Chemical Modification of α-Subunit Tryptophan Residues in *Schizosaccharomyces pombe* Mitochondrial F_1 Adenosine 5′-Triphosphatase: Differential Reactivity and Role in Activity†

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ABSTRACT: Chemical modification of mitochondrial F_1-ATPase from *Schizosaccharomyces pombe* by the tryptophan-specific reagent *N*-bromosuccinimide (NBS) at pH 5.0 in the presence of 20% glycerol produced a characteristic lowering in both enzyme absorbance at 280 nm and intrinsic fluorescence at 332 nm that varied with NBS/F_1 molar ratio up to a value of 130. Fluorometric titration of tryptophans and correlation to residual ATPase activity showed that modification of three reactive residues among the seven present on α- and ε-subunits did not markedly modify the enzyme activity but efficiently released endogenous ATP and abolished the fluorescence quenching related to GDP or ATP binding to the catalytic site. Additional modification of one, less reactive, tryptophan altered both negative cooperativity of ATP hydrolysis and sensitivity to azide inhibition and produced a nearly complete inactivation at high NBS/F_1 molar ratio. NBS-induced inactivation of F_1 was favored by catalytic-site saturation with GDP or low ATP concentration and on the contrary was prevented by noncatalytic-site saturation with ADP or high ATP concentration. When reactive tryptophans were selectively modified by NBS in the presence of ADP, and subunits were isolated after guanidine hydrochloride dissociation by one-step purification on reversed-phase HPLC, the absorbance of α-subunit at 280 nm was decreased, whereas that of ε-subunit was unchanged. Cyanogen bromide cleavage of α-subunit and fragments separation by reversed-phase HPLC showed that one peptide of 3 kDa apparent molecular mass had decreased absorbance. N-Terminal sequencing allowed its identification to fragment 255–282 that contains tryptophan^{257}.

Mitochondrial F_1[1] is a solubilizable moiety of the ATPase–ATP synthase complex responsible for ATP synthesis during oxidative phosphorylation. Purified F_1 only retains the ability to hydrolyze ATP. It is composed of five types of subunits with the stoichiometry $\alpha_3\beta_3\gamma_1\delta_1\epsilon_1$, with the two major subunits α and β containing the nucleotide binding sites. Catalytic sites bind guanine as well as adenine nucleotides and are assumed to be mainly located on β-subunits, whereas noncatalytic, regulatory, sites are specific for adenine nucleotides and are assumed to be shared between α- and β-subunits. A number of reactive residues of β-subunit have been revealed by various chemical modifiers to be involved in both catalysis and regulation of ATP hydrolysis [for a review, see Vignais and Lunardi (1985), Senior (1988), and Futai et al. (1989)].

In contrast, very few reactive groups have been characterized on α-subunit: cysteine residues with *N*-ethylmaleimide in mitochondrial F_1 (Godinot et al., 1981; Tamura & Wang, 1983; Falson et al., 1986) and one lysine residue with luciferine yellow in the chloroplast enzyme (Nalin et al., 1985). Other residues could only be modified using nucleotide affinity labels (Tagaya et al., 1988; Rao et al., 1988; Verburg & Allison, 1990). Recent results have shown that mitochondrial F_1 from the yeast *Schizosaccharomyces pombe* contains a total of seven tryptophan residues: one on the ε-subunit and two per α-subunit (Divita et al., 1991). The two residues on α-subunit, at positions 190 and 257 of the sequence (Falson et al., 1991), are predicted to be strategically located, in close proximity to the nucleotide-site elements defined by Walker et al. (1982). Indeed, intrinsic fluorescence studies have allowed differentiation of nucleotide binding to catalytic and noncatalytic sites and monitoring of related enzyme conformational changes involving critical interactions between α and β subunits (Divita et al., 1992).

The present work describes specific chemical modification of these tryptophan residues by NBS in order to determine their respective reactivity and their role in enzyme activity and interaction with nucleotides at catalytic and noncatalytic sites. The results indicate that Trp^{257} is very reactive to NBS and its fluorescence is quenched by nucleotide binding to the catalytic site. In contrast, Trp^{190} has a low reactivity and appears to interact with ADP or ATP at the noncatalytic site; its oxidation by NBS produces complete loss of enzyme activity.

EXPERIMENTAL PROCEDURES

Materials. *N*-Bromosuccinimide (NBS) was from Sigma. Nucleotides were from Boehringer Mannheim; their purity was controlled by high-pressure liquid chromatography (HPLC) using a Partisil PXS (10–25) SAX column from Whatman, as previously described (Di Pietro, 1988). Nucleosil C_8 cartridge (300 Å, 5 μm, 4.6 × 30 mm) was from Brownlee. HPLC was performed with a Waters apparatus consisting of two M510 pumps, an U6K injector, and a 990 photodiode array detector. Ultraviolet spectra were scanned

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[1] Abbreviations: ATPase, adenosine 5′-triphosphatase (E.C. 3.6.1.3); CNBr, cyanogen bromide; EDTA, ethylenediaminetetraacetic acid; F_1, soluble mitochondrial ATPase; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-pressure liquid chromatography; Mes, morpholinoethanesulfonic acid; NBS, *N*-bromosuccinimide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol.

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using a Beckman DU7 spectrophotometer. HPLC-grade acetonitrile and trifluoroacetic acid were from SDS. The micro BCA protein assay reagent was from Pierce.

Protein Preparations. F_1 was purified as previously described (Falson et al., 1986, 1989) from a large-scale glycerol culture of *S. pombe* wild strain (972 h^-) in the presence of 0.05% antifoam (Sigma). The pure enzyme at 6 mg of protein/mL of 100 mM Tris-HCl, pH 7.5, containing 50% glycerol, 5 mM EDTA, 10 mM *p*-aminobenzamidine, 10 mM ϵ-amino-*n*-caproic acid, and 1 mM phenylmethanesulfonyl fluoride was kept frozen in liquid nitrogen. Just before use, aliquots were thawed at 30 °C in a water bath and equilibrated with the adequate buffer by centrifugation–elution through Sephadex G-50 fine columns (Penefsky, 1977). A molecular mass of 380 kDa was assumed for pure F_1 (Falson et al., 1986).

The quantitative method of subunit purification from pig heart F_1 (Gagliardi et al., 1991) could not be successfully applied to our yeast enzyme that contained high amounts of endogenous nucleotides (Di Pietro et al., 1989). Therefore, the subunits were separated and isolated after F_1 was dissociated with 6 M guanidine hydrochloride during overnight incubation at 30 °C in the presence of 50 mM dithiothreitol. The HPLC separation is detailed in the Results section. Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to Laemmli (1970). Protein concentration was determined using either the modified Lowry procedure of Weinstein and Bensadoun (1976) or the bicinchoninic acid (micro BCA) method (Smith et al., 1985) with bovine serum albumin as the standard.

CNBr cleavage of α-subunit (0.15 mg of protein) was performed in 400 μL of 70% (v/v) formic acid, 5% β-mercaptoethanol, and 0.35 mM free L-tryptophan by incubation with CNBr (50-fold molar excess relative to methionine residues) under nitrogen flux and in the dark, for 24 h at room temperature. The molecular mass of α-subunit from *S. pombe*, containing 10 methionine residues, was taken as 55.557 kDa (Falson et al., 1991). The samples were submitted to three washing cycles of 15-fold dilution with Milli-Q water and lyophilization and finally kept at −80 °C. They were then dissolved in 0.5 mL of 50 mM Tris-HCl, pH 7.5, containing 6 M guanidine hydrochloride and 50 mM dithiothreitol, incubated for 2 h at 30 °C, and loaded on a reversed-phase Nucleosil C_8 cartridge. The fragments were separated with a linear gradient of acetonitrile in the presence of 0.1% trifluoroacetic acid, monitored by absorbance at 280 nm, and collected. The main peaks obtained were lyophilized and analyzed by polyacrylamide electrophoresis on high-density gels (containing 20% acrylamide and 10% glycerol) in the presence of sodium dodecyl sulfate.

N-Terminal sequencing of peptides was performed by automatic Edman degradation using a 470A/SF gas-phase sequencer coupled to a 120A/01 amino acid analyzer (Applied Biosystems). Identification of fragments was achieved by comparison with the theoretical ones predicted from α-subunit sequence analysis with the ANTHEPROT program (Geourjon et al., 1991).

Chemical Modification by NBS. F_1 was equilibrated by centrifugation–elution in 50 mM Tris–acetate, 20% glycerol, and 1 mM EDTA, pH 5.0, mixed with a freshly prepared solution of NBS, and incubated in the dark. After 10 min at 30 °C, residual ATPase activity was measured in aliquots. Alternatively, unreacted NBS was neutralized by a 10-fold molar excess of free L-tryptophan or *N*-acetyltryptophanamide and the modified enzyme was equilibrated by centrifugation–elution in the appropriate buffer for either spectral analysis (ultraviolet absorbance and fluorescence), tryptophan and endogenous nucleotide titrations, kinetic parameter measurements, or subunit dissociation to localize the modified residues.

Spectra Analysis. Ultraviolet difference spectra were recorded on a Beckman DU-7 spectrophotometer using a 0.4 μM F_1 solution in 50 mM Tris–acetate, 20% glycerol, and 1 mM EDTA, pH 5.0, and the same buffer without protein in the reference. Emission fluorescence spectra were recorded at 30.0 ± 0.1 °C upon excitation at 295 nm using a Bio-Logic spectrofluorometer equipped with a 150-W xenon lamp, as previously (Divita et al., 1991). The cuvette contained 1 mL of 0.4 μM F_1 in the same buffer at pH 5.0 and the values were corrected for buffer blank (no protein present). The nucleotide-induced quenching of fluorescence was studied by the decrease of fluorescence intensity at 332 nm in the presence of increasing amounts of either GDP or ATP, upon excitation at 300 nm. The values were corrected for the nucleotide inner effect, which was determined under the same conditions with bovine serum albumin and *N*-acetyltryptophanamide, as previously detailed (Divita et al., 1992). Tryptophan titration, based on quantitative emission fluorescence at 350 nm in the presence of 6 M guanidine hydrochloride (Pajot et al., 1976), was performed as previously described using free L-tryptophan as the standard (Divita et al., 1991).

Nucleotide Titration and Hydrolysis. Endogenous nucleotides were titrated, after enzyme thermal denaturation, by HPLC on a strong anion-exchange column as detailed (Di Pietro et al., 1988; Penin et al., 1988).

The ATPase activity was assayed in 0.62 mL of 50 mM Hepes–KOH, pH 8.0, in the presence or absence of 20 mM bicarbonate by spectrophotometric recording of the oxidation of reduced nicotinamide adenine dinucleotide, using an ATP-regenerating system composed of phosphoenolpyruvate, pyruvate kinase, and lactate dehydrogenase as detailed (Falson et al., 1989; Jault et al., 1991). To determine K_m and V_{max}, the assay medium contained 1 mM excess $MgCl_2$ and increasing equal concentrations of ATP and $MgCl_2$ in the range 0.035–3.3 mM. The activity is expressed as units per milligram of protein; 1 unit = 1 μmol of ATP hydrolyzed/min. The concentration of ATP stock solutions was controlled by absorbance at 259 nm (ϵ = 15 400 M^{-1} cm^{-1}).

RESULTS

Effects of NBS Modification on F_1 Spectral Properties. Incubation of F_1 with NBS at pH 5.0, in 50 mM Tris–acetate, 20% glycerol, and 1 mM EDTA, produced characteristic changes of its spectral properties depending on the NBS/F_1 molar ratio (Figure 1). The ultraviolet absorbance peak at 280 nm was significantly lowered, whereas an increased absorbance was observed at 250 nm (panel A). A pronounced effect was produced on the emission spectrum of intrinsic fluorescence, characterized by a large decrease in intensity at 332 nm, the maximal emission wavelength that remained apparently unchanged (panel B).

The dependence on NBS/F_1 molar ratio is detailed in Figure 2. The low decrease in absorbance at 280 nm appeared to be correlated with NBS/F_1 molar ratio up to a value of 130, but the maximal effect represented only 14% of the initial value (panel A). In contrast, a high fluorescence extinction of 64% was produced by 130 NBS/F_1. Titration of unmodified tryptophan residues, by a fluorometric method using denaturing conditions in the presence of guanidine hydrochloride, indicated that a residual amount of 2.9 residues was recovered from the total initial amount of 7.0 and therefore that up to 4.1 residues could be oxidized by NBS under the above

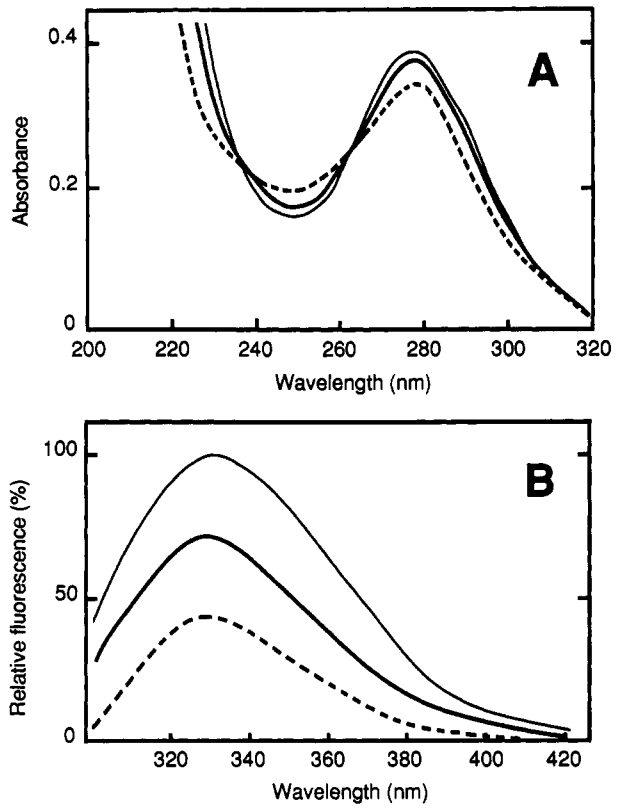

FIGURE 1: Effects of NBS modification on F_1 spectral properties. F_1 (0.15 mg of protein/mL) was equilibrated in 50 mM Tris–acetate and 20% glycerol, pH 5.0, and mixed in the dark in the absence of NBS (thin line) or in the presence of a 25- (thick line) or 80-fold (- - -) molar excess. After a 10-min incubation at 30 °C, unreacted NBS was neutralized by a 10-fold molar excess of free L-tryptophan or *N*-acetyltryptophanamide and then eliminated by centrifugation–elution. The eluates were used to record ultraviolet absorbance spectra (A) and fluorescence emission spectra upon excitation at 295 nm (B).

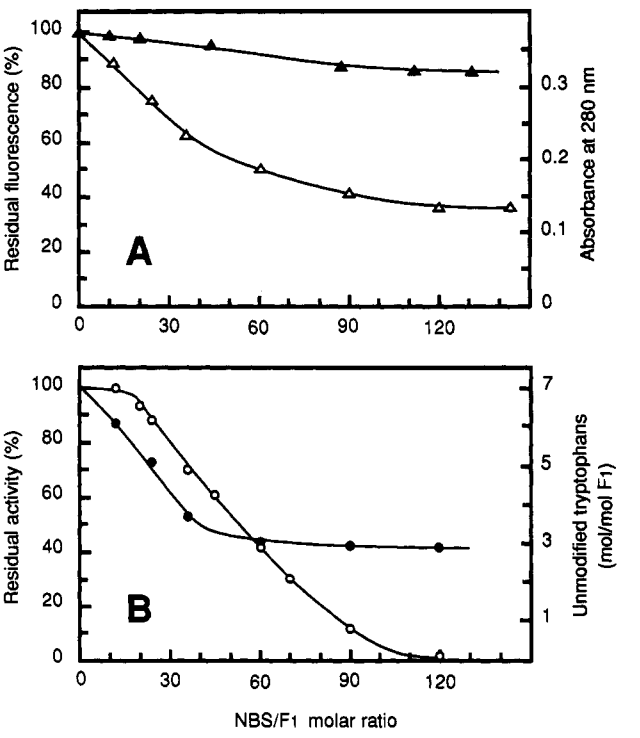

FIGURE 2: Concentration-dependent effects of NBS modification. (A) The variations of F_1 spectral properties were studied as in Figure 1 except that a series of NBS concentrations were tested. The residual absorbance at 280 nm (▲) and fluorescence emission at 332 nm (△) were referred to the corresponding controls without NBS. (B) The residual ATPase activity V_i of eluates (○) was measured on 1-μL aliquots in 50 mM Hepes–KOH, pH 8.0, containing 20 mM sodium bicarbonate and saturating 3.3 mM ATP, and compared to the control activity V_0 in the absence of NBS. The eluates were then mixed with 6 M guanidine hydrochloride and incubated overnight at 30 °C; titration of unmodified tryptophan residues (●) was performed by fluorescence emission at 350 nm using free L-tryptophan as the standard (see Experimental Procedures).

conditions (panel B). Correlation to the enzyme inactivation monitored by residual ATPase activity at pH 8.0 in the presence of 20 mM bicarbonate evidenced at least two differently related classes of residues producing a sigmoidal inactivation curve. The modification of the three most reactive residues by 33 NBS/F_1 produced a limited 24% inactivation (Figure 2B). In contrast, the modification of the fourth residue produced a very pronounced loss of activity leading to nearly complete inactivation. The latter did not produce any red shift of the emission spectrum (see Figure 1B); it was neither due to nor accompanied by enzyme precipitation and/or subunit dissociation as controlled by polyacrylamide gel electrophoresis under nondenaturing conditions (not shown here).

Interdependence between Tryptophan Modification by NBS and Enzyme/Nucleotide Interactions. Table I shows that oxidation of the most reactive tryptophan residues by 20 NBS/F_1 released all the endogenous ATP initially present (1.8 mol/mol of F_1) as monitored by centrifugation–elution, whereas it did not modify endogenous ADP. Modification of all four accessible tryptophan residues by 120 NBS/F_1 did not reduce the amount of endogenous ADP, which appeared completely unexchangeable.

As previously observed, F_1 intrinsic fluorescence emission at 332 nm was quenched by GDP or ATP binding to the catalytic site (Divita et al., 1992). Figure 3 shows that oxidation of the most reactive tryptophan residues by 30 NBS/F_1 producing a 30% decrease of enzyme intrinsic fluorescence completely abolished (panel B) the concentration-dependent

Table I: Release of Endogenous Nucleotides by Modification with NBS[a]

NBS/F_1 molar ratio	endogenous nucleotides[b] (mol/mol of F_1)		
	ATP	ADP	ATP + ADP
0	1.8	1.85	3.65
20	nd	2.0	2.0
120	nd	1.95	1.95

[a] F_1 (0.4 mg of protein/mL) was incubated for 10 min in the absence or the presence of NBS at a molar ratio of either 20, modifying only the most reactive tryptophans, or 120, modifying all reactive tryptophans. [b] The samples were then submitted to centrifugation–elution through Sephadex G50 fine columns and analyzed for their content of remaining bound endogenous nucleotides by anion-exchange HPLC (see Experimental Procedures); nd, not detectable. Results are the mean of three separate experiments; the relative error is around 5%.

nucleotide quenching, which reached a value of 24% or 15%, respectively, with 45 μM GDP or ATP at pH 5.0 (panel A).

The effects produced by tryptophan modification with increasing NBS concentrations on kinetic parameters of ATPase activity are indicated in Figure 4. Modification of the most reactive residues by 20 NBS/F_1 produced a limited change of the kinetics obtained in the presence of bicarbonate that gave linear double-reciprocal plots: the V_{max} of 171 units/mg of protein in the unmodified control was hardly lowered (7%), whereas the K_m was significantly increased from 107 to 155 μM. More extensive modification by 45 NBS/F_1 decreased the V_{max} by 40% without further changing the K_m (panel A). When the activity was measured in the absence

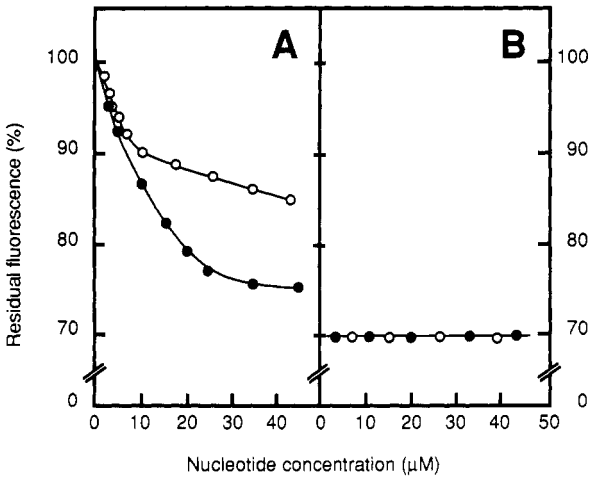

FIGURE 3: Effects of NBS modification on nucleotide-induced quenching of intrinsic fluorescence. F_1 at 0.4 μM was incubated in the absence (A) or the presence (B) of a 30-fold molar excess of NBS as described in Figure 1. The eluates were diluted with 50 mM Tris–acetate, 20% glycerol, and 1 mM EDTA, pH 5.0, and analyzed for fluorescence emission at 332 mm, upon excitation at 300 nm, in the presence of increasing amounts of either ATP (○) or GDP (●) (see Experimental Procedures).

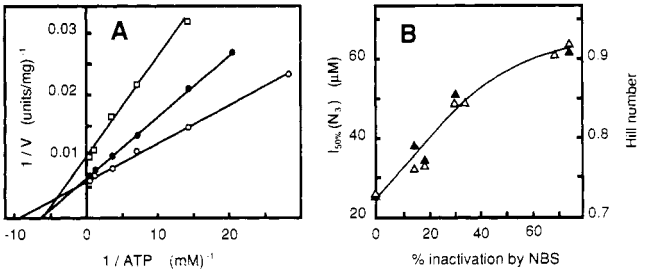

FIGURE 4: Effects of NBS modification on parameters of ATPase activity and inhibition by azide. F_1 at 0.8 μM was incubated for 10 min at pH 5.0 in the presence of NBS, up to a 70-fold molar excess; 0.5 mM free L-tryptophan was then added and the samples were submitted to centrifugation elution in 50 mM Tris (Mes) and 20% glycerol, pH 7.5. (A) Aliquots of 0.5–2 μL were withdrawn to study the substrate dependence of ATPase activity at pH 8.0 and 30 °C in the presence of 20 mM bicarbonate as an activating anion: control without NBS (○), 20 NBS/F_1 (●), and 45 NBS/F_1 (□). (B) Kinetics were performed in the absence of bicarbonate and the Hill number values were graphically estimated (not shown here) and plotted (▲) as a function of NBS-induced inactivation estimated at 1.7 mM ATP. The same ATP concentration was used to measure the $I_{50\%}$ (N_3^-) values (△) in the presence of increasing NaN_3 concentrations up to 100 μM.

of bicarbonate, a negative cooperativity with a Hill number of 0.73 was observed; the percent inactivation produced by increasing NBS concentrations, as monitored by the residual activity at 1.7 mM ATP, was correlated to alteration of negative cooperativity, the Hill number being increased to 0.92 at 74% inactivation by 70 NBS/F_1 (panel B). The same correlation was observed with sensitivity to reversible inhibition by azide, the $I_{50\%}$ value being increased from 26 μM in the unmodified control to 64 μM at 74% inactivation.

A 10-min incubation with increasing concentrations of nucleotides markedly affected the extent of inactivation produced by NBS (Figure 5A). Under conditions where 35 NBS/F_1 produced 30% inactivation, prior addition of GDP greatly increased the inactivation, which approached completion at 20 μM with a half-maximal effect around 8 μM. An opposite effect was obtained with ADP, which afforded a very efficient protection with a half-maximal effect around 3 μM. Dual effects were obtained with ATP since a 2 μM concentration increased the NBS-dependent inactivation from

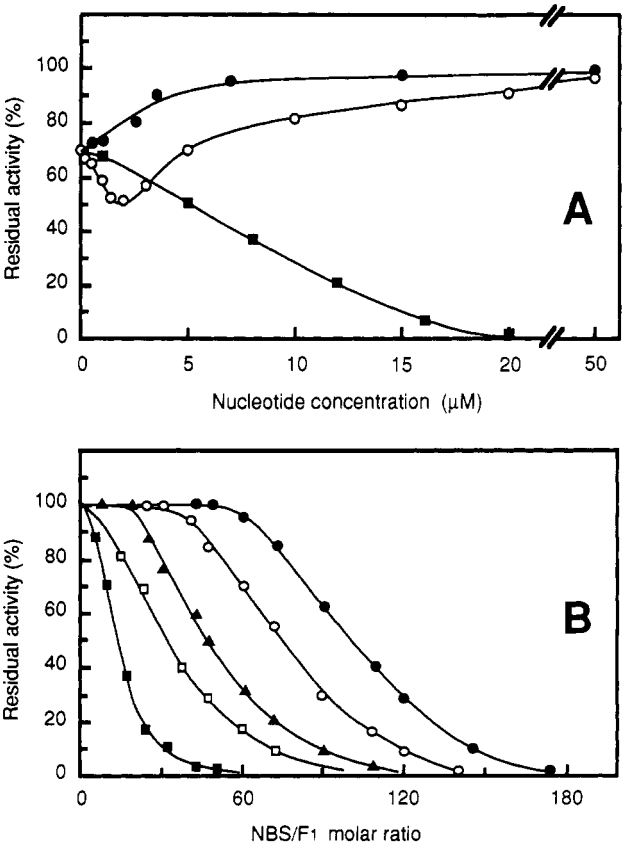

FIGURE 5: Effects of preincubation with nucleotides on NBS-induced inactivation of F_1. The enzyme at 0.4 μM was incubated for 10 min at pH 5.0 with the indicated concentration of either ADP (●), ATP (○), or GDP (■) and then mixed with NBS and further incubated for 10 min. The residual activity was measured as in Figure 2B on 1-μL aliquots. (A) Increasing nucleotide concentrations at a constant NBS/F_1 molar ratio of 35. (B) Constant nucleotide concentration [20 μM of either GDP (■), ATP (○), or ADP (●) or 2 μM ATP (□)] and increasing NBS/F_1 molar ratios; a control without nucleotide (▲) was performed under the same conditions.

30% to 48% whereas higher concentrations protected against inactivation, similarly to ADP. The protection was near complete at 50 μM ATP or ADP.

Convergent observations were obtained when the experiments were conducted with a 20 μM nucleotide concentration during preincubation and increasing NBS/F_1 molar ratios during modification (Figure 5B). The molar ratio value producing half-maximal inactivation in the absence of nucleotide (49 mol of NBS/mol of F_1) was greatly reduced (16 mol of NBS/mol of F_1) in the presence of GDP, which essentially abolished the sigmoidal pattern of the inactivation curve. In contrast, the value was increased to 98 or 73 mol of NBS/mol of F_1, respectively, in the presence of ADP or ATP that increased sigmoidicity. When a low 2 μM ATP concentration was used, a significant decrease of NBS/F_1 molar ratio to a value of 33 was observed.

Identification of Modified Tryptophan Residues. When F_1 was incubated overnight with 6 M guanidine hydrochloride and 50 mM dithiothreitol, all subunits could be quantitatively separated by a one-step method using reversed-phase HPLC on a Nucleosil C_8 cartridge with a discontinuous 25–51% acetonitrile gradient in 0.1% trifluoroacetic acid, as illustrated in Figure 6. Five absorbance peaks, denoted I, II, III, IV, and V, were obtained at 215 nm with respective percent acetonitrile values of 32%, 39%, 46%, 48%, and 51% and with quite distinct retention times ranging from 16 to 49 min. Polyacrylamide gel electrophoresis under denaturating conditions showed that

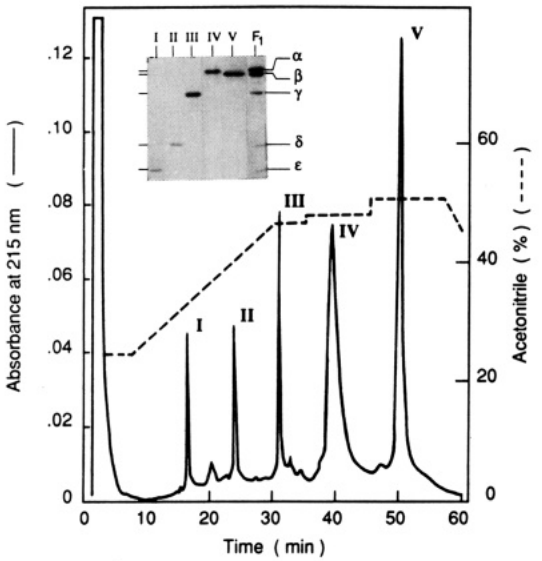

FIGURE 6: One-step separation of the five F_1 subunits by reversed-phase HPLC. F_1 (0.5 mg of protein) was mixed with 6 M guanidine hydrochloride and 50 mM dithiothreitol and incubated overnight at 30 °C. After centrifugation for 2 min at 9000*g*, the supernatant fluids were filtered through 0.45-μm filters and loaded on a reversed-phase Nucleosil C_8 cartridge at a flow rate of 1 mL/min. Subunit separation was performed using a discontinuous gradient of acetonitrile in trifluoroacetic acid as indicated by the dotted line, and elution was monitored by absorbance at 215 nm. The five elution peaks, denoted I, II, III, IV and V, were collected and lyophilized; aliquots were analyzed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate by reference to whole F_1.

all peaks contained pure subunits with the respective order ε (peak I), δ (II), γ (III), α (IV), and β (V). Protein assays in the corresponding eluates gave recovery yields ranging from near 100% for the ε-subunit to 61% for the α-subunit. When compared to peak V (β-subunit), peak IV appeared broader, suggesting that α-subunit contained some α′-peptide, generated by gentle proteolysis of a few N-terminal amino acids, as previously observed (Falson et al., 1986; Divita et al., 1991). No significant difference in elution pattern was observed when F_1 was preincubated with 20 μM ADP and then mixed with a 20-fold molar excess of NBS in order to modify only the most reactive tryptophan residues, not directly involved in enzyme activity. All subunits from NBS-modified F_1 exhibited the same behavior in polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate as that shown for the unmodified enzyme, and no evidence for fragmented subunit, which might arise from possible NBS-dependent cleavage, was observed.

When the elution was monitored by absorbance at 280 nm (Figure 7), peaks I and IV appeared more intense than the other ones due to the fact that only the ε- and α-subunits contain tryptophan residues. The elution pattern obtained with NBS-modified F_1 was superimposable to that of unmodified enzyme except for peak IV, which showed a markedly decreased absorbance. This indicated a selective modification of α-subunit whereas neither ε nor any other subunit was modified during the incubation with NBS.

Modified and unmodified α-subunits were eluted from corresponding peaks IV and cleaved by CNBr. Separation of the fragments was achieved by reversed-phase HPLC on a Nucleosil C_8 cartridge (Figure 8): three main peaks, denoted 1, 2, and 3, were monitored from unmodified α-subunit by absorbance at 280 nm; they were eluted at respective percent acetonitrile values of 18%, 37%, and 38%. Analysis of the elution pattern from NBS-modified α-subunit evidenced that the absorbance of peak 1 was markedly reduced while the

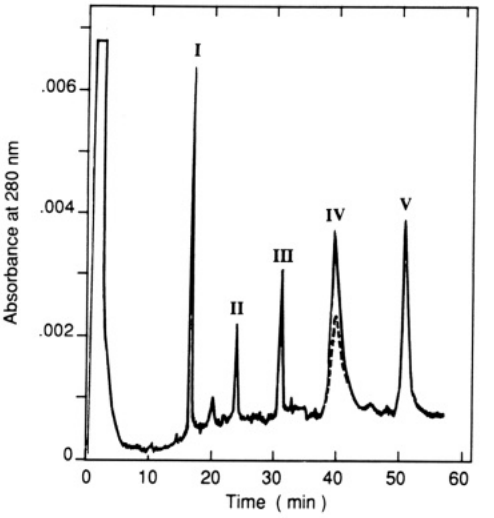

FIGURE 7: Effects of NBS modification on subunit absorbance at 280 nm. F_1 was incubated with 20 μM ADP in the presence or absence of a 20-fold molar excess of NBS and mixed after 10 min with a 100-fold molar excess of free L-tryptophan and 5 mM dithiothreitol. After centrifugation–elution, the eluates were submitted to guanidine hydrochloride denaturation and to HPLC subunit separation under the conditions of Figure 6. Elution absorbance was monitored at 280 nm; the pattern of NBS-modified F_1 (dotted line) superimposed upon that of unmodified enzyme (solid line) except at the level of peak IV, corresponding to α-subunit.

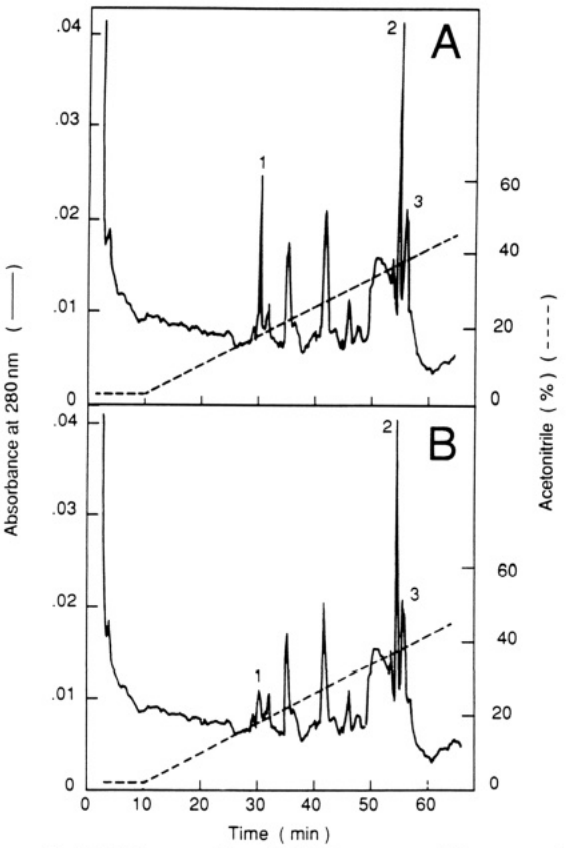

FIGURE 8: HPLC separation of CNBr-generated fragments from α-subunit: effects of NBS modification on fragment absorbance at 280 mm. The NBS-modified and native α-subunits obtained in Figure 7 (0.15 mg of protein) were lyophilized and submitted to CNBr cleavage as described under Experimental Procedures. The fragments were separated on the reversed-phase Nucleosil C_8 cartridge using a linear gradient of acetonitrile in trifluoroacetic acid, as indicated by the dotted line, and monitored by absorbance at 280 nm. (A) Unmodified α-subunit; (B) NBS-modified α-subunit.

other peaks were unchanged. Polyacrylamide gel electrophoresis (not shown here) indicated that peak 1 contained a

Table II: N-Terminal Sequence of α-Subunit Fragment with Decreased Absorbance upon NBS Modification[a]

fragment	sequence
NBS-modified fragment	
N-terminal sequence	GEWFRDNGKHG...
predicted fragment 225–282	GEWFRDNGKHGLVVY-DDLSKQAVAYRQM
contaminating fragment	
N-terminal sequence	AEKAAPTEVPS...
predicted fragment 1–26	AEKAAPTEVPSILEERI-RGAYNQAQM

[a] The peak 1 obtained in Figure 8 from unmodified α-subunit fragments (about 2 μg of protein) was collected, lyophilized, and submitted to sequencing by automatic Edman degradation. The two obtained N-terminal sequences were identified by comparison with predicted fragments. Modified tryptophan residue (**W**) is indicated in boldface type.

peptide with an apparent molecular mass around 3 kDa whereas peak 2 essentially contained a 10-kDa component and peak 3 was composed of three different peptides with molecular masses of 5–10 kDa.

Peak 1 from unmodified α-subunit was submitted to 11 cycles of protein N-terminal sequencing by automatic Edman degradation. The results obtained in Table II indicate that the determined sequence, GEWFRDNGKHG..., unambiguously corresponds to the predicted fragment 255–282, containing Trp^{257} and with a calculated molecular mass of 3.254 kDa. The other predicted α-subunit fragment 158–254 containing Trp 190 has a completely different sequence and a much higher molecular mass (>10 kDa). Also present in peak 1 was a contaminating peptide with the sequence AEKAAPTEVPS..., which corresponded to the N-terminal α-subunit, tryptophan-free, fragment 1–26 with a molecular mass of 2.861 kDa.

DISCUSSION

Chemical modification of α-subunit tryptophan residues in *S. pombe* F_1 is shown here to bring original information concerning their respective reactivity, contribution to intrinsic fluorescence, and role in nucleotide binding and enzyme activity.

Chemical Modification of Tryptophan Residues by NBS. Oxidation of the tryptophan indole moiety into oxindole by NBS is particularly interesting since the additional steric hindrance of the substituted group is very limited, contrary to other chemical modifiers (Lundblad & Noyes, 1984), and since the modified residue becomes totally nonfluorescent (Imoto et al., 1971; Bannister et al., 1972). Tryptophan residues may be rapidly (Ohnishi et al., 1989) and specifically modified at acidic pH, which increases both reactivity (Spande & Witkop, 1967b) and selectivity (Lundblad & Noyes, 1984).

These conditions are found with our yeast F_1 preparation, which contains as much as 3.6 mol of endogenous nucleotides/mol (Falson et al., 1989) and is quite stable down to pH 5.0 (Divita et al., 1992). The NBS-dependent inactivation reaches its maximal effect after a few minutes of incubation and is not accompanied by protein precipitation provided 20% glycerol is included in the incubation buffer. No subunit dissociation is observed as controlled by the absence of free subunit in polyacrylamide gel electrophoresis under nondenaturating conditions; this is consistent with both the lack of red shift of the fluorescence emission spectrum and the only partial removal of endogenous nucleotides. No peptide bond cleavage is apparent under the nondenaturating incubation conditions used, either, as monitored by subsequent analysis of both F_1 and isolated subunits on polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate; this agrees with the observation that proteins are much less susceptible than model peptides to NBS-dependent tryptophan modification and cleavage, which additionally requires denaturating conditions and long incubation times (Ramachandran & Witkop, 1967; Shechter et al., 1976).

Specific modification of tryptophan residues is warranted by a characteristic decrease in both absorbance at 280 nm (Spande & Witkop, 1967a) and fluorescence emission. The absorbance decrease is limited with F_1, due to the high amount of tyrosine and phenylalanine residues, and has been reported to be a qualitative, rather than a quantitative, method with other native proteins (O'Gorman & Matthews, 1977; Sartin et al., 1980). We therefore have used the fluorometric method of Pajot et al. (1976) in the presence of guanidine hydrochloride, which was previously shown to allow accurate titration of F_1 tryptophan residues (Divita et al., 1991). Titration of modified residues as a function of NBS/F_1 molar ratio and correlation to the induced extent of enzyme inactivation evidence at least three classes of residues.

Properties of α-Subunit Tryptophan^{257}. A first class is composed of three reactive residues that would appear to be located in a rather exposed environment on the protein surface, according to the criteria defined by Spande and Witkop (1967b). Selective labeling with a low NBS/F_1 molar ratio in the presence of ADP and one-step separations of F_1 subunits and of CNBr-generated α-subunit fragments by reversed-phase HPLC allow the unambiguous identification of Trp^{257} present in fragment 255–282. Progressive extinction by modification with increasing NBS/F_1 molar ratios indicates that these Trp^{257} residues exhibit a lower-than-average contribution to the native F_1 intrinsic fluorescence since a 35% decrease is produced by modification of the three Trp^{257} amounting to 43% relative to the total amount of seven residues.

Selective modification of Trp^{257} totally prevents the fluorescence quenching normally produced by GDP or ATP binding to catalytic site that is assumed to be mainly located on the β-subunit. Whatever fluorescence is directly or indirectly quenched by the nucleotide, the results indicate that Trp^{257} reports the effects of catalytic site occupancy on the α-subunit interaction in yeast F_1. This agrees with the recent crystallographic structure of rat liver F_1 showing that its two α-subunit cysteine residues, corresponding to Cys^{202} and Cys^{252} of *S. pombe* positioned near Trp^{190} and Trp^{257}, are located in close proximity to the β-subunits (Bianchet et al., 1991).

Although these essential α/β-subunit contacts might be located at a distance from the catalytic site, the higher fluorescence quenching produced by guanine than by adenine and hypoxanthine nucleotides in yeast F_1 suggests a short distance between Trp^{257} and the nucleotide base, possibly involving hydrophobic interaction and allowing a Förster resonance energy transfer (Divita et al., 1992). Some affinity-labeling experiments concluded a shared position of the catalytic site between α- and β-subunits (Williams & Coleman, 1982; Schäfer & Dose, 1984), and α-subunit Lys^{201} in *Escherichia coli* (corresponding to Lys^{210} in *S. pombe*) was proposed to interact with the phosphate chain moiety of nucleotide bound at the catalytic site (Tagaya et al., 1988). Trp^{257} seems not to be directly involved in the enzyme activity since (i) its modification by NBS only produces moderate changes in V_{max} and K_m for ATP and (ii) it is not conserved during evolution, although the hydrophobic character of the residue is conserved from bacteria to mammals (Walker et

al., 1985). However, it is worthwhile mentioning that the α-subunit sequence surrounding W^{257}, EWFRD, has great homology with sequences present in the β-subunit from mitochondrial F_1-ATPases and in the a-subunit from vacuolar ATPases (Zimniak et al., 1988; Gogarten et al., 1989), suggesting that it occupies a critical position. Indeed, its oxidation by NBS is found here to produce a decrease in nucleotide affinity, possibly due to lowered hydrophobicity, that appears to be sufficient to promote release of endogenous ATP as monitored by centrifugation–elution. This ATP is therefore bound at catalytic exchangeable sites, which agrees with previous results showing that incubation with Mg^{2+} ions promoted hydrolysis and exchange of endogenous ATP in both wild-type and mutant enzymes (Falson et al., 1989).

Properties of α-Subunit Tryptophan^{190}. A second, less reactive class is constituted of one tryptophan residue at position 190. Its oxidation by NBS markedly decreases the negative cooperativity of ATP hydrolysis and concomitant high sensitivity to azide inhibition in addition to the limited effects produced by oxidation of Trp^{257}. Therefore, Trp^{190} chemical modification appears to have similar consequences as the vicinal $Gln^{173} \rightarrow$ Leu point mutation, which was shown to lower the affinity of ADP binding to the noncatalytic regulatory site (Jault et al., 1991). Chemical modification of an unidentified essential cysteine of α-subunit was previously found to inhibit ATPase activity, abolish bicarbonate activation, and lower nucleotide binding (Falson et al., 1986). The present results suggest Cys^{202}, another vicinal residue, as a possible candidate. The total inactivation produced by the complete modification of one Trp^{190} by NBS indicates an essential involvement of the noncatalytic regulatory site in the enzyme activity. This is consistent with its affinity labeling by various adenine nucleotide analogues which produced a large enzyme inactivation (Di Pietro et al., 1981; Fellous et al., 1984; Bullough & Allison, 1986; Cross et al., 1987; Verburg & Allison, 1990). Under the present conditions, exogenous ADP produces a very efficient protection against NBS-dependent inactivation with a half-maximal concentration (around 3 μM) similar to the K_D of radiolabeled ADP binding (2.2–2.7 μM) to the noncatalytic site (Divita et al., 1992); this further supports the location of Trp^{190} within or very close to this site. The fact that no endogenous ADP is released upon NBS modification of the single Trp^{190} indicates that the residue is located in a vacant asymmetric noncatalytic site, as previously characterized with other F_1 preparations (Di Pietro et al., 1981; Kironde & Cross, 1986). Since the two nonexchangeable sites remain saturated by endogenous ADP, all three ADP sites appear to be occupied during the protection experiment in the presence of added ADP.

In contrast, the reactivity of Trp^{190} toward NBS is greatly increased by saturation of catalytic site by GDP, with a concentration for half-maximal effect (8 μM) comparable to the value producing half-maximal quenching of F_1 intrinsic fluorescence (6 μM). This evidences a conformational change mediated by interactions between catalytic and noncatalytic sites. The dual effects produced by ATP on Trp^{190} reactivity indicate a high-affinity ATP binding to catalytic site and a lower affinity binding to the noncatalytic site, which is consistent with conclusions drawn from quenching of intrinsic fluorescence (Divita et al., 1992). The fact that the modification of a single Trp^{190}, among the three of the enzyme, is sufficient to induce a complete loss of activity constitutes an additional indication of α-subunits' structural and functional asymmetry or of strong negative cooperativity. Indeed, previous results revealed both an ATP-dependent decreased accessibility of one-third of the α-subunit tryptophans (Divita et al., 1991) and the chemical modification of only one essential α-subunit cysteine (Falson et al., 1986). A similar conclusion was drawn from various chemical (Tagaya et al., 1988; Verburg & Allison, 1990) and immunochemical (Moradi-Améli et al., 1989) modifications in different species.

A third class contains the three remaining residues, two Trp^{190} in α-subunits and one Trp in the ε-subunit, that appear not to be accessible to NBS. On the basis of the efficient protective effect observed upon ADP binding to a vacant noncatalytic site, it may be assumed that the two endogenous ADP are bound to noncatalytic nonexchangeable sites and are responsible for the lack of reactivity of the two Trp^{190}. Main occupation of noncatalytic sites by endogenous ADP has also been reported for other mitochondrial enzymes from either pig heart (Penin et al., 1979) or beef heart (Kironde & Cross, 1986; Bullough et al., 1988). This contrasts with bacterial enzymes, where the noncatalytic sites contain ATP whereas catalytic exchangeable sites contain ADP (Issartel et al., 1986) and even GDP (Mileykovskaya et al., 1992). The lack of Trp accessibility in *S. pombe* ε-subunit to NBS is likely due to tight association with the δ-subunit (Divita et al., 1991), as also observed in the pig heart enzyme (Penin et al., 1990). The contribution of both low- and nonreactive Trp^{190} to the total intrinsic fluorescence of native F_1 appears to be almost 2-fold higher than that of the reactive Trp^{257} residues; this is consistent with the classification of Burnstein et al. (1973), showing that completely exposed tryptophan residues exhibit lower quantum yields than residues located at protein surface in limited contact with water. The ε-subunit Trp, due to the tight interaction with δ-subunit, has previously been shown to produce a very minor contribution to the fluorescence intensity measured at 332 nm (Penin et al., 1990; Divita et al., 1991). The marked overall heterogeneity of *S. pombe* F_1 tryptophan residues toward reactivity with NBS agrees with the large bandwidth at half-height of the fluorescence emission spectrum (Divita et al., 1991) as discussed by Burstein et al. (1973).

At present, work is in progress to determine the distances between α-subunit tryptophan residues and either catalytic or noncatalytic sites using fluorescence resonance energy transfer with suitable fluorescent nucleotide analogues.

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