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Mitochondrial ATP Synthase Complex: Interaction of Its F_1 Adenosinetriphosphatase Moiety with the Heavy Atom Iodine†

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ABSTRACT: Studies were carried out to determine whether a simple electron-dense "heavy atom" like iodine could be introduced selectively into one or more of the subunits of the mitochondrial ATP synthase complex of rat liver. Surprisingly, very low amounts of iodine are incorporated into the isolated F_1 moiety of this complex under conditions which result in a marked loss of catalytic activity. ATPase activity is inactivated in a concentration-dependent manner at pH 7.5 with half-maximal inactivation occurring at about 40 μM iodine. A maximum of only 10 atoms of iodine are incorporated per F_1 molecule under conditions where inhibition of ATPase activity is linearly related to iodine incorporation. The molecular size of F_1 after iodination is unchanged, indicating that inactivation is due to modification of essential amino acid residues rather than subunit dissociation. Treatment of F_1 with 20-50 μM [^{125}I]iodine followed sequentially by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography showed that the β subunit is preferentially labeled. Significantly, about two atoms of iodine per β subunit are incorporated. Some iodine amounting to less than 23% of the total radioactivity placed on the gels is recovered in the α and γ subunits whereas no radioactivity is detected in the δ and ϵ subunits. Iodination of F_1 appears to modify essential residues other than those involved in substrate or product binding per se. Thus, nucleotide binding to F_1 is unaltered by iodine, and neither phosphate, MgADP, nor MgATP protects F_1 against inhibition by this agent. Rather, loss of ATPase activity upon iodination appears to be associated with one or more pH-sensitive groups. It seems likely that these groups are tyrosine both because tyrosine is the principal amino acid involved in the iodination of proteins and because the pK of its phenolic hydroxyl groups is dramatically altered by iodination of the associated benzene ring. These studies represent the first attempt to introduce a heavy atom into an F_1-ATPase preparation in a selective manner. The results show that at low concentrations iodine does react preferentially with β subunits of the rat liver enzyme while inactivating the catalytic capacity of the intact complex. These findings may prove useful in future studies directed at understanding structural-functional relationships within ATP synthase complexes.

Enzymes involved in ATP synthesis in biological systems consist of two major components: one called F_0 which directs protons to the second component called F_1 [for recent reviews, see Senior (1979), Dunn and Heppel (1981), Amzel and Pedersen (1983), Senior and Wise (1983), Wang (1983), and Hatefi (1985)]. It is the F_1 moiety which contains binding sites for Mg^{2+}, ADP, and P_i as well as amino acid residues essential for dehydration of the latter two substrates to give ATP. Work in a number of laboratories has focused on elucidating the structure of F_1 and relating its structural features to its function. Significantly, the F_1 moieties from bacteria, chloroplasts, and mitochondria have a molecular weight of 360000-380000 and contain five different types of polypeptide chains in the stoichiometric ratio $\alpha_3\beta_3\gamma\delta\epsilon$ (Catterall et al., 1973; Kagawa et al., 1976; Foster & Fillingame, 1979; Yoshida et al., 1979). The purified β subunit has been shown from one species, *Rhodospirillum rubrum* (Harris et al., 1985), to catalyze a very low turnover of ATP in the hydrolytic reaction, indicating that this subunit contains one or more amino acid residues involved in the catalytic event.

To date, only the F_1 moiety of the rat liver ATP synthase has been crystallized in a form which is amenable to X-ray crystallography (Amel & Pedersen, 1978). A 9-Å map has

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been published (Amzel et al., 1982), and a higher resolution map is likely to be forthcoming as the rat liver F_1 crystals diffract to 3.5 Å (Amzel & Pedersen, 1978). At 9 Å, rat liver F_1 is shown to be an apparent dimer with each 190-kilodalton (kDa)[1] half of the dimer containing three roughly equivalent masses.

Although X-ray crystallographic studies have already contributed significantly to our understanding of the quaternary structure of F_1, the identities of the subunits within the crystallographic map of the enzyme are still unknown, as is the location of catalytic sites. The present study was carried out to determine whether a simple electron-dense ("heavy atom") label like iodine could be selectively introduced into one or more of the subunits of F_1. Significantly, we were able to effect preferential labeling of the β subunit at low concentrations of iodine, the iodine being targeted under the conditions chosen to residues essential for ATP hydrolytic activity. These findings, described in detail below, indicate that the selective iodination approach could be of importance in future studies directed at understanding structural–functional relationships within ATP synthase complexes.

Experimental Procedures

Materials

AMP-PNP, elemental iodine, sodium iodide, EDTA, phosphoenolpyruvate, NADH, Tris, MES, MOPS, Tricine, sucrose, HEPES, pyruvate kinase, lactate dehydrogenase, and defatted bovine serum albumin were obtained from Sigma Chemical Co. Acrylamide, methylenebis(acrylamide), Coomassie Blue R-250, and TEMED were from Bio-Rad. Ultrapure urea was obtained from Schwarz/Mann. Sephadex G-50 and protein calibration standards were obtained from Pharmacia. ATP was purchased from P-L Biochemicals. $N^{125}I$ (specific activity 15.0 mCi/μg) was obtained from Amersham Corp. whereas [^{3}H]AMP-PNP was purchased from ICN Radiochemicals. [^{3}H]ADP was from New England Nuclear. XAR-5 film was purchased from Kodak. Pierce protein assay reagent was purchased from Pierce Chemical Co. All other chemicals were of the highest purity commercially available.

Methods

Preparation of Mitochondria. Rat liver mitochondria were isolated by the high-yield procedure described by Bustamante et al. (1977) in a medium (H medium) containing 220 mM D-mannitol, 70 mM sucrose, 5 mM HEPES, and 0.5 mg/mL defatted bovine serum albumin, pH 7.4.

Isolation of F_1-ATPase. F_1 was prepared from rat liver mitochondria as described by Catterall and Pedersen (1971) with the modifications described in Pedersen et al. (1981). F_1 was stored lyophilized from 250 mM KP_i/5 mM EDTA, pH 7.5 at −20 °C.

Iodination of F_1. An iodinating solution of iodine (I_2) in dimethyl sulfoxide and NaI in aqueous solution was prepared such that the I_2:I^- ratio was 1:5 (1 mM I_2 and 5 mM NaI). Unless indicated otherwise, F_1-ATPase (0.5 mg/mL) was dissolved in 250 mM KP_i/5 mM EDTA, pH 7.5. The reaction was initiated by adding the appropriate amount of the iodine/iodide solution with a Hamilton syringe. In each experiment, the final concentration of Me_2SO was less than 0.1%. The reaction was allowed to proceed at room temperature (23–26 °C) for 15 min, and then dithiothreitol (final concentration 1 mM) was added to quench unreacted iodine. The iodinated enzyme was subjected to gel filtration (Sephadex G-50, "fine") in a centrifuge column equilibrated with 50 mM Tris–sulfate, 100 mM Na_2SO_4, and 5 mM EDTA, pH 7.5 (see Nucleotide Binding Assay for centrifugation details). Aliquots were used for the determination of ATPase activity, protein content, and radioactivity. When the binding of radioactive iodine was determined, the iodine/iodide stock solution was equilibrated with a trace amount of $Na^{125}I$ (final specific activity = 900–1000 cpm/nanoatom).

Gel Electrophoresis in SDS. Slab gel electrophoresis was carried out either by the method of Laemmli (1970) in the presence of 4 M urea or by the method described by Weber and Osborn (1969). In either case, the gels were subjected to electrophoresis until the bromophenol blue dye front was 0.5 cm from the bottom of the gel. Gels were fixed in 25% 2-propanol/10% acetic acid for 2 h, stained with 0.2% Coomassie Blue in 15% methanol and 7.5% acetic acid for a minimum of 2 h, and finally destained in 20% methanol/7.5% acetic acid.

Measurement of Radioactivity and Autoradiography. Radioactivity was measured in a γ-counter whereas autoradiography was carried out for a period ranging from 5 to 12 days in the presence of one intensifying screen, using Kodak XAR-5 X-ray film.

Assay for ATPase Activity. ATPase activity was measured either by monitoring the release of ADP with a coupled spectrophotometric assay or by monitoring the release of P_i. In the spectrophotometric assay, ADP production was coupled to the oxidation of NADH via the pyruvate kinase and lactate dehydrogenase reactions (Pullman et al., 1960). The decrease in absorption of NADH was monitored at 340 nm. Unless indicated otherwise, the reaction contained in a volume of 1 mL, at pH 7.5 and 25 °C, 4.0 mM ATP, 50 mM Tris-HCl (or Tris–bicarbonate) 4.8 mM $MgCl_2$, 0.4 mM NADH, 0.6 mM phosphoenolpyruvate, 1 unit of pyruvate kinase (NH_4SO_4 suspension), and 1 unit of lactate dehydrogenase (NH_4SO_4 suspension). The reaction was started by adding 1 μg of F_1-ATPase. Initial rates were used for the calculation of specific activity. In the phosphate release assay, P_i produced in the ATPase reaction was monitored colorimetrically as described by Gomori (1962). The assay medium contained in a final volume of 1 mL either 50 mM sodium acetate (pH 4–5), 60 mM MES (pH 5–6), and 50 mM MOPS (pH 6–7) or 50 mM Tricine (pH 7–8), together with 5 mM $MgCl_2$ and 10 μg of F_1. The reaction was started by adding ATP to a final concentration of 5 mM. After 2 min at 30 °C, the reaction was terminated with 0.1 mL of 2.5 M $HClO_4$. The entire volume was taken for the determination of P_i.

Nucleotide Binding Assay. Binding of AMP-PNP or ADP to unmodified or iodinated F_1 was determined by the centrifugation–column technique (Garrett & Penefsky, 1975). Prior to the binding assay, F_1, in 250 mM KP_i + 5 mM EDTA, pH 7.5, was precipitated once with ammonium sulfate exactly as described previously (Pedersen et al., 1981). The pellet was dissolved in 50 mM Tris-HCl, pH 7.6, and used directly in the binding assay. The binding assay mixture contained in a total volume of 100 μL 5 mM AMP-PNP (with 5 mM Co^{2+}) or ADP (0.2 μCi of ^{3}H-nucleotide), 50 mM Tris-HCl, pH 7.6, and 125 μg of F_1. After incubation at 25 °C for 20 min, the

[1] Abbreviations: AMP-PNP, 5′-adenylyl imidodiphosphate; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; Tricine, *N*-[tris(hydroxymethyl)methyl]glycine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; kDa, kilodalton(s); EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane; Me_2SO, dimethyl sulfoxide; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis(5-phenyl-2-oxazolyl)benzene.

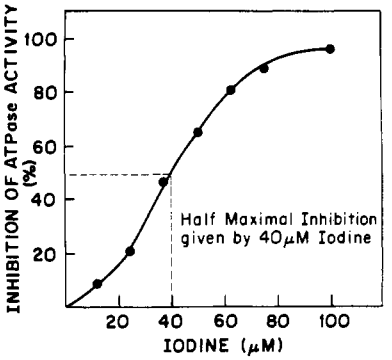

FIGURE 1: Dependence of the inhibition of F_1-ATPase activity on iodine concentration. F_1-ATPase, 50 μg, was dissolved in 250 mM KP_i buffer and 5 mM EDTA, pH 7.5, and then reacted with the iodinating mixture exactly as described under Methods. Iodine concentrations are based upon the molar amount of iodine (I_2) added to the stock solution. After the column centrifugation step, samples were assayed for protein concentration and ATPase activity by using the spectrophotometric assay (see Methods). Values represent duplicates from two different experiments.

entire reaction mixture was loaded onto a Sephadex G-50 "fine" column (1 cm^3 tuberculin syringe with a filter at the bottom) preequilibrated with 50 mM Tris-HCl, pH 7.6, and precentrifuged for 2 min at 2500 rpm in an IEC Model NH-SII clinical centrifuge. Centrifugation of the reaction mixture was carried out for 2 min at 2500 rpm to separate nucleotide bound to the enzyme from free nucleotide. A control incubation without F_1 was always carried out to assure that "spillover" of the unbound nucleotide did not elute with F_1. Radioactivity was assessed on the entire eluting fraction dissolved in 10 mL of a liquid scintillation cocktail containing 15.2 g of PPO and 0.38 g of POPOP in 3.8 L of toluene and 2.28 L of ethanol.

Determination of Protein. Protein was determined either by the Lowry method (Lowry et al., 1951) or by the method of Bradford (1976) using the protein assay dye supplied by Pierce. In both cases, bovine serum albumin was used as a standard.

RESULTS

Dependence of the Inhibition of F_1-ATPase Activity on Iodine Concentration. Rat liver F_1 was iodinated at room temperature in a stabilizing buffer consisting of 250 mM KP_i + 5 mM EDTA, pH 7.5. The enzyme is stable indefinitely under the latter conditions. As shown in Figure 1, half-maximal inhibition of ATPase activity is achieved at 40 μM iodine whereas maximal inhibition (~95%) is achieved at 100 μM. Significantly, inhibition of F_1-ATPase activity does not start to plateau until the iodine concentration exceeds 60 μM, suggesting that amino acid residues critical for ATPase activity may be rather specifically modified below this concentration.

Experiments were carried out also to determine whether iodination of F_1 is more pronounced in other buffer systems. In data not presented here, it was shown that, in the five different buffer systems tested, half-maximal (or near half-maximal) inhibition of F_1-ATPase activity is achieved also at or near 40 μM iodine. Because of its long-term stabilizing properties, the P_i buffer system noted above was chosen for subsequent studies.

Comparison of the Molecular, Kinetic, and Nucleotide Binding Properties of Native and Iodinated F_1. Results presented in Figure 2 show that the buffer volume required to elute the iodinated F_1-ATPase, from a Waters HPLC I-300 "sizing" column, is identical with that of the unmodified enzyme. A single protein profile is obtained in both cases appearing shortly after the excluded volume. Thus, loss of ATPase activity upon iodination results from titrating critical residues within the oligomeric complex rather than from dissociating the enzyme into its subunits. It is interesting to note also from Figure 2 that the total absorption at 280 nm is significantly less for iodinated F_1 than for the identical amount of unmodified F_1, a finding consistent with modification of aromatic residues.

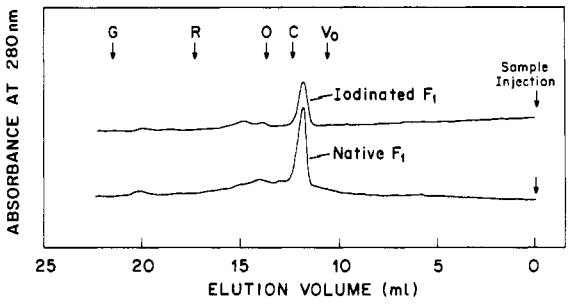

FIGURE 2: Elution profiles of native and iodinated F_1 obtained from chromatography on a Waters HPLC I-300 column. F_1 was iodinated with 100 μM iodine exactly as described under Methods. Native F_1 and iodinated F_1 (15 μg of each) were then subjected to chromatography at 25 °C in 250 mM KP_i and 5 mM EDTA, pH 7.5, at a flow rate of 0.5 mL/min. Protein was monitored at 280 nm by using a Waters 440 absorbance detector. Arrows denote the void volume (V_0) obtained with Blue Dextran and the peak elution volumes of the following standards: C, catalase (230 kDa); O, ovalbumin (45 kDa); R, ribonuclease (13.6 kDa); and G, guanosine (283 Da). Over 95% of the original ATPase activity in the native F_1 sample added to the column was recovered after elution in the protein peak.

Table I: Comparison of the Kinetic Properties of Native and Iodinated F_1-ATPase[a]

	Tris-HCl buffer		Tris–bicarbonate buffer	
in assay	K_m (mM)	V_{max} (μmol of ATP hydrolyzed min^{-1} mg^{-1})	K_m (mM)	V_{max} (μmol of ATP hydrolyzed min^{-1} mg^{-1})
F_1-ATPase	0.40	32.5	0.65	48.5
iodinated F_1-ATPase (40 μM I_2)	0.45	19.5	0.50	28.5

[a] Iodination of F_1 using 40 μM iodine was carried out exactly as indicated under Methods. The native and iodinated enzymes were then assayed in either Tris-HCl or Tris–bicarbonate buffer using the spectrophotometric procedure described under Methods. The ATP:Mg ratio was maintained at 1.0 for all concentrations of ATP which varied from 0.25 to 10 mM. Under these conditions, velocity vs. ATP concentration curves were hyperbolic both when F_1 was assayed in Tris-HCl buffer and when F_1 was assayed in Tris–bicarbonate buffer. Apparent K_m and V_{max} values were obtained directly from these Michaelis–Menten plots. Values represent averages of duplicate determinations.

Table I compares the K_m and V_{max} values of control F_1 (unmodified F_1) with those of a preparation which has been treated with 40 μM iodine. Velocity vs. ATP curves were derived from experiments conducted in both Tris-HCl buffer and the activating buffer Tris–bicarbonate (Pedersen, 1976). The results indicate that iodine behaves like a typical covalent inhibitor, which at suboptimal concentration inhibits part of the enzyme population while leaving the remainder intact. Consequently, as indicated by data in the table, V_{max} is lowered but the apparent K_m remains the same.

Experiments presented in Tables II and III indicate that the inhibitory effects of iodine are not due to its direct interaction at sites involved in either substrate or product binding. Both the iodinated and unmodified F_1 preparations bind 2.5–3 mol of AMP-PNP (a nonhydrolyzable ATP analogue) and about 1 mol of ADP per mole of F_1 (Table II). Moreover,

Table II: Comparison of the Nucleotide Binding Properties of Native and Iodinated F_1-ATPase[a]

in assay	ATPase activity remaining (%)	mol of nucleotide/mol of F_1	
		AMP-PNP	ADP
F_1-ATPase	(100)	2.9	0.93
iodinated F_1-ATPase (50 μM I_2)	43.0	2.5	1.0
iodinated F_1-ATPase (100 μM I_2)	5.3	2.8	1.1

[a] Iodination of F_1 was carried out exactly as indicated under Methods. The native and iodinated enzymes were then assayed for the binding of AMP-PNP or ADP by the column centrifugation method also described in exact detail under Methods. Nucleotides were present in the binding assay at a concentration of 5 mM. Values represent averages of duplicate determinations.

Table III: Inability of P_i, MgADP, and MgATP To Protect F_1-ATPase Activity against Inhibition by Iodine[a]

components in prior incubation	ATPase activity (μmol of ATP hydrolyzed min^{-1} mg^{-1})
F_1-ATPase + KP_i	21.4
F_1-ATPase + KP_i + I_2	7.4
F_1-ATPase + KP_i + MgADP	14.8
F_1-ATPase + KP_i + MgADP + I_2	0
F_1-ATPase + KP_i + MgATP	16.0
F_1-ATPase + KP_i + MgATP + I_2	0

[a] Iodination of F_1 in the presence of KP_i and in the absence or presence of 5 mM nucleotide and 5 mM $MgCl_2$ was carried out exactly as indicated under Methods. The final concentration of iodine was 70 μM. ATPase activity was assayed by the spectrophotometric procedure also described under Methods. Results are averages of duplicate determinations.

neither P_i, MgADP, nor MgATP protects F_1 against inhibition by iodine (Table III). In fact, the latter nucleotide–metal complexes appear to promote a slow inhibition of F_1-ATPase activity in the absence of iodine.

Subunit Labeling Patterns of F_1 at Different Iodine Concentrations. Results of experiments presented in Figure 3 show that at concentrations of iodine from 20 to 50 μM there is preferential labeling of the β subunit. At 20 μM iodine, there is no apparent labeling of subunits other than β. At 30, 40, and 50 μM iodine, the β subunit continues to be labeled preferentially with small amounts of labeling of the α and γ subunits now becoming apparent. At 80 μM iodine, although the β subunit remains the subunit most heavily labeled, there appears to be much more labeling of the α and γ subunits. At no concentration of iodine is there detectable labeling of the δ and ϵ subunits of rat liver F_1.

Stoichiometry of Labeling F_1 with Iodine. As shown in Figure 4, labeling of F_1 by iodine proceeds in a linear fashion until about 10 atoms of iodine/molecule of F_1 have been incorporated. The linear portion of the curve corresponds to the concentration range of 20–50 μM iodine where the β subunit of F_1 is preferentially labeled (see Figure 3, lanes 2–9). Significantly, it is this concentration range where F_1-ATPase activity is inhibited in an almost linear manner by iodine (see Figure 1). At 80 μM iodine, greater than 20 atoms of iodine/molecule of F_1 are incoporated. However, at this higher iodine concentration, inhibition of F_1-ATPase activity starts to plateau (Figure 1), with enhanced labeling of α and γ subunits now becoming apparent (see Figure 3, lane 10).

In order to estimate the number of atoms of iodine incorporated in β subunits, under selective labeling conditions F_1 was iodinated with [^{125}I]iodine to give approximately 90% inhibition. Autoradiograms of the subsequent SDS gel patterns were then analyzed by using an Ultroscan laser densitometer

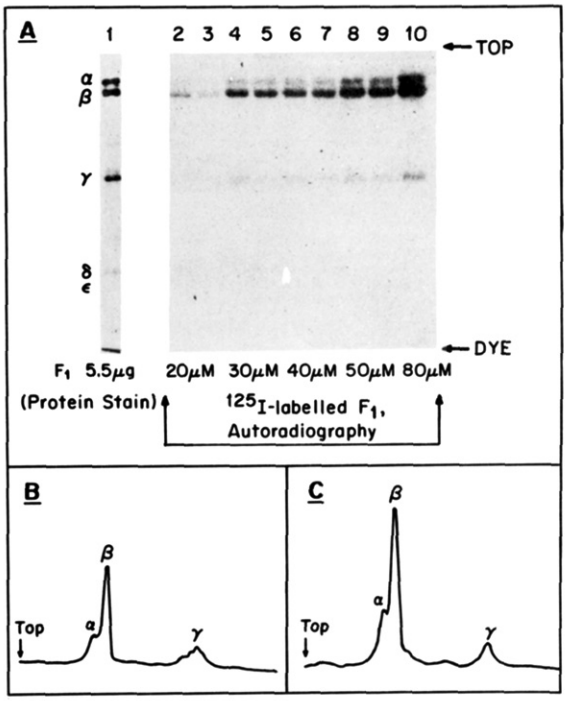

FIGURE 3: Subunit labeling patterns of F_1 at different iodine concentrations. F_1 was labeled with [^{125}I]iodine at the indicated concentrations exactly as described under Methods. After the column centrifugation step, protein was determined, and the samples (5.5 μg) were then subjected to SDS gel electrophoresis and autoradiography, also as described under Methods. (A) (Lane 1) Control F_1 subjected to SDS gel electrophoresis by the method of Laemmli (1970) and then stained with Coomassie Blue; (lanes 2–10) autoradiograms of F_1 samples labeled with the indicated concentration of [^{125}I]iodine and then subjected to SDS gel electrophoresis by the method of Laemmli (1970). (B and C) Densitometric tracing at 525 nm of autoradiograms from lanes 4 and 8 in (A).

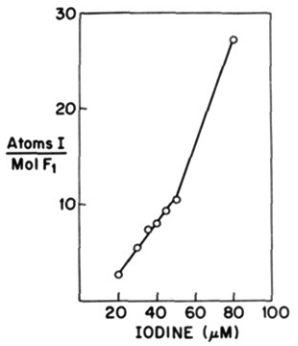

FIGURE 4: Stoichiometry of the labeling of F_1 with iodine. F_1 was iodinated with [^{125}I]iodine at the indicated concentrations exactly as described under Methods. After the column centrifugation step, separate aliquots were taken for protein determinations and radioactivity measurements. Each point represents the average of at least three separate experiments.

interfaced with a recording integrator for calculating relative areas. The results showed that of the 8.2 atoms of iodine/molecule of F_1 recovered in F_1 subunits, 6.1 were in β subunits and 1.4 and 0.7 in α and γ subunits, respectively.

Dependence of the Hydrolytic Activity of Native and Iodinated F_1 on pH. Results presented in Figure 5 show that when the pH of the ATPase assay medium (in this case, the P_i release assay) is lowered below 6, F_1, whether unmodified or iodinated, retains the capacity to hydrolyze ATP at a low rate (>3 μmol min^{-1} mg^{-1}). In contrast, as the pH is raised, there is a rapid increase in the specific activity of the unmodified F_1 while the iodinated enzyme continues to exhibit a low specific rate of ATP hydrolysis. These results indicate

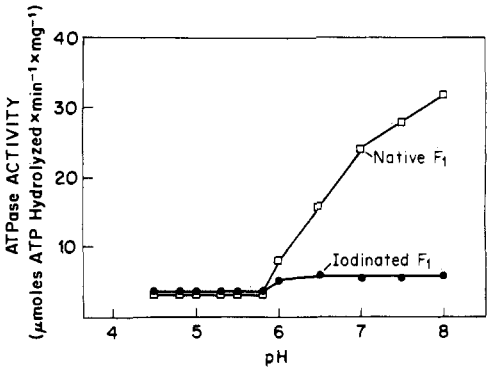

FIGURE 5: Dependence of the hydrolytic activity of native and iodinated F_1 on pH. F_1 was incubated with sufficient iodine to maximally inhibit the ATPase activity of the enzyme (>90%). After column centrifugation, the iodinated and native enzymes were assayed for ATPase activity at different pH values in the buffers described under Methods. The P_i release assay described under Methods was used in these experiments to quantify ATPase activity.

that iodine labels one or more types of amino acid residues which are not critical for F_1 to cleave the terminal phosphate of ATP per se, but which are critical in facilitating the bond-breaking event.

DISCUSSION

Results of experiments described here represent the first detailed study of the interaction of iodine with an ATP synthase preparation. In sharp contrast to the iodination of many proteins where iodine in radioactive form is introduced in large quantities without altering catalytic activity, iodination of the F_1 moiety of rat liver ATP synthase results in just the opposite response. That is, the introduction of only a relatively few atoms of iodine markedly inhibits the catalytic activity of the enzyme. Significantly, inhibition of the ATPase activity of F_1 is paralleled by preferential labeling of the β subunit (Figures 1 and 3). As there is considerable evidence in the literature (Esch & Allison, 1978; Williams & Coleman, 1982; Andrews et al., 1983; Ho & Wang 1983; Hollemans et al., 1983) that β subunits of F_1 contain ATP hydrolytic sites, it seems likely that iodine modifies amino acid residues essential for the normal function of these sites.

It seems unlikely that the small amount of iodination of α and γ subunits at concentrations ≤ 50 μM contributes significantly to the loss of ATPase activity. At 20 μM iodine, these subunits are not labeled even though ATPase activity is partially inhibited (Figures 1 and 3A). Moreover, in the range of 30–50 μM iodine, F_1-ATPase activity undergoes its sharpest loss while the small amount of iodine appearing in the γ subunit remains essentially constant (Figure 1 and Figure 3B,C). Therefore, it seems reasonable to conclude that most, if not all, loss of ATPase activity observed in these studies at iodine concentrations ≤ 50 μM is due to reaction of this molecule with essential amino acid residues on β subunits.

Essential amino acid residues labeled with iodine do not appear to be involved directly in binding substrates or products of the F_1 molecule. Thus, neither P_i, MgATP, or MgADP protects F_1-ATPase activity against inhibition by iodine (Table III), and nucleotide binding to F_1 is nearly identical for the iodinated and unmodified enzymes (Table II). These results indicate that those amino acid residues modified by iodine may play some other role within the β subunit, either directly in the catalytic event or in maintaining the proper three-dimensional structure of the catalytic site. However, a role of such amino acids in binding nucleotide at the active site cannot be totally excluded as iodine is a small molecule, which unlike many other covalent labeling agents does not possess a bulky structure which might interfere with substrate or product binding.

Among those amino acids within the β subunit of F_1 which are candidates for iodine modification, tyrosine residues seem most likely for several reasons. First, of those amino acid residues in proteins which have been shown to interact with iodine, tyrosine is the principal amino acid residue involved (Koshland et al., 1963; Dube et al., 1964; Seon et al., 1970). Although cysteine residues can be iodinated also, the rat liver F_1 β subunit, similar to the bovine heart F_1 β subunit (Walker et al., 1985), contains no cysteines.[2] Second, six tyrosyl residues are conserved within the amino acid sequences of F_1 β subunits from different species (Hollemans et al., 1983; Runswick & Walker, 1983). Significantly, and consistent with results presented here (Tables II and III), these conserved tyrosyl residues are at locations somewhat peripheral to or completely removed from conserved amino acid sequences thought to play a role in nucleotide binding (Walker et al., 1982; Fry et al., 1986). Finally, several covalent modification studies (Esh & Allison, 1978; Andrews et al., 1983; Ho & Wang, 1983) have implicated a role for β subunit tyrosines in the catalytic function of the F_1 molecule.

The functional group of tyrosine is a hydroxyl group, the pK of which may vary within the microenvironment of a protein but which is in the range 9.8–10.4 (Dixon & Webb, 1979). Iodination of tyrosine results first in the formation of monoiodotyrosine and then diiodotyrosine, both of which exhibit significantly lower pK values ($\sim$8.5 and 6.8, respectively) than tyrosine (Bolton, 1985). Therefore, if iodination of tyrosine residues within the β subunit of F_1 is, in fact, the cause of the enzyme's inability to hydrolyze ATP, this would imply that the protonated form of tyrosine residues is critical for the catalytic event. Above pH 7, one would predict that tyrosine residues within the β subunit of unmodified F_1 would be substantially protonated and, therefore, active in catalysis whereas in the iodinated F_1 they would be much less protonated and, therefore, much less active in catalysis. Such is the case for unmodified and iodinated F_1 at pH values above 7 (Figure 5).

Prior results of Dorgan and Schuster (1981) provide suggestive evidence that at least one deprotonated tyrosine residue may be involved in ATP hydrolysis by F_1. Therefore, it is possible that two different classes of tyrosine residues located in different microenvironments of the enzyme may be involved in catalysis. One such class of tyrosines may be mostly protonated at physiological pH and the other significantly deprotonated. This view explains both why an increase in pH results in an increase in ATPase activity (i.e., by deprotonating the class of tyrosines with the lower pK) and why iodine inhibits the enzyme (i.e., by lowering the pK of the class of tyrosines with the more normally high pK).

Studies reported here indicate also that an iodine-resistant residue may help promote ATP hydrolysis catalyzed by F_1. Thus, at pH values below 6, both the maximally iodinated F_1 and the unmodified enzyme still retain the capacity to hydrolyze ATP at a low rate (Figure 5).

F_1 from only one other source (bovine heart) has been examined for its interaction with iodine (Penefsky, 1967). Although that study was very abbreviated and constituted part of a larger study with other covalent labeling agents, it is, nevertheless, of interest to compare it with results obtained here on rat liver F_1. Iodination of the bovine heart enzyme

[2] D. Garboczi, A. Fox, S. Holloway-Gerring, and P. L. Pedersen, unpublished results.

carried out in 20 mM KP_i, 2 mM EDTA, and 4 mM ATP did result in inhibition of ATPase activity. However, maximal inhibition required 764 μM iodine as compared to 100 μM for the rat liver enzyme reported in this study. Unfortunately, the subunit(s) labeled in the bovine enzyme were never identified, but the fact that 8–9 sulfhydryl groups/mol of F_1 were titrated indicates that subunits other than β were being substantially labeled. This is because it is now known from amino acid sequencing data that the β subunit of bovine heart F_1 contains no cysteine residues (Walker et al., 1985). It seems clear, therefore, that the iodination of bovine heart F_1 as described above results in a significantly different and less specific labeling pattern than that reported here for the rat liver enzyme.

In summary, we have shown that a number of iodine atoms, at the minimum six, can be introduced rather specifically into the β or catalytic subunit of the rat liver ATP synthase molecule. As iodine is an electron-dense heavy atom, these studies may prove valuable in identifying essential amino acid residues within the three-dimensional structure of rat liver F_1 when a $\leq$3.5-Å map of the enzyme becomes available.

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