measured at pH 5.0 by quenching of intrinsic fluorescence. A hyperbolic saturation curve was obtained, with extrapolated values of 28% for maximal quenching and of 62 μM for the concentration producing the half-maximal effect. GMP binding was very dependent on the pH since it could hardly be detected at pH 7.5 where a 195 μM concentration produced only 6% quenching, as compared to 26% at pH 5.0. In contrast, the binding of AMP appeared to be biphasic, similar to that of ADP, both at pH 5.0 and at pH 7.5 (Figure 6B). Up to 40 μM, no quenching was observed at any pH. The quenching increased at higher AMP concentrations to reach a maximal value of 11% at pH 5.0, with a 130 μM concentration for half-maximal effect. The increase was lower at pH 7.5: 400 μM AMP produced 8% quenching, as compared to 11% at pH 5.0, and a 230 μM concentration was required to produce the same quenching as 130 μM at pH 5.0.

Changes in the Tryptophan Environment by Nucleotide Binding. In the absence of added nucleotides, all tryptophans were accessible to both acrylamide and iodide (f_a = 1.0) with Stern–Volmer constants, K_{sv}, of 4.0 and 1.8 M^{-1}, respectively (Table I). Addition of guanine nucleotides or analogues markedly lowered the accessibility to both quenchers: K_{sv} for acrylamide was reduced to 1.4–1.5 M^{-1} by GDP or GTP or to 2.2 M^{-1} by GMP-P(NH)P, whereas the fraction accessible to iodide was half-reduced (f_a = 0.5). ITP and IDP produced similar effects to GTP and GDP with respect to the accessibility to iodide, although they were less efficient in reducing the K_{sv} for acrylamide. High-affinity binding of ADP did not produce any marked effect whereas additional lower-affinity binding produced the same half-reduction of the fraction accessible to iodide as guanine or hypoxanthine nucleotides and a significant decrease of the K_{sv} for acrylamide. In contrast, high-affinity binding of ATP already produced such effects, and additional, lower-affinity, binding produced a one-third reduction of the fraction accessible to acrylamide. The adenine nucleotide analogue AMP-P(NH)P at high concentration produced the same characteristic effects as guanine or hypoxanthine nucleotides and as ADP at high concentration.

Discussion

The intrinsic tryptophan fluorescence of mitochondrial F_1 from the yeast *S. pombe* is shown here to be a very sensitive probe to monitor differential nucleotide binding to catalytic and to noncatalytic sites, specific conformational changes induced by selective-site saturation, and critical interactions between α and β subunits.

F_1 Intrinsic Fluorescence: A Very Sensitive Probe for Nucleotide Binding. The present method of fluorescence quenching has allowed for the first time the detection and direct characterization of GMP and AMP binding. GMP binding, with an apparent affinity about 10-fold lower than that of GDP, was not detected previously. Direct measurement of AMP binding was not realized in earlier works; however, it was indirectly inferred since high AMP concentration afforded some protection against labeling and inactivation of beef heart F_1 by FSBεA (Verburg & Allison, 1990) and since its 2′,3′-*O*-(2,4,6-trinitrophenyl) fluorescent derivative showed detectable binding (Grubmeyer & Penefsky, 1981). The sensitivity is especially high with our yeast F_1 prepared through poly(ethylene glycol) precipitation, containing high amounts of endogenous nucleotides (3.6 mol/mol; Falson et al., 1989), and quite stable in the presence of 20% glycerol down to pH 5.0. The results with GDP indicate that nucleotide affinity at the catalytic site is about 10-fold increased from pH 7.5 to 5.0 and low apparent K_d values of 6 and 62 μM could be measured respectively for GDP and GMP at pH 5.0.

Even at pH 7.5, quenching of the F_1 intrinsic tryptophan fluorescence is shown to be more sensitive than other more commonly used methods and allows better characterization of low-affinity nucleotide binding. Indeed, the binding of radiolabeled ADP, when measured by centifugation–elution, does not reach complete saturation in the absence of anion, as previously observed for both wild-type and mutant *S. pombe* enzymes (Falson et al., 1989). The limits of the centrifugation–elution method to retain loosely-bound nucleotides have been discussed for the beef heart enzyme (Lunardi et al., 1987). Under our conditions, the low-affinity binding of radiolabeled ADP is not detected at all in the presence of bicarbonate, which decreases about 2-fold its affinity, as opposed to the quenching of tryptophan fluorescence which leads to a saturation curve. On the contrary, the intrinsic tyrosine fluorescence in beef heart F_1 was also inefficient for measuring low-affinity binding since only one high-affinity site could be detected (Tiedge et al., 1982).

The presently observed sensitive variations of *S. pombe* F_1 intrinsic fluorescence are actually due to nucleotide binding since we have taken great care to correct for the nucleotide inner-filter effect. The latter was minimized by excitation at 300 nm, and the residual effect was quantitatively determined under the same experimental conditions with several references.

Monitoring of Differential Nucleotide Binding to Catalytic and Noncatalytic Sites. All the present results clearly indicate that nucleotide saturation of the catalytic site of hydrolysis produces a significant quenching of intrinsic fluorescence. A

hyperbolic quenching saturation curve is obtained with guanine or hypoxanthine nucleotides, which are assumed to bind selectively to the catalytic site of *S. pombe* F_1 since they produced significant protection against affinity labeling of the catalytic site by 5′-[(*p*-fluorosulfonyl)benzoyl]guanosine but not against that of the noncatalytic site by 5′-[(*p*-fluorosulfonyl)benzoyl]adenosine (Jault et al., 1991). On the contrary, saturation of the adenine-specific noncatalytic site by low ADP concentration (apparent K_d of 2.2–2.7 μM as estimated from centrifugation–elution) does not produce any quenching but induces a marked positive cooperativity for ADP binding to the lower-affinity catalytic site correlated to the quenching of fluorescence. Different effects are observed with ATP: the first high-affinity binding produces a marked fluorescence quenching, indicative of binding to the catalytic site, whereas the second one, with lower affinity, does not quench, which indicates binding to the noncatalytic site. It is interesting to recall that ADP and ATP also behaved differently toward quenching of the extrinsic fluorescence produced by aurovertin binding to beef heart F_1 (Chang & Penefsky, 1973). The present results with *S. pombe* F_1 indicate that the adenine-specific noncatalytic site has higher affinity for ADP than for ATP, which agrees with the observation that, in pig heart F_1, a noncatalytic regulatory site requires lower amounts of ADP to induce hysteretic inhibition than of ATP to reverse it (Di Pietro et al., 1980). Saturation of the *S. pombe* F_1 noncatalytic site by ATP can also be detected through the limited tryptophan accessibility to acrylamide. Despite its lower affinity, AMP behaves like ADP and therefore exhibits better affinity for the noncatalytic, as compared to the catalytic, site. The biphasic saturation curve of AMP-P(NH)P confirms that the analogue also binds to both types of sites. It cannot be excluded that some AMP-P(NH)P is hydrolyzed during the course of the experiments as observed by Tomaszek and Schuster (1986). However, similar biphasic saturation curves were obtained with the nonhydrolyzable analogue adenosine 5′-(β,γ-methylenetriphosphate). The facts that AMP-P(NH)P produces a lower maximal quenching of fluorescence and that it does not reveal tryptophan heterogeneity toward acrylamide, contrary to ATP, indicate that the analogue does not perfectly fit the ATP site and/or is unable to induce the same enzyme conformational change. A similar difference has been observed in the case of myosin (Konrad & Goody, 1982) and phosphofructokinase (Berger & Evans, 1991). One can wonder whether the two types of sites, catalytic and noncatalytic, of *S. pombe* F_1 which bind ATP, ADP, or AMP-P(NH)P correspond to the two proposed vicinal, catalytic and noncatalytic, sites of beef heart F_1 that bind diadenosine oligophosphate compounds (Vogel & Cross, 1991).

Information allowing better understanding of anion effects has been obtained. Bicarbonate is shown to 2-fold lower ADP affinity to the catalytic site and the concomitant quenching of fluorescence. This favors the conclusion that bicarbonate activation of F_1 ATPase activity and abolition of negative cooperativity (Ebel & Lardy, 1975) are correlated to an increased rate of product ADP release from the catalytic site, a rate-limiting step of the ATPase reaction in the absence of anion. This would explain why the single-point mutation $\beta Gln^{170} \rightarrow$ Tyr in the catalytic site of *S. pombe* R4.3 strain (Falson et al., 1991a), which increases the affinity for ADP, requires high bicarbonate concentration to produce both maximal activation of ATPase activity (Jault et al., 1989) and complete abolition of negative cooperativity (Falson et al., 1989). Another activating anion, 2,4-dinitrophenolate, was also shown to lower ADP interaction at the catalytic site by kinetics with beef heart F_1 (Harris et al., 1981). In contrast, bicarbonate does not produce any apparent modification of ADP binding at the noncatalytic site of *S. pombe* F_1, which agrees with its lack of effect toward the ADP binding responsible for hysteretic inhibition of pig heart F_1 (Baubichon et al., 1982). The inhibitory anion azide produces opposite effects, as compared to bicarbonate. It increases the affinity for ADP to the catalytic site, the concentration producing half-maximal quenching of which is 2-fold lower; the azide inhibition is therefore related to decreased, rate-limiting, release of product ADP from the catalytic site during the ATPase reaction. This is quite consistent with previous results showing that (i) azide is able to produce a bicarbonate-sensitive negative cooperativity of ATPase activity (Jault et al., 1991), (ii) the catalytic-site mutation of the R4.3 strain produces both higher affinity for ADP and higher sensitivity to azide inhibition (Falson et al, 1989), and (iii) detailed kinetic analysis concludes to a mixed-type, i.e., at least partly competitive, inhibition of azide (Daggett et al., 1985). Of course, the present results do not exclude the possibility of an additional azide effect, undetectable under our conditions, on the ADP interaction at the noncatalytic site. As a control, the same concentrations of bicarbonate and azide, which do not or hardly modify F_1 ITPase activity, are shown not to produce any detectable modification of IDP binding as measured by fluorescence quenching.

Another new finding of the present work is that nucleotide saturation of the catalytic site is highly dependent on pH, the affinity being markedly increased at acidic values. This allows us to quantify high-affinity binding for GDP (apparent K_d = 6 μM at pH 5.0) and to detect low-affinity binding of GMP and AMP (as discussed above); the affinity for ADP and ATP is also increased. Similarly, the K_m for ATP hydrolysis was found to decrease when the pH was lowered from 8.8 to 7.5 (J.-M. Jault and A. Di Pietro, unpublished results), and radiolabeled ATP showed an increased binding at acidic pHs (Harris et al., 1978). Such a pH dependence of nucleotide binding to the catalytic site might be of physiological relevance with respect to activation of ATP synthesis by the membrane-bound enzyme since a local acidification due to proton gradient utilization is expected to occur. One can wonder whether this is correlated to the observation that the F_1 intrinsic fluorescence, even in the absence of added nucleotide, is quenched at acidic pH values. Similar effects in lysozyme (Lehrer, 1971) and in barnase (Loewenthal et al., 1991) have been interpreted as quenching due to protonation of a vicinal amino acid; in contrast, a conformational change has been proposed to occur in β_1-bungarotoxin (Chu & Chen, 1989). It is important to recall that the binding of inorganic phosphate in the γ-phosphate position of the ATP site (Penefsky, 1977) and its synergy with ADP to induce hysteretic inhibition (Di Pietro et al., 1986) are similarly dependent on pH.

The maximal quenching of fluorescence produced by catalytic-site saturation depends on the base of the nucleotide with the following order: guanine > adenine > hypoxanthine. This correlates to the spectral overlap of the base absorption red-edge with the F_1 tryptophan fluorescence spectrum and suggests a Förster energy-transfer mechanism, as proposed in the case of creatine kinase (Vasak et al., 1979).

Conformational Changes Induced by Selective-Site Saturation and Critical Interactions between α and β Subunits. Selective nucleotide saturation of the catalytic site produces marked changes in the tryptophan environment characterized by lowering in accessibility to external quenchers. Half of the tryptophans are no longer accessible to iodide, a charged

nonpenetrating quencher reacting with surface residues. In the presence of acrylamide, a neutral penetrating quencher reacting with all residues except for the most hydrophobic, all tryptophans remain accessible, but the binding of either GDP, GTP, GMP-P(NH)P, IDP, ITP, ADP or AMP-P(NH)P to the catalytic site makes half of the tryptophans, i.e., three among the six located on α subunits, more hydrophobic. Each nucleotide appears to produce specific effects: GDP and GTP decrease K_{sv} toward both acrylamide and iodide whereas the GTP analogue GMP-P(NH)P is somewhat less efficient, perhaps due to imperfect fitting to the site. The increased hydrophobicity of half of the tryptophans appears less pronounced with IDP or ITP since the decrease in K_{sv} for acrylamide is smaller than with guanine nucleotides. In the presence of adenine nucleotides or analogues, fully selective site saturation is rarely reached, due to cooperativity between binding sites. It may be assumed that the noncatalytic site is essentially saturated at 10 μM ADP; under these conditions, no apparent change in accessibility to either acrylamide or iodide is detected. At high ADP concentration saturating both noncatalytic and catalytic sites, the same features as those characterizing selective catalytic-site saturation by guanine or hypoxanthine nucleotides are essentially observed. Preferential occupation of the catalytic site by 10 μM ATP also produces similar effects; in contrast, saturation of both types of sites at 300 μM ATP makes one-third of the tryptophans no longer accessible to acrylamide, which suggests that two tryptophans get a very hydrophobic environment. A quite different conformational change was produced by inorganic phosphate binding which made all tryptophans slightly more hydrophobic (Divita et al., 1991).

Since the F_1 catalytic site is generally recognized to be, at least mainly, located on the tryptophan-free β subunit, the marked fluorescence quenching and environment change of tryptophans, which belong to the α subunit, show that critical interactions exist between α and β subunits and strongly suggest that these tryptophans are located within or very near these subunit contacts. Up to now, critical α/β-subunit interactions could only be detected by using aurovertin, an extrinsic fluorescent probe, and α-subunit-mutated enzymes from *Escherichia coli* essentially devoid of activity (Wise et al., 1981; Kanazawa et al., 1984). In contrast, our system has the great advantage of being noninvasive, of allowing work with the wild-type, fully active, enzyme, and of using tryptophan which is known to be a very powerful intrinsic reporter. Indeed, tryptophan residues are often located at protein/protein contacts [for a review, see Janin and Chothia (1990)] as this appears to be the case for the mitochondrial F_1 ϵ subunit interacting with δ subunit in both pig heart (Penin et al., 1990) and *S. pombe* (Divita et al., 1991). An attractive possibility would be a shared location of the catalytic site between α and β subunits, consistent with possible Förster energy transfer between tryptophan and the base of bound nucleotide, although a distant conformational change cannot be excluded.

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