Intrinsic Tryptophan Fluorescence of *Schizosaccharomyces pombe* Mitochondrial F_1-ATPase. A Powerful Probe for Phosphate and Nucleotide Interactions†

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

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

Received September 24, 1990

ABSTRACT: Mitochondrial F_1 from the yeast *Schizosaccharomyces pombe*, in contrast to the mammalian enzyme, exhibits a characteristic intrinsic tryptophan fluorescence with a maximal excitation at 291 nm and a maximal emission at 332 nm. Low values of Stern–Volmer quenching constants, 4.0 M^{-1} or 1.8 M^{-1}, respectively, in the presence of either acrylamide or iodide, indicate that tryptophans are mainly buried inside the native enzyme. Upon subunit dissociation and unfolding by 6 M guanidine hydrochloride (Gdn·HCl), the maximal emission is shifted to 354 nm, a value very similar to that obtained with *N*-acetyltryptophanamide, a solute-tryptophan model compound. The tryptophan content of each isolated subunit has been estimated by fluorescence titration in the presence of Gdn·HCl with free tryptophan as a standard. Two tryptophans and one tryptophan are found respectively in the α and ϵ subunits, whereas none is detected in the β, γ, and δ subunits. These subunit contents are consistent with the total of seven tryptophans estimated for native F_1 with $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ stoichiometry. The maximal emission of the isolated ϵ subunit is markedly blue-shifted to 310–312 nm by interaction with the isolated δ subunit, which suggests that the ϵ subunit tryptophan might be a very minor contributor to the native F_1 fluorescence measured at 332 nm. This fluorescence is very sensitive to phosphate, which produces a marked blue shift indicative of tryptophans in a more hydrophobic environment. On the other hand, ADP and ATP quench the maximal emission at 332 nm, lower tryptophan accessibility to acrylamide, and reveal tryptophan heterogeneity.

Mitochondrial F_1[1] is a solubilizable moiety of the ATPase–ATP synthase complex that catalyzes ATP synthesis during oxidative phosphorylations. Purified F_1 is composed of five types of subunits with a $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ stoichiometry and is able to hydrolyze ATP [see the review of Godinot and Di Pietro (1986)]. The two major subunits α and β are known to contain nucleotide-binding sites, whereas only the β subunit has been reported to bind phosphate or its analogues [for a review, see Vignais and Lunardi (1985); Wang (1988); Ysern et al. (1988)].

Nucleotide- and phosphate-induced conformational changes of the enzyme have been characterized in our laboratory by a number of approaches including accessibility to proteases (Di Pietro et al., 1983), circular dichroism (Roux et al., 1984), and reactivity to various chemical reagents (Di Pietro et al., 1979; Fellous et al., 1984; Falson et al., 1986) and monoclonal antibodies (Gautheron & Godinot, 1988).

Another powerful method currently used to monitor protein conformational changes is intrinsic tryptophan fluorescence, since tryptophan is highly fluorescent compared to the other aromatic amino acids, and its fluorescence is very sensitive to a wide variety of environmental conditions (Creed, 1984). Unfortunately, mammalian F_1 contains only one tryptophan on its ϵ subunit (Walker et al., 1985), the fluorescence of which has been shown to be considerably altered by its tight association with the δ subunit (Penin et al., 1990). This would explain why the intrinsic fluorescence spectrum of mammalian F_1, which contains about a hundred tyrosines, looks like a typical tyrosine spectrum (Tiedge et al., 1982; Baracca et al., 1986; Penin et al., 1990).

The present work, on the contrary, shows that mitochondrial F_1 from the yeast *Schizosaccharomyces pombe* exhibits a characteristic intrinsic tryptophan fluorescence. This fluorescence is due to the presence of two tryptophans on each α subunit that are weakly accessible to external medium and chemical quenchers. Important conformational changes are induced by phosphate, which produces a more hydrophobic microenvironment, and by nucleotides, which behave as quenchers and reveal tryptophan heterogeneity.

Experimental Procedures

Materials. Guanidine hydrochloride (Gdn·HCl), sodium iodide, and sodium thiosulfate were from Merck. *N*-Acetyltryptophanamide and tryptophan-containing model proteins were from Sigma. Molecular weight marker proteins were from Serva, and free L-tryptophan was RP from Prolabo. Acrylamide and SP-Trisacryl came from Pharmacia LKB. The HPLC C4 Aquapor Butyl 300-Å column came from Touzard & Matignon, and Centriprep concentrators were from Amicon.

Biological Preparations. Purified F_1 from a large-scale glycerol culture of *S. pombe* wild strain $(972h)^-$ was obtained as previously described (Falson et al., 1986) with subsequent modifications including poly(ethylene glycol) precipitation (Falson et al., 1987, 1989). The pure enzyme at 4 mg of protein/mL in 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 rapidly thawed at 30 °C and equilibrated in the indicated buffer by

† Financial support from the CNRS, UMR 24, is acknowledged.

[1] Abbreviations: ADP, adenosine 5′-diphosphate; ATP, adenosine 5′-triphosphate; ATPase, adenosine-5′-triphosphatase (EC 3.6.1.3); EDTA, ethylenediaminetetraacetic acid; F_1, solubilized mitochondrial ATPase; Gdn·HCl, guanidine hydrochloride; HPLC, high-pressure liquid chromatography; Mes, 4-morpholineethanesulfonic acid; NATA, *N*-acetyltryptophanamide; P_i, inorganic phosphate; SDS, sodium dodecyl sulfate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

0006-2960/91/0430-3256$02.50/0

centrifugation–elution through Sephadex G-50 Fine columns (Penefsky, 1977).

Isolated subunits were prepared from 6 mg of F_1 equilibrated in 100 mM P_i and 1 mM EDTA, pH 7.5, mixed with 6 M guanidine hydrochloride (Gdn·HCl), 50 mM dithiothreitol, and 50 mM Tris-HCl (final concentrations), pH 8.0, and incubated overnight at 35 °C. The smaller subunits ϵ, δ, and γ were purified by reverse-phase HPLC with a C4 Aquapor Butyl 300-Å column using a linear 25%–60% acetonitrile gradient in 0.1% trifluoroacetic acid–water; they eluted respectively at 31%, 38%, and 44% acetonitrile, whereas the α and β subunits coeluted at around 50%. The different peaks were collected and lyophilized. To separate the α and β subunits, the lyophilysate was solubilized in 8 M deionized urea, 17 mM sodium acetate, 100 mM β-mercaptoethanol, and 0.02% sodium azide, pH 5.0, incubated overnight at 35 °C, and submitted to ion-exchange chromatography on SP-Trisacryl in the same buffer. The β subunit was collected in the void volume, whereas the α subunit was retained on the column and subsequently eluted at 0.2 M NaCl when a linear 0–0.5 M NaCl gradient was applied. The eluates were first 10-fold diluted and then concentrated by centrifugation on a Centriprep apparatus equipped with YM-10 ultrafiltration membranes. The concentrates were mixed with 6 M Gdn·HCl and 50 mM DTT (final concentrations) and submitted to reverse-phase HPLC as above; the α and β subunits were eluted respectively at 49% and 47% of the acetonitrile gradient in 0.1% trifluoroacetic acid–water and lyophilized.

Polyacrylamide gel electrophoresis in the presence of SDS was performed according to the method of Laemmli (1970). Protein concentration was estimated either by the modified Lowry procedure of Bensadoun and Weinstein (1976) or by the bicinchoninic acid method (Smith et al., 1985) with bovine serum albumin as a standard. A molecular mass of 380 kDa was assumed for pure F_1 (Falson et al., 1986). The ATPase activity was measured by spectrophotometric recording of NADH oxidation in the presence of an ATP-regenerating system with pyruvate kinase and lactate dehydrogenase as auxiliary enzymes, as previously described (Falson et al., 1986).

Fluorescence. Fluorescence measurements were performed at 30 ± 0.1 °C with a Bio-Logic spectrofluorometer equipped with a 150-W xenon lamp. The tryptophan content of isolated subunits and of native F_1 was determined by the method of Pajot (1976) on the basis of fluorescence measurements in the presence of 6 M Gdn·HCl. Free tryptophan, or alternatively NATA, was used as the standard, and the validity of the method was controlled on various tryptophan-containing model proteins: bovine serum albumin [66 kDa, $\epsilon_{280} = 42 \times 10^3$ M^{-1} cm^{-1}; King and Spencer (1970)], ovalbumin [45 kDa, Mc Reynolds et al. (1978)], bovine trypsin [24 kDa, $\epsilon_{280} = 36.8 \times 10^3$ M^{-1} cm^{-1}; Robinson et al. (1971)], and egg lysozyme [14.3 kDa, $\epsilon_{280} = 37.8 \times 10^3$ M^{-1} cm^{-1}; Canfield (1963)]. The medium (1.5-mL final volume) contained either the isolated F_1 subunit (3.6–14 μM), the model protein (0.8–1 μM), or native F_1 (0.25–0.5 μM) previously incubated overnight with 6 M Gdn·HCl at room temperature. Excitation was performed at 295 nm and emission was recorded at 350 nm. Standard experiments were conducted under the same conditions with increasing free tryptophan (or alternatively NATA) concentrations up to 15 μM. Under these conditions, it was determined by use of internal standards that the fluorescence values due to free tryptophan and proteins (model proteins or F_1 subunits) were additive.

Intrinsic fluorescence of native F_1 or of isolated subunits was assayed in 20 mM Tris–Mes and 20% glycerol, pH 7.5. The medium contained 2 mM $MgCl_2$ except when ATP was present; then a 1 mM excess of EDTA was added to avoid ATP hydrolysis. Excitation was routinely performed at 295 nm and emission was recorded in the range 300–450 nm. Both emission and excitation spectra were corrected for the buffer blank and for the variations of the lamp emission power. When nucleotides were present, the excitation was performed at 300 nm in order to minimize the inner-filter effect. The remaining effect (less than 5% at 300 μM nucleotide) was estimated in control experiments in the presence of either bovine serum albumin or NATA (which do not interact with nucleotides), and F_1 fluorescence emission was corrected accordingly.

Quenching experiments in the presence of sodium iodide or acrylamide were performed by adding successive aliquots of 6–8 M stock solutions up to a 0.26–0.32 M final concentration. The sodium iodide stock solution contained 0.1 mM sodium thiosulfate to prevent I_3^- formation, and it was determined by using NaCl that ionic strength up to 0.26 M did not significantly modify the fluorescence emission of native F_1. In the experiments with acrylamide, excitation was performed at either 295 or 300 nm in the absence or the presence of nucleotides, respectively. In the former case, corrections for the inner-filter effect due to acrylamide absorption were calculated according to Calhoun (1983). The values were also corrected for dilution (never exceeding 5%) and for the buffer blank (no protein present).

The fluorescence quenching data obtained in the presence of either iodide or acrylamide were analyzed according to the Stern–Volmer equation, which considers two types of quenching, collisional and static (Eftink & Ghiron, 1976, 1981):

$$F_0/F = (1 + K_{SV}[Q])e^{V[Q]} \quad (1)$$

where F_0 and F are the fluorescence intensities in the absence or the presence of quencher, respectively, K_{SV} is the collisional Stern–Volmer constant, [Q] is the quencher concentration, and V is the static quenching constant. A plot of F_0/F vs [Q] is used to describe the process and gives an upward curvature.

If there is only collisional quenching (no static quenching), eq 1 becomes

$$F_0/F = 1 + K_{SV}[Q] \quad (2)$$

and the plot is linear for a homogeneous population of emitting fluorophores. On the contrary, a downward curvature indicates fluorophore heterogeneity; only a fraction of the fluorophore is accessible to the quencher. The modified Stern–Volmer relationship of Lehrer (1971) allows one to get a linear plot:

$$F_0/(F_0 - F) = 1/([Q]f_aK_Q) + 1/f_a \quad (3)$$

where f_a represents the fractional number of accessible fluorophores and K_Q their collisional constant. The plot of $F_0/(F_0 - F)$ vs $1/[Q]$ allows graphical determination of f_a and K_Q.

Results

Intrinsic Fluorescence of F_1-ATPase. Limited Accessibility of Tryptophans. Native mitochondrial F_1 purified from *S. pombe* showed a typical intrinsic tryptophan fluorescence with a maximal excitation at 291 nm (Figure 1A). The maximal emission spectrum (Figure 1B) was centered at 332 nm with a bandwidth (at half-height) of about 60 nm. When 6 M Gdn·HCl was added to dissociate and unfold F_1 subunits, which produced a complete loss of ATPase activity, the excitation spectrum was not modified, whereas the maximal emission wavelength was shifted to 354 nm. A very similar value of 356 nm was obtained under the same conditions with NATA, a solute-tryptophan model compound, whether Gdn·HCl was present or not (data not shown).

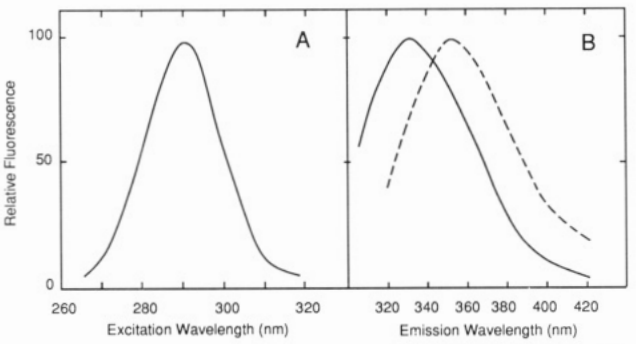

FIGURE 1: Intrinsic fluorescence of native or unfolded F_1. The fluorometer cuvette contained a 0.2 mg/mL protein solution of F_1 either native (solid line) or unfolded by a previous overnight incubation with 6 M Gdn·HCl (dashed line). (A) Excitation spectrum with or without Gdn·HCl, the emission being measured at 332 or 354 nm, respectively, for native or unfolded F_1. (B) Emission spectrum, the excitation wavelength being 291 nm.

Table I: Increased Accessibility of F_1 Tryptophans to Quenchers upon Gdn·HCl Addition: Comparison with NATA[a]

	K_{SV} (M^{-1})		
	F_1-ATPase		
quencher	native	unfolded	NATA[b]
acrylamide	4.0	13	18
iodide	1.8	3.2	6.0

[a] F_1 either native or unfolded by a previous incubation with 6 M Gdn·HCl was mixed with increasing amounts of either acrylamide or iodide. The excitation was performed at 295 nm and emission was measured at either 332 or 355 nm, respectively, for native or unfolded F_1. Stern–Volmer plots were linear and allowed graphical estimation of K_{SV} (see Experimental Procedures). [b] Controls under the same experimental conditions were conducted with NATA instead of F_1 and emission was measured at 355 nm. The same results were obtained whether 6 M Gdn·HCl was present or not.

Table I shows that tryptophans in native F_1 exhibited a limited accessibility to acrylamide, a penetrating uncharged quencher. A relatively low apparent Stern–Volmer quenching constant, K_{SV}, of 4.0 M^{-1} was obtained as compared to the value of 18 M^{-1} obtained under the same conditions with NATA. Subunit dissociation and unfolding in the presence of Gdn·HCl largely increased tryptophan accessibility (K_{SV} = 13 M^{-1}). The tryptophans were even less accessible to iodide, a polar anionic quencher exhibiting a K_{SV} of 1.8 M^{-1}. The latter value was increased up to 3.2 M^{-1} by addition of Gdn·HCl but remained lower than that of NATA under comparable conditions (6.0 M^{-1}). Native F_1 activity was barely affected by the presence of acrylamide; its ATPase activity was only 15% inhibited by a 30-min incubation with 0.32 M acrylamide. On the contrary, it was much more sensitive to 0.32 M iodide (around 75% inhibition). The inhibition was even more extended and 5-fold more rapid when 0.1 mM thiosulfate, routinely used to prevent I_3^- formation, was omitted. Thiosulfate alone had no effect on the enzyme activity.

Location and Titration of Tryptophans on Isolated Subunits. F_1 subunits were dissociated by either Gdn·HCl or urea and then purified by HPLC as detailed under Experimental Procedures. Isolated ϵ, δ, γ, and β subunits appeared as single bands when analyzed by Coomassie blue staining after SDS–polyacrylamide gel electrophoresis (Figure 2A). Isolated α subunits contained some faint additional component of slightly higher mobility, in too low an amount to be clearly differentiated, which appeared during subunit purification. This faint component looked like the α' component, which generally arises from gentle proteolysis of a few N-terminal amino acids

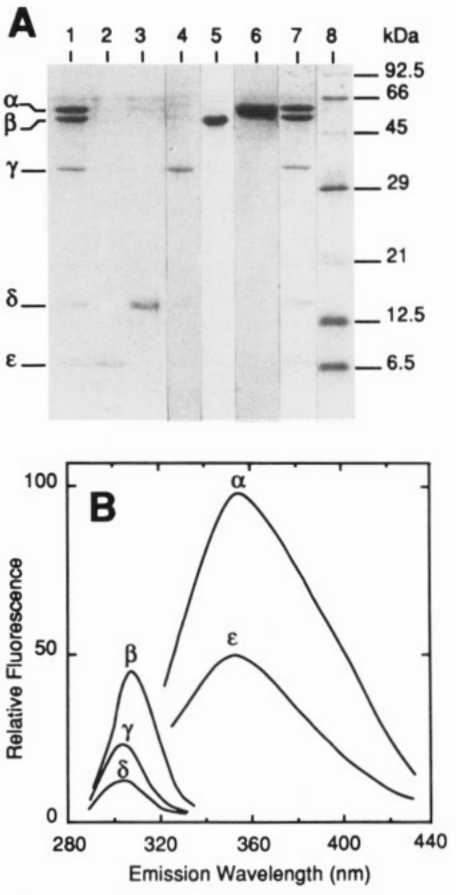

FIGURE 2: Electrophoresis and intrinsic fluorescence of isolated F_1 subunits. F_1 was dissociated by incubation with Gdn·HCl, and the minor subunits ϵ, δ, and γ were purified by reverse-phase HPLC with an acetonitrile gradient. The major subunits α and β were further separated by cation-exchange chromatography in the presence of urea (see Experimental Procedures). (A) SDS–polyacrylamide gel electrophoretogram of isolated F_1 subunits: lanes 1 and 7, starting F_1; lanes 2–6, purified subunits ϵ, δ, γ, β, and α, respectively; lane 8, mixture of protein molecular weight standards composed of, from top to bottom, phosphorylase B, bovine serum albumin, egg albumin, carbonic anhydrase, soybean trypsin inhibitor, cytochrome *c*, and bovine lung trypsin inhibitor, the corresponding molecular weight values being indicated on the right side of the Figure. (B) Emission fluorescence of 3.6 μM of each isolated subunit in the presence of Gdn·HCl. The excitation was performed at 295 nm, and the emission spectrum was recorded in the range 290–430 nm.

of the α subunit as already observed in some F_1 preparations from *S. pombe* (Falson et al., 1986) and other species (Di Pietro et al., 1983, and references therein). The apparent molecular masses of subunits, as estimated by comparison with marker proteins' mobility, were the following: ϵ, 7.5 kDa; δ, 15 kDa; γ, 32 kDa; β, 50 kDa; α, 56 kDa. The emission fluorescence spectra of isolated subunits, in the presence of 6 M Gdn·HCl and upon excitation at 295 nm, are shown in Figure 2B. Two subunits, namely α and ϵ, showed a characteristic tryptophan emission spectrum with a maximum centered at 354 or 356 nm, respectively. The α subunit's spectrum was much more intense than that of the ϵ subunit at the same molar concentration. In both cases, the maximal wavelength of excitation was 292 nm (not shown here). On the contrary, the three other subunits, namely β, γ, and δ, showed a very low intensity emission peak centered around 308 nm, characteristic of tyrosine fluorescence. Their maximum wavelength of excitation was in the range 278–280 nm. Table II shows a tryptophan titration of isolated F_1 subunits and of native F_1 by fluorescence emission at 350 nm in the presence of Gdn·HCl and with free tryptophan as the standard.

Table II: Tryptophan Content of F_1 Subunits As Titrated by Fluorescence in the Presence of Gdn·HCl[a]

protein	tryptophan content (mol/mol)
model proteins[b]	
bovine serum albumin (2)[c]	1.85
ovalbumin (3)[d]	3.0
bovine trypsin (4)[e]	4.0
egg lysozyme (6)[f]	6.1
F_1 subunits	
α	2.3
ϵ	0.9
native F_1 ($\alpha_3\beta_3\gamma_1\epsilon_1$)	6.9

[a] Various model proteins, purified F_1 subunits, or native F_1 was incubated overnight in the presence of 6 M Gdn·HCl. The samples were excited at 295 nm and emission was measured at 350 nm. A calibration was performed with increasing free tryptophan concentrations under the same experimental conditions (see Experimental Procedures). [b] The theoretical amounts of tryptophan in model proteins are indicated in parentheses. [c] King and Spencer (1970). [d] Mc Reynolds et al. (1978). [e] Mikes et al. (1966). [f] Canfield (1963).

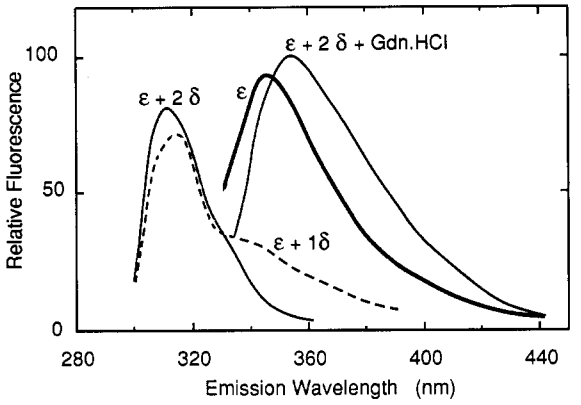

FIGURE 3: Modifications of ϵ subunit intrinsic fluorescence by interaction with the δ subunit and dissociation of the complex with Gdn·HCl. Upon excitation at 295 nm, the fluorescence emission spectrum of 13.3 μM purified ϵ subunit was recorded either before (ϵ spectrum) or after reconstitution for 1 h with purified δ subunit in equimolecular amounts (ϵ + 1δ) or a 2-fold excess (ϵ + 2δ). In the latter experiment, 3 M Gdn·HCl was added, and a new emission spectrum was recorded 1 h later (ϵ + 2δ + Gdn·HCl).

The results indicated that isolated α and ϵ subunits contained respectively about 2.3 and 0.9 mol of tryptophan/mol, whereas no tryptophan was detected in isolated β, γ, and δ subunits. An experimental value of 6.9 mol of tryptophan/mol was obtained for the native F_1 (with $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ stoichiometry). The validity of the titration method was controlled with model proteins like bovine serum albumin, ovalbumin, bovine trypsin, and egg lysozyme known to contain respectively 2, 3, 4, and 6 tryptophans: experimental values of 1.85, 3.0, 4.0, and 6.1 tryptophans were obtained under the present titration conditions. About 10% lower values were obtained for all proteins when NATA, instead of free tryptophan, was used as the standard.

It is shown in Figure 3 that in the absence of Gdn·HCl the isolated ϵ subunit exhibited a maximum of emission at 344 nm. This maximum was markedly blue-shifted to 310–312 nm by addition of the isolated δ subunit. For equimolecular amounts of δ and ϵ subunits, a shoulder remained at 344 nm, which amounted to 32% of the initial intensity. With a 2-fold excess of the added δ subunit, the peak at 310–312 nm was further increased and the shoulder at 344 nm decreased to less than 15% of the initial value. Addition of 6 M Gdn·HCl, to dissociate the $\delta\epsilon$ complex and unfold both subunits, produced an important red shift of the emission maximum up to 355 nm.

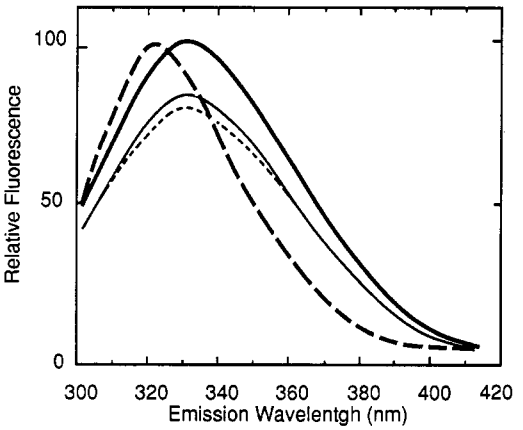

FIGURE 4: Modifications of native F_1 intrinsic fluorescence by addition of phosphate or nucleotides. Native F_1 was incubated for 30 min in the absence of effector (thick solid line) or in the presence of either 200 μM P_i (thick dashed line), 300 μM ADP (thin solid line), or 300 μM ATP (thin dashed line). The emission spectra were recorded upon excitation at 300 nm.

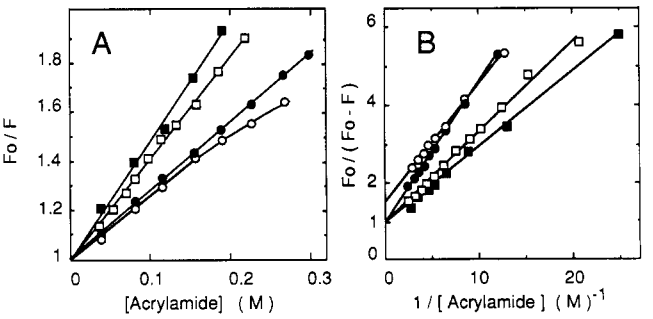

FIGURE 5: Acrylamide quenching of native F_1 intrinsic fluorescence. Effects of phosphate and nucleotides are shown. Native F_1 was first incubated in the absence of effector (□) or in the presence of either phosphate (■), ADP (●), or ATP (○) as in Figure 4 and then mixed with increasing amounts of acrylamide (see Experimental Procedures). The fluorescence emission was measured at 332 nm upon excitation at 300 nm. (A) Stern–Volmer plots. (B) Modified Stern–Volmer plots according to Lehrer (1971).

Phosphate- and Nucleotide-Induced Conformational Changes of Native F_1. The emission spectrum of native F_1 was significantly blue-shifted by incubation with 200 μM P_i: the maximal wavelength of emission changed from 332 to 323 nm, whereas the intensity was not modified (Figure 4). On the contrary, the presence of ADP or ATP lowered the maximal intensity of fluorescence, which remained centered at 332 nm. The maximal quenching effect was 14% or 17%, respectively, with ADP or ATP at 300 μM.

The acrylamide quenching of native F_1 fluorescence at 332 nm was also differently modified by phosphate or nucleotides (Figure 5). Linear Stern–Volmer plots in the presence of increasing acrylamide concentrations up to 0.2–0.3 M allowed the calculation of a Stern–Volmer constant, K_{SV}, of 4.0 M^{-1} for the control enzyme in the absence of effector (Figure 5A). Addition of phosphate significantly increased the slope of the straight line (K_{SV} = 4.9 M^{-1}). On the contrary, ADP markedly decreased the slope and gave a K_{SV} value of 2.7 M^{-1}. In the presence of ATP, a downward curvature was observed, which was indicative of tryptophan heterogeneity toward acrylamide accessibility. Modified Stern–Volmer plots (Lehrer, 1971) confirmed the ATP-induced tryptophan heterogeneity with an accessible fraction f_a of 0.65 (Figure 5B). The slope of the straight line allowed the calculation of an apparent K_Q constant of 2.6 M^{-1}. On the contrary, no apparent heterogeneity was observed in the other assays (f_a = 1.0), indicating that all tryptophans are accessible to acrylamide.

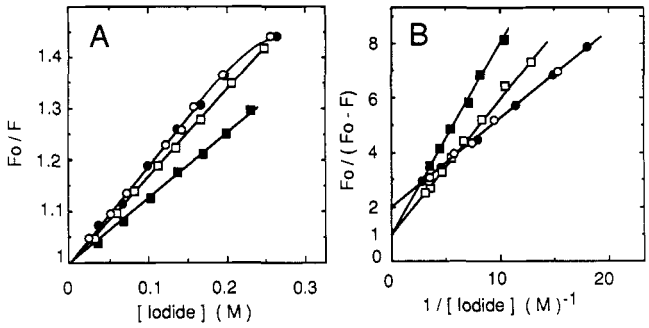

FIGURE 6: Iodide quenching of native F_1 intrinsic fluorescence. The conditions were the same as in Figure 5 except that iodide replaced acrylamide.

The same effectors differently modified iodide quenching of native F_1 fluorescence (Figure 6). The low K_{SV} of 1.75 M^{-1}, calculated from a linear Stern–Volmer plot of the control assay, was further decreased by P_i addition (1.3 M^{-1}) (Figure 6A). The presence of either ADP or ATP produced the same downwardly curved plot. A marked heterogeneity of tryptophan accessibility to iodide in the presence of nucleotides was observed in the representation of Lehrer, with $f_a = 0.5$ (Figure 6B). The K_Q value of 1.8 M^{-1} in the presence of nucleotides was not significantly different from that of the control. All tryptophans were accessible to iodide in the control without effector or in the presence of P_i ($f_a = 1.0$).

Discussion

The present paper demonstrates for the first time that intrinsic fluorescence of mitochondrial native F_1 from *S. pombe* can be used as a very powerful probe to monitor phosphate- and nucleotide-dependent enzyme conformational changes.

S. pombe F_1 contains seven tryptophan residues and exhibits a characteristic intrinsic tryptophan fluorescence as opposed to the mammalian enzyme. The isolated α subunit shows an emission spectrum centered at 354 nm in the presence of 6 M Gdn·HCl. Two tryptophans per α subunit are present as estimated by the titration method of Pajot (1976), based on quantitative emission fluorescence in the presence of 6 M Gdn·HCl with free tryptophan, rather than NATA, as the standard. This method, the validity of which has been determined under our conditions on various model proteins, appears better suited than that of Sasaki et al. (1975), where urea treatment or enzymatic digestion of lysozyme were poorly efficient in unmasking tryptophans. In addition, urea did not change the low fraction of lysozyme tryptophans accessible to iodide quenching, in contrast to Gdn·HCl, which made the tryptophans completely accessible (Lehrer, 1971). Very recently, it has been shown that various tryptophans that exhibited very different fluorescence properties in the native β subunit of succinyl-CoA synthetase are made equivalent in the presence of Gdn·HCl (Nishimura et al., 1990). The ratio of about two tryptophans per α subunit from *S. pombe* F_1 is based on an apparent molecular mass of 56 kDa as estimated from SDS–polyacrylamide gel electrophoresis. This ratio agrees quite well with the sequencing of the *atp*1 gene encoding the *S. pombe* α subunit, which shows the presence of two tryptophans (Falson et al., 1988). Both tryptophans are conserved in the *Saccharomyces cerevisiae* α subunit which however contains an additional tryptophan near its C-terminal end (Takeda et al., 1986). According to the known $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$ stoichiometry for F_1, a total of six tryptophans are present on the α subunits of *S. pombe* F_1.

The seventh tryptophan is located on the ϵ subunit, which also exhibits a characteristic intrinsic tryptophan fluorescence when isolated. Indeed, one tryptophan per isolated ϵ subunit is found by the same titration method, under denaturating conditions, when an apparent molecular mass of 7.5 kDa is used. In the absence of Gdn·HCl, the maximal emission of the isolated ϵ subunit at 344 nm is considerably shifted to 310–312 nm by interaction with the added δ subunit. This indicates that the tryptophan of the ϵ subunit is involved in tight interaction with the δ subunit. A similar interaction has been described for the corresponding subunits from pig heart and has been proposed to occur in native F_1 (Penin et al., 1990). This would explain why mammalian F_1 exhibits a characteristic intrinsic tyrosine fluorescence with a maximal emission around 310 nm (Tiedge et al., 1982; Baracca et al., 1986; Penin et al., 1990), a faint shoulder above 330 nm being occasionally observed (Keira et al., 1978).

The single ϵ subunit tryptophan can therefore be assumed to produce a very minor contribution to the *S. pombe* native F_1 fluorescence emission at 332 nm, as opposed to the six tryptophans located on the α subunits. Since the mitochondrial ϵ subunit from *S. pombe* appears similar to that from mammalian F_1 in both tryptophan content (Walker et al., 1985) and interaction with the δ subunit (Penin et al., 1990), this suggests structural as well as functional homology between the various mitochondrial ϵ subunits. Similarly, the mitochondrial ϵ subunit from *S. cerevisiae* also contains one tryptophan (Pevec et al., 1984). This contrasts with the bacterial ϵ subunit, which does not contain any tryptophan and exhibits some homology with the mitochondrial δ subunit (Walker et al., 1985; Ysern et al., 1988).

The tryptophans of native F_1 appear rather buried inside the molecule, as revealed by the low wavelength of maximal emission, 332 nm (Burstein et al., 1973), and the low quenching constants for both acrylamide and iodide as compared to NATA under the same experimental conditions. Acrylamide has been shown not to strongly interact with proteins (Eftink & Ghiron, 1987). On the contrary, iodide is well-known as a protein-structure-perturbing agent (Tonomura et al., 1962). *S. pombe* F_1, although exhibiting a limited accessibility to iodide (K_{SV} = 1.8 M^{-1}), is largely inhibited. The increase in inhibition observed when thiosulfate is omitted is likely due to iodination of some amino acids, as observed for mammalian F_1 (Penefsky, 1967; Petrone et al., 1987). High concentrations of iodide, in the absence of thiosulfate, have been found to promote mammalian F_1 dissociation at 0 °C but not at 25 °C (Penefsky & Warner, 1965). Our experiments with *S. pombe* F_1, performed at 30 °C with added thiosulfate and in the presence of glycerol and Mg^{2+} as stabilizing agents, might prevent subunit dissociation at least up to 0.25 M iodide. Indeed, subunit dissociation would have produced a break in Stern–Volmer plots with a much higher K_{SV} and a red shift in the maximum of the emission spectrum; such effects are not observed under our conditions. Since none of the Stern–Volmer plots in the presence of either acrylamide or iodide shows an upward curvature, this indicates that no significant static quenching is produced and that the whole observed quenching is collisional (Eftink & Ghiron, 1981).

Native F_1 intrinsic fluorescence is shown to be very sensitive to the presence of substrates and products, which produce interesting enzyme conformational changes. A significant 9-nm blue shift is produced by 200 μM phosphate at pH 7.5. This concentration is consistent with high-affinity phosphate binding (Penefsky, 1977) occurring to a β subunit (Lauquin et al., 1980) and synergic with ADP to produce hysteretic inhibition (Di Pietro et al., 1980, 1986) in mammalian F_1. Phosphate binding to *S. pombe* F_1 lowers tryptophan acces-

sibility to the charged quencher iodide, whereas it increases the accessibility to the neutral quencher acrylamide; it therefore produces a more hydrophobic environment for the tryptophans. This might be of physiological relevance in regard to activation of ATP synthesis, which requires the formation of a hydrophobic catalytic center. A comparable blue shift has also been observed upon substrate addition to lysozyme (Lehrer & Fasman, 1967) and to avidin (Kurzban et al., 1989).

On the contrary, the presence of 300 μM ADP or ATP is shown here to produce an important quenching and to lower tryptophan accessibility to both acrylamide and iodide. Since both nucleotides produce a similar important quenching without changing the maximal emission wavelength, an attractive interpretation would be a resonance energy transfer between tryptophan and the adjacent adenine chromophore, as also suggested for creatine kinase (Messmer & Kagi, 1985), although a nucleotide-induced distant conformational change of the enzyme cannot be excluded. ADP and ATP behave similarly in revealing a marked tryptophan heterogeneity toward iodide, with half of the tryptophans becoming completely inaccessible. Some heterogeneity in tryptophan polarity seems to exist even in the absence of exogenous nucleotides as revealed by the rather large bandwidth, around 60 nm, with respect to the low wavelength, 332 nm, of the emission spectrum (Burstein et al., 1973). Nucleotide addition would increase this heterogeneity, which becomes quite apparent in the presence of iodide. ATP also reveals an important tryptophan heterogeneity in the presence of acrylamide, one-third of the tryptophans being no longer accessible. Since F_1 contains three α subunits, our results show an ATP-induced asymmetry of α subunits, which might be of special significance with respect to the enzyme mechanism. This is consistent with previous conclusions drawn from chemical modification of a single α subunit with an essential thiol in *S. pombe* F_1 (Falson et al., 1986) and immunochemical modifications with an anti-α monoclonal antibody in mammalian F_1 (Moradi-Améli et al., 1989).

The hydrophobic character of the two tryptophans from the *S. pombe* α subunit seems very important since both residues are conserved in the other yeast, *S. cerevisiae* (Takeda et al., 1986), or substituted by either phenylalanine or tyrosine in beef heart (Walker et al., 1985). These residues are located very near two conserved amino acid sequences proposed to belong to the nucleotide-binding site (Walker et al., 1985), which is quite consistent with our results showing nucleotide quenching in *S. pombe* F_1. Since effectors known to bind at least partly to the tryptophan-free β subunit are shown here to markedly modify the environment of α subunit tryptophans, this strongly suggests that these tryptophans are located very near critical interactions between the α and β subunits. Similar effects have been observed with succinyl-CoA synthetase, where it was concluded that at least one tryptophan was located at the subunit interface, close to the catalytic site (Prasad et al., 1983).

In conclusion, our results with *S. pombe* native F_1 show that α subunit tryptophan fluorescence is a very powerful intrinsic reporter of events directly related to enzyme mechanism since three different enzyme conformational changes are produced by three different physiological ligands. Work is in progress to discriminate, by intrinsic fluorescence, nucleotide binding to catalytic or noncatalytic sites and to monitor interactions between these sites.

ACKNOWLEDGMENTS

Thanks are due to J. C. Brochon, LURE Orsay, for critical reading of the manuscript. F. Penin and D. Gagliardi are acknowledged for help in subunit purification, and C. Van Herrewege is acknowledged for the drawings.

REFERENCES

Baracca, A., Curatola, G., Parenti-Castelli, G., & Solaini, S. (1986) *Biochem. Biophys. Res. Commun. 136*, 891–898.
Bensadoun, A., & Weinstein, D. (1976) *Anal. Biochem. 70*, 241–250.
Burstein, E. A., Vedenkina, N. S., & Ivkova, M. N. (1973) *Photochem. Photobiol. 18*, 263–279.
Calhoun, D. B., Vanderkooi, J. M., & Englander, S. W. (1983) *Biochemistry 22*, 1533–1539.
Canfield, R. E. (1963) *J. Biol. Chem. 238*, 2698–2707.
Creed, D. (1984) *Photochem. Photobiol. 39*, 537–562.
Di Pietro, A., Godinot, C., Martin, J. C., & Gautheron, D. C. (1979) *Biochemistry 18*, 1738–1745.
Di Pietro, A., Penin, F., Godinot, C., & Gautheron, D. C. (1980) *Biochemistry 19*, 5671–5678.
Di Pietro, A., Godinot, C., & Gautheron, D. C. (1983) *Biochemistry 22*, 785–792.
Di Pietro, A., Fellous, G., Godinot, C., & Gautheron, D. C. (1986) *Biochim. Biophys. Acta 851*, 283–294.
Di Pietro, A., Jault, J.-M., Falson, P., Divita, G., & Gautheron, D. C. (1989) *Biochimie 71*, 931–940.
Eftink, M. R., & Ghiron, C. A. (1981) *Anal. Biochem. 114*, 199–227.
Eftink, M. R., & Ghiron, C. A. (1987) *Biochim. Biophys. Acta 916*, 343–349.
Falson, P., Di Pietro, A., & Gautheron, D. C. (1986) *J. Biol. Chem. 261*, 7151–7159.
Falson, P., Di Pietro, A., Darbouret, D., Jault, J.-M., Gautheron, D. C., Boutry, M., & Goffeau, A. (1987) *Biochem. Biophys. Res. Commun. 148*, 1182–1188.
Falson, P., Boutry, M., & Goffeau, A. (1988) *Eur. Bioenerg. Conf. 5*, 266.
Falson, P., Di Pietro, A., Jault, J.-M., Gautheron, D. C., & Boutry, M. (1989) *Biochim. Biophys. Acta 975*, 119–126.
Fellous, G., Godinot, C., Baubichon, H., Di Pietro, A., & Gautheron, D. C. (1984) *Biochemistry 23*, 5294–5299.
Gautheron, D. C., & Godinot, C. (1988) *J. Bioenerg. Biomembr. 20*, 451–468.
Godinot, C., & Di Pietro, A. (1986) *Biochimie 68*, 367–373.
Keira, T., Godinot, C., & Gautheron, D. C. (1978) *Experientia 34*, 1548–1549.
King, T. P., & Spencer, M. (1970) *J. Biol. Chem. 245*, 6134–6148.
Kurzban, G. P., Gitlin, G., Bayer, E. A., Wilchek, M., & Horowitz, P. M. (1989) *Biochemistry 28*, 8537–8542.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lauquin, G. J. M., Pougeois, R., & Vignais, P. V. (1980) *Biochemistry 19*, 4620–4626.
Lehrer, S. S. (1971) *Biochemistry 10*, 3254–3262.
Lehrer, S. S., & Fasman, G. D. (1967) *J. Biol. Chem. 242*, 4646–4651.
Mc Reynolds, L., O'Malley, B. W., Nisbet, A. D., Forthergill, J. E., Gruol, D., Fields, S., Robertson, M., & Braunlee, G. G. (1978) *Nature 273*, 723–728.
Messmer, C. H., & Kägi, J. H. R. (1985) *Biochemistry 24*, 7172–7178.
Mikes, O., Holeysovsky, V., Tomasek, V., & Sorm, F. (1966) *Biochem. Biophys. Res. Commun. 24*, 346–352.
Moradi-Améli, M., Julliard, J. H., & Godinot, C. (1989) *J. Biol. Chem. 264*, 1361–1367.
Nishimura, J. S., Mann, C. J., Ybarra, J., Mitchell, T., & Horrowitz, P. M. (1990) *Biochemistry 29*, 862–865.

Pajot, P. (1976) *Eur. J. Biochem. 63*, 263-269.
Penefsky, H. S. (1967) *J. Biol. Chem. 242*, 5789-5795.
Penefsky, H. S. (1977) *J. Biol. Chem. 252*, 2891-2899.
Penefsky, H. S., & Warner, R. C. (1965) *J. Biol. Chem. 240*, 4694-4702.
Penin, F., Deléage, G., Gagliardi, D., Roux, B., & Gautheron, D. C. (1990) *Biochemistry 29*, 9358-9364.
Petrone, G., Garboczi, D. N., & Pedersen, P. L. (1987) *Biochemistry 26*, 4016-4021.
Pevec, B., Fassold, E., Uwira, N., & Hess, B. (1984) *Eur. Bioenerg. Conf. 3*, 351-352.
Prasad, A. R. S., Nishimura, J. S., & Horowitz, P. M. (1983) *Biochemistry 22*, 4272-4275.
Robinson, N. C., Tye, R. W., Neurath, H., & Walsh, K. A. (1971) *Biochemistry 10*, 2743-2747.
Roux, B., Fellous, G., & Godinot, C. (1984) *Biochemistry 23*, 534-537.
Sasaki, T., Abrams, B., & Horecker, B. L. (1975) *Anal. Biochem. 65*, 396-404.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76-85.
Takeda, K., Chen, W. J., Saltzgaber, J., & Douglas, M. G. (1986) *J. Biol. Chem. 261*, 15126-15133.
Tiedge, H., Lücken, U., Weber, J., & Schäfer, G. (1982) *Eur. J. Biochem. 127*, 291-299.
Tonomura, Y., Sekiya, K., & Imamura, K. (1962) *J. Biol. Chem. 237*, 3110-3115.
Vignais, P. V., & Lunardi, J. (1985) *Annu. Rev. Biochem. 54*, 977-1014.
Walker, J. E., Fearnley, I. M., Gay, N. J., Gibson, B. W., Northrop, F. D., Powell, S. J., Runswick, M. J., Saraste, M., & Tybulewicz, V. L. J. (1985) *J. Mol. Biol. 184*, 677-701.
Wang, J. H. (1988) *J. Bioenerg. Biomembr. 20*, 407-422.
Ysern, X., Amzel, L. M., & Pedersen, P. L. (1988) *J. Bioenerg. Biomembr. 20*, 423-450.

Structural Features of an Exocyclic Adduct Positioned opposite an Abasic Site in a DNA Duplex†

Michael Kouchakdjian,‡ Moises Eisenberg,§ Francis Johnson,§ Arthur P. Grollman,*,§ and Dinshaw J. Patel*,‡

Department of Biochemistry and Molecular Biophysics, College of Physicians and Surgeons, Columbia University, New York, New York 10032, and Department of Pharmacological Sciences, State University of New York at Stony Brook, Stony Brook, New York 11794

Received November 8, 1990; Revised Manuscript Received December 17, 1990

ABSTRACT: Structural studies have been extended to dual lesions where an exocyclic adduct is positioned opposite an abasic site in the center of a DNA oligomer duplex. NMR and energy minimization studies were performed on the 1,N^2-propanodeoxyguanosine exocyclic adduct (X) positioned opposite a tetrahydrofuran abasic site (F) with the dual lesions located in the center of the (C1-A2-T3-G4-*X5*-G6-T7-A8-C9)·(G10-T11-A12-C13-*F14*-C15-A16-T17-G18) X·F 9-mer duplex. Two-dimensional NMR experiments establish that the X·F 9-mer helix is right-handed with Watson–Crick A·T and G·C base pairing on either side of the lesion site. NOEs are detected from the methylene protons of the exocyclic ring of X5 to the imino protons of G4·C15 and G6·C13 which flank the lesion site, as well as to the H1′ and H1″ protons of the cross strand F14 tetrahydrofuran moiety. These NMR results establish that the exocyclic adduct X5 is positioned between flanking G4·C15 and G6·C13 base pairs and directed toward the abasic lesion F14 on the partner strand. These studies establish that the exocyclic ring of the 1,N^2-propanodeoxyguanosine adduct fits into the cavity generated by the abasic site.

Basu and Essigmann (1988), Harris et al. (1988), Singer and Grunberger (1983), and Singer and Bartsch (1986) have reviewed the structural and biological consequences of DNA-damaging agents. Our laboratories have approached this problem through NMR structural studies on site-specifically modified lesions in DNA oligomer duplexes. Early efforts were focused on O^6alkG lesions (Patel et al., 1986a–c; Kalnik et al., 1989b,c), O^4alkT lesions (Kalnik et al., 1988b,c), abasic sites (Kalnik et al., 1988a, 1989a), and exocyclic adducts (Kouchakdjian et al., 1989, 1990). These studies, along with contributions on abasic sites by others (Pochet et al., 1986; Raap et al., 1987; Cuniasse et al., 1987, 1989), provide insights into the conformation and pairing alignments at the lesion site and the interplay between hydrogen-bonding and hydrophobic interactions modulated by the pH of the solution (Kouchakdjian et al., 1990; Norman et al., 1989). Structural studies of DNA lesions in solution were undertaken within the same DNA sequence context as biological studies conducted in parallel in an attempt to understand the molecular basis of chemical mutagenesis and carcinogenesis.

NMR has been used to characterize structural features of modified DNA duplexes containing stable abasic sites (Kalnik et al., 1988, 1989a) and stable exocyclic adducts (Kouchakdjian et al., 1989, 1990). Oligonucleotides containing abasic sites have been shown to misincorporate dNTP's in reactions catalyzed by DNA polymerases (Takeshita et al., 1987). By determining the structural and biological properties of such lesions, we hope to understand the molecular basis of

†This research was supported by NIH Grants CA-46533 (abasic sites) and CA-49982 (exocyclic adducts) to D.J.P. and NIH Grants CA-17395 and CA-47995 to A.P.G.
‡Columbia University.
§State University of New York at Stony Brook.

0006-2960/91/0430-3262$02.50/0