Structural Alterations and Inhibition of Unisite and Multisite ATP Hydrolysis in Soluble Mitochondrial F1 by Guanidinium Chloride

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ABSTRACT: The effect of guanidinium chloride (GdnHCl) on the ATPase activity and structure of soluble mitochondrial F1 was studied. At high ATP concentrations, hydrolysis is carried by the three catalytic sites of F1; this reaction was strongly inhibited by GdnHCl concentrations of <50 mM. With substoichiometric ATP concentrations, hydrolysis is catalyzed exclusively by the site with the highest affinity. Under these conditions, ATP binding and hydrolysis took place with GdnHCl concentrations of >100 mM; albeit at the latter concentration, the rate of hydrolysis of bound ATP was lower. Similar results were obtained with urea, although nearly 10-fold higher concentrations were required to inhibit multisite hydrolysis. GdnHCl inhibited multisite ATPase activity by diminishing the V_{max} of the reaction without significant alterations of the K_m for MgATP. GdnHCl prevented the effect of excess ATP on hydrolysis of ATP that was already bound to the high-affinity catalytic site. With and without 100 mM GdnHCl and 100 μM [^{3}H]ATP in the medium, F1 bound 1.6 and 2 adenine nucleotides per F1, respectively. The effect of GdnHCl on some structural features of F1 was also examined. GdnHCl at concentrations that inhibit multisite ATP hydrolysis did not affect the exposure of the cysteines of F1, nor its intrinsic fluorescence. With 100 mM GdnHCl, a concentration at which unisite ATP hydrolysis was still observed, 0.7 cysteine per F1 became solvent-exposed and small changes in its intrinsic fluorescence of F1 were detected. GdnHCl concentrations on the order of 500 mM were required to induce important decreases in intrinsic fluorescence. These changes accompanied inhibition of unisite ATP hydrolysis. The overall data indicate that increasing concentrations of GdnHCl bring about distinct and sequential alterations in the function and structure of F1. With respect to the function of F1, the results show that at low GdnHCl concentrations, only the high-affinity site expresses catalytic activity, and that inhibition of multisite catalysis is due to alterations in the transmission of events between catalytic sites.

ATP synthesis in mitochondria, chloroplasts, and the plasma membrane of bacteria is catalyzed by the ATP synthase with the energy of electrochemical H^+ gradients derived from electron transport. The enzyme consists of two multisubunit components (for reviews, see refs *1*−*3*), Fo, which is embedded in the membrane, and F1,[1] which can be obtained in a soluble form. F1 possesses the catalytic machinery to hydrolyze ATP and, under certain conditions, the synthesis of ATP (*4*−*8*). F1 consists of five different subunits which in order of decreasing molecular weight are α_3, β_3, γ, δ, and ϵ. F1 has three catalytic sites that are located in the interface between the α and β subunits.

At high ATP concentrations, steady-state ATP hydrolysis is carried out by the three catalytic sites of F1. The generally accepted view is that the three sites are equivalent but that, depending on their conformation, they acquire different characteristics with respect to affinity for substrates and products, and ability to carry out catalysis. In the binding change mechanism (*9*), it is visualized that during steady-state hydrolysis, tightly bound substrates exist in one of the catalytic sites; the binding of substrate to another site induces product release from the former site, and simultaneously, another site acquires the characteristics of the high-affinity catalytic site. The binding change mechanism is substantiated by kinetic experiments (*2*) and by the crystal structure of F1 (*10*) that shows that the three catalytic sites of F1 exist in different conformations. More recently, it was documented that during hydrolysis of ATP, the central γ subunit rotates within the core of the α and β subunits (*11*−*15*). It is relevant to the present paper that the modeling of F1 shows that during steady-state catalysis, the enzyme undergoes extensive conformational changes and multiple rearrangements of its subunits (*15*).

F1 also catalyzes hydrolysis of substoichiometric concentrations of ATP (*16*, *17*). The kinetics of hydrolysis and the arrangements that F1 undergoes during catalysis differ drastically from those in which the substrate is at high concentrations (*18*). When substoichiometric ATP concentrations are mixed with F1, ATP binds exclusively and rapidly to the catalytic site with the highest affinity (*16*, *17*). At that site, ATP is rapidly hydrolyzed and an equilibrium between ATP and ADP and P_i is established (*16*, *17*). F1 from *Escherichia coli* in which the γ subunit was cross-linked to

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[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; F1, soluble catalytic component of the ATP synthase; GdnHCl, guanidinium chloride; MOPS, 3-(*N*-morpholino)propanesulfonic acid.

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one of the β subunits was able to carry out unisite ATP hydrolysis (*19*). Hence, it is clear that the conformational changes and subunit arrangements that F1 undergoes during unisite catalysis are much less extensive than in multisite hydrolysis.

Denaturants, such as GdnHCl and urea, have been powerful tools in ascertaining the kinetics and energetics of the events that lead to formation of native structures. When proteins are exposed to increasing denaturant concentrations, it is generally observed that their structure does not suffer drastic changes until a certain concentration is reached (*20*, *21*). These findings illustrate that protein denaturation is a cooperative phenomenon that involves the transition from the native to the unfolded state in a single step, i.e., the two-state model. However, the question has been raised as to whether in the range of denaturant concentrations in which the protein does not exhibit large structural alterations, it, nonetheless, undergoes discrete structural modifications. Indeed in several enzymes, titration with denaturants brings about alterations in catalytic activity before large structural changes take place (*21*). Tsou (*20*, *21*) reviewed the data and presented convincing evidence that, at least in several enzymes, catalysis is significantly more sensitive to denaturant action than the rest of the enzyme.

Because F1 is able to carry out unisite and multisite catalysis, it seemed to be an excellent system for probing how denaturants affect in the same enzyme two different expressions of catalysis, and how these effects relate to alterations of enzyme structure. Therefore, we determined the effect of GdnHCl on multisite and unisite catalysis and on some structural features of F1. The results showed that in F1 GdnHCl induces a sequence of events that initially comprise subtle structural perturbations and abolition of multisite hydrolysis. In a following step, the protein undergoes extensive structural alterations and inhibition of unisite catalysis. In the final step, the protein is completely unfolded.

MATERIALS AND METHODS

F1 from bovine heart mitochondria was prepared as described elsewhere (*22*); the enzyme was maintained at 4 °C in 50% saturation NH_4SO_4, 2 mM ATP, and 2 mM EDTA. Before the experiments, the suspension was centrifuged, and the pellet was dissolved in 40 mM MOPS, 2 mM phosphate, and 10 mM $MgCl_2$ (pH 6.8) and filtered by centrifugation through Sephadex colums. The amount of protein was determined in the eluate by method of Lowry (*23*). In spectrophotometric assays (*24*), the enzyme exhibited an activity of about 70 μmol min^{-1} mg^{-1} at 20 °C in a medium (pH 8.0) that contained 25 mM Tris-SO_4, 3 mM $MgSO_4$, 3 mM ATP, 3 units of pyruvate kinase, 6 units of lactate dehydrogenase, and 0.2 mM NADH. At pH 6.8, its activity was around 35 μmol min^{-1} mg^{-1}.

[γ-^{32}P]ATP was prepared from [^{32}P]phosphate (Amersham) according to the method of Glynn and Chappel (*25*). Its specific activity was $5-7 \times 10^7$ cpm/nmol.

Unisite ATP Hydrolysis. In all experiments, this reaction was studied at a ratio of 0.3 mol of [γ-^{32}P]ATP/mol of F1 in reaction medium that contained 40 mM MOPS, 2 mM phosphate, and 10 mM $MgCl_2$ (pH 6.8). The reaction was arrested at the indicated times with 6% trichloroacetic acid (final concentration). This was followed by the addition of 0.5 mL of 3.3% ammonium molybdate in 3.75 N H_2SO_4. After mixing, 1 mL of a 1:1 isobutanol/benzene mixture was added. The mixture was vigorously stirred for ~1 min. The organic phase was eliminated. The latter procedure was repeated two times. The amount of radioactivity in the aqueous phase was determined so the amount of remaining [γ-^{32}P]ATP could be calculated. At the concentrations that were used, GdnHCl and urea did not interfere with the extraction procedure. In some experiments, the amount of radioactivity in the organic phase of the first extraction was measured to determine the amount of [^{32}P]phosphate.

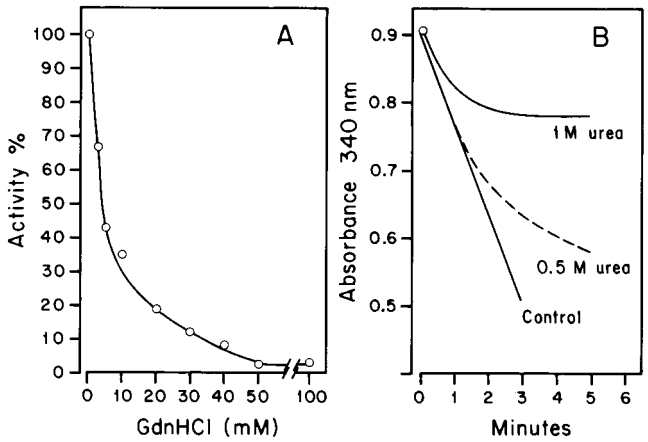

FIGURE 1: Effect of GdnHCl and urea on multisite ATPase activity of F1. Activity with the indicated concentrations of GdnHCl (A) and urea (B) was determined by the spectrophotometric method (see Materials and Methods). In the experiments with GdnHCl, the traces were linear with time; results are expressed as the percentage of the rate of the activity of the control (26 μmol min^{-1} mg^{-1}). With urea (B), ATPase activity decreased with time; the traces are shown.

Multisite ATP Hydrolysis. The experiments in Figure 1 were carried out with the spectrophotometric assay. The composition of the medium was identical to that used for the assay of unisite hydrolysis, except for ATP which was at a concentration of 3 mM and F1 at 1 μg/mL. ATPase activity was also assayed by determination of the amount of inorganic phosphate according to the method of Sumner (*26*). Essentially the same results were obtained by the two methods.

Binding of [γ-^{32}P]ATP and $^{32}P_i$ to F1. Unless otherwise indicated, F1 was incubated in the medium used for measuring the level of unisite [γ-^{32}P]ATP hydrolysis. At the indicated times, the mixtures were filtered by centrifugation through Sephadex columns equilibrated with the same medium (except for [γ-^{32}P]ATP). In the experiments with GdnHCl, the columns were equilibrated with a medium that contained GdnHCl. The eluate was received in 0.1 mL of 10% trichloroacetic acid and [γ-^{32}P]ATP, and the amount of $^{32}P_i$ was determined as described above. Protein in the eluates was assayed by the method of Lowry (*23*); in this case, the eluates were not received in trichloroacetic acid.

Reactivity of the Cysteines of F1 to 5,5-Dithiobisnitrobenzoic Acid. Because at pH 6.8 the reactivity of DTNB with sulfhydryl groups is poor, the experiments were performed at pH 8.0. F1 (0.6−0.8 mg/mL) was incubated in a medium that contained 25 mM Tris-sulfate, 3 mM $MgCl_2$, 30 mM K_2SO_4 (pH 8.0), and 2.5 mM DTNB. After introduction of F1, the changes in absorbance at 412 nm were recorded in

a cell thermostated at 25 °C. For the calculation of the level of cysteines, an absorption coefficient of 13 600 M^{-1} cm^{-1} was used.

Changes in the Intrinsic Fluorescence of F1. For the assays, F1 was introduced at a final concentration of 50 μg/mL in 3 mL of a pH 6.8 mixture that contained 40 mM Bis-tris, 2 mM phosphate, 10 mM $MgCl_2$, and the indicated concentrations of GdnHCl. The samples were excited at 280 nm, and the emission spectra from 295 to 400 nm were recorded. In all cases, recordings for mixtures without protein were taken. The latter were subtracted from the samples that contained F1 using the software of the fluorometer (Shimatzu RF 5000). It was also determined if GdnHCl interferes with the fluorescence of tyrosine, it was found that with and without 6.0 M GdnHCl, tyrosine exhibited the same fluorescence.

RESULTS

Beharry and Bragg (*27*) reported that at saturating ATP concentrations, 300 mM GdnHCl inhibits the ATPase activity of F1. We confirmed their observations, but in addition, we found that GdnHCl in the concentration range of 0−50 mM produced a progressive inhibition of hydrolysis; with 50 mM GdNHCl, hydrolysis was almost fully abolished (Figure 1A). In the experiments whose results are depicted in Figure 1, hydrolysis was assessed in a coupled system that contained pyruvate kinase and lactate dehydrogenase. With GdnHCl, the activity traces were linear with time until NADH became limiting. It is also noted that 100 mM GdnHCl did not affect the activity of the two latter enzymes. Similar results were obtained when hydrolysis was assessed by a phosphate assay by a colorimetric method or by $^{32}P_i$ formation from [γ-^{32}P]-ATP (not shown). It is also pointed out that the effect of GdnHCl was not an unspecific salt effect, since 100 mM KCl did not affect significantly the hydrolytic reaction.

The effect of urea on multisite and unisite hydrolysis was also determined to ascertain if the effect of GdnHCl was related to its denaturing action. Like GdnHCl, urea induced inhibition of multisite hydrolysis (Figure 1B). However, concentrations on the order of 1 M were needed to induce an inhibition comparable to that observed with 100 mM GdnHCl. In this regard, it is recalled that in comparison to GdnHCl levels, much higher urea concentrations are generally needed to induce protein denaturation. It is also relevant that the effect of urea on F1 was not immediate; Figure 1B shows the decay of the ATPase activity of F1 as function of time with 0.5 and 1 M urea. The decrease in the rate of the reaction was not due to inhibition of the coupling enzymes, since the addition of excess ADP at the end of recording induced an abrupt decrease in absorbance at 340 nm (not shown).

When F1 is mixed with substoichiometric [γ-^{32}P]ATP concentrations, there is an initial burst of hydrolysis that is followed by a relatively slow hydrolytic phase (see ref *28* and Figure 2). GdnHCl (100 mM) and urea at a concentration of 1 M did not affect the initial hydrolytic burst, and the denaturants only slowed the subsequent hydrolytic phase (Figure 2). It is important to note that with the two denaturants, hydrolysis proceeded until [γ-^{32}P]ATP was exhausted. Thus, it would appear that GdnHCl and urea are effective inhibitors of multisite, but not of unisite, ATP hydrolysis. Further studies of denaturant action on F1 were carried out with only GdnHCl.

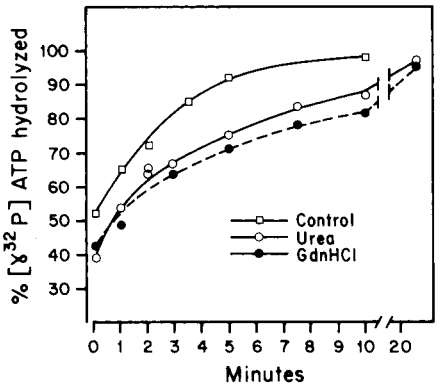

FIGURE 2: Effect of GdnHCl and urea on unisite [γ-^{32}P]ATP hydrolysis. The standard mixture for unisite [γ-^{32}P]ATP hydrolysis was employed. It also contained 100 mM GdnHCl or 1 M urea as shown. At the indicated times, the reaction was arrested and the amount of remaining [γ-^{32}P]ATP was determined.

Table 1: Effect of Guanidinium Chloride on the Kinetics of F1[a]

GdnHCl (mM)	K_m (μM)	V_{max} (μmol min^{-1} mg^{-1})
0	330 ± 48	30 ± 3
2.5	280 ± 25	21 ± 2
5	279 ± 62	16 ± 2
7.5	263 ± 10	12 ± 1
10	338 ± 61	11 ± 1

[a] ATPase activity was measured at concentrations of ATP that ranged from 0.5 to 3 mM and the indicated concentrations of GdnHCl by the spectrophotometric method. The K_m and V_{max} were calculated from linear Lineweaver−Burk plots. Average of three experiments with three different F1 preparations.

To ascertain if the effect of 100 mM GdnHCl was reversible, we incubated F1 with GdnHCl for 5 min. At this time, an aliquot was withdrawn into the mixture for an assay of multisite ATP hydrolysis. In this step, GdnHCl was diluted 30-fold. The activity of the enzyme was similar to that observed in a medium with 3.3 mM GdnHCl. Thus, the inhibiting effect of GdnHCl on multisite ATP hydrolysis was reversible.

The type of inhibition that GdnHCl exerts on multisite catalysis was determined by measurements of hydrolysis at several ATP and GdnHCl concentrations. Lineweaver−Burk plots of the data showed that the K_m for MgATP was not importantly altered by GdnHCl; the main effect of GdnHCl was on the V_{max} of the reaction (Table 1).

Cold Chase Experiments with and without GdnHCl. In confirmation of previous findings (*29*), we observed that the addition of high ATP concentrations to F1 undergoing unisite [γ-^{32}P]ATP hydrolysis led to rapid hydrolysis of the previously bound [γ-^{32}P]ATP (Figure 3A). The rapid hydrolysis of [γ-^{32}P]ATP bound to the high-affinity catalytic site results from cooperative effects triggered by the filling of the other catalytic sites of F1 by ATP. In mixtures that contained 100 mM GdnHCl, the addition of excess ATP to F1 that had been carrying out unisite [γ-^{32}P]ATP hydrolysis for a few seconds caused a relatively small burst of [γ-^{32}P]ATP hydrolysis (compare in panels A and B of Figure 3). The results also show that even though the system contained a substantial amount of [γ-^{32}P]ATP, the extent of the hydrolytic burst of [γ-^{32}P]ATP induced by nonradioactive ATP decreased with time until only a small response was observed.

Table 2: Effect of Guanidinium Chloride on the Binding of ATP to F1[a]

Experiment A

conditions	[γ-^{32}P]ATP/F1	bound		
		[γ-^{32}P]ATP/F1	$^{32}P_i$/F1	ATP/P_i
F1 and [γ-^{32}P]ATP for 10 s	0.20 ± 0.01	0.16 ± 0.03	0.1 ± 0.01	1.7 ± 0.3
F1, [γ-^{32}P]ATP, and GdnHCl for 10 s	0.23 ± 0.01	0.19 ± 0.03	0.07 ± 0.01	2.6 ± 0.6

Experiment B

conditions	[γ-^{32}P]ATP/F1	bound [γ-^{32}P]ATP/F1
F1 and [γ-^{32}P]ATP for 10 s	0.21 ± 0.01	0.18 ± 0.02
F1 and [γ-^{32}P]ATP for 15 s	0.20 ± 0.01	0.19 ± 0.02
F1 and [γ-^{32}P]ATP for 10 s → ATP for 5 s	0.10 ± 0.01	0.02 ± 0.01
F1, [γ-^{32}P]ATP, and GdnHCl for 10 s	0.23 ± 0.02	0.22 ± 0.01
F1, [γ-^{32}P]ATP, and GdnHCl for 15 s	0.24 ± 0.03	0.22 ± 0.02
F1, [γ-^{32}P]ATP, and GdnHCl for 10 s → ATP for 5 s	0.15 ± 0.03	0.17 ± 0.01

[a] In experiment A, F1 was incubated under conditions for unisite [γ-^{32}P]ATP hydrolysis with and without 100 mM GdnHCl in a final volume of 0.1 mL. After 10 s, the reaction was arrested with trichloroacetic acid (6% final concentration) and the amount of [γ-^{32}P]ATP determined. From this value, the ratio of [γ-^{32}P]ATP/F1 in the mixture was calculated. The experiment also included identical samples that after incubation for 10 s were filtered by centrifugation through Sephadex columns. The eluates were received in 0.1 mL of 12% trichloroacetic acid, and the amount of [γ-^{32}P]ATP and $^{32}P_i$ was determined. From the data, the ratios of [γ-^{32}P]ATP/F1 and $^{32}P_i$/F1 were calculated; these are termed bound [γ-^{32}P]ATP/F1 and $^{32}P_i$/F1. The ratios of bound [γ-^{32}P]ATP/$^{32}P_i$ are also shown. In experiment B, F1 was incubated as in experiment A without and with 100 mM GdnHCl as indicated. After incubation for 10 or 15 s, the reaction was arrested with trichloroacetic acid. The experiment also included mixtures that were supplemented with 5 mM ATP after incubation for 10 s; this was followed by the addition of acid 5 s later. In all cases, the amount of [γ-^{32}P]ATP was determined. This is termed [γ-^{32}P]ATP/F1. The experiment also included identical samples that after 10 and 15 s and after the addition of 5 mM ATP were filtered by centrifugation and received in trichloroacetic acid. In the latter eluates, the amounts of [γ-^{32}P]ATP and protein were determined. From the data, the ratios of bound [γ-^{32}P]ATP/F1 were calculated. The average results of three different experiments and the standard deviation are also shown.

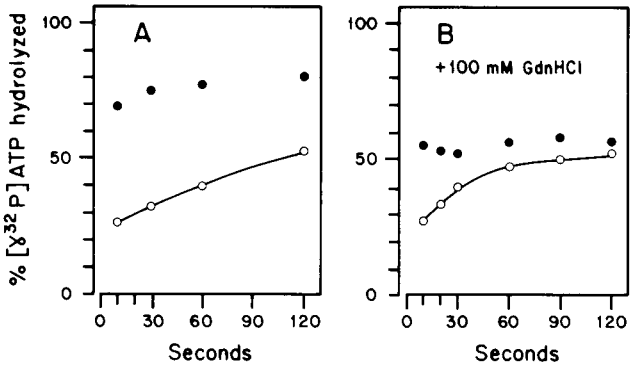

FIGURE 3: Effect of high concentrations of ATP in F1 undergoing unisite [γ-^{32}P]ATP in a medium without (A) and with 100 mM GdnHCl (B). F1 was allowed to carry out unisite [γ-^{32}P]ATP hydrolysis (○). At the indicated times, 5 mM ATP dissolved in buffer with and without 100 mM GdnHCl was added. Ten seconds later, the reaction was arrested and the amount of [γ-^{32}P]ATP was determined (●).

The latter effect of GdnHCl could indicate that not all the [γ-^{32}P]ATP that was introduced under unisite conditions became enzyme-bound. As a consequence, only the fraction of bound [γ-^{32}P]ATP would be hydrolyzed when high ATP concentrations were added (Figure 3). Accordingly, F1 was mixed with [γ-^{32}P]ATP at a ratio of 0.3 mol of [γ-^{32}P]ATP/mol of F1 in a medium with 100 mM GdnHCl; after 10 s, the mixture was filtered through centrifuge columns. In the eluate, the amounts of $^{32}P_i$ and [γ-^{32}P]ATP bound to F1 were determined. At the time of filtration, an identical sample was quenched with trichloroacetic acid and the amounts of [γ-^{32}P]-ATP and $^{32}P_i$ were measured. In a medium with GdnHCl, essentially all the [γ-^{32}P]ATP that existed in the system was enzyme-bound (Table 2, experiment A). Thus, the failure of excess ATP to induce hydrolysis of previously bound [γ-^{32}P]ATP was not due to hindrances in the binding of [γ-^{32}P]ATP to F1. The data also show that F1 contained $^{32}P_i$, indicating that GdnHCl did not modify the affinity of the catalytic site for P_i. In this regard, it is interesting that the ratio of the amount of [γ-^{32}P]ATP to $^{32}P_i$ at the catalytic site was higher with GdnHCl (Table 2, experiment A).

It was also considered that in a medium with GdnHCl, [γ-^{32}P]ATP bound to the high-affinity catalytic site was not hydrolyzed upon addition of excess ATP, because it could have undergone exchange with medium ATP. However, results of experiment B of Table 2 show that after the addition of high ATP concentrations to F1 undergoing unisite [γ-^{32}P]ATP hydrolysis, F1 still possessed bound [γ-^{32}P]ATP.

Effect of GdnHCl on the Binding of Adenine Nucleotides to F1. Up to this point, the experiments indicate that F1 incubated with GdnHCl fails to express the catalytic events that "normally" occur when F1 is exposed to high concentrations of ATP. This detrimental effect of GdnHCl could be due to impairments in the binding of ATP to the sites with lower affinity, or to perturbation in the transmission of events between catalytic sites. Thus, we studied if GdnHCl affects the binding of ATP to F1 when ATP is introduced at relatively high concentrations. F1 was incubated with 100 μM [^{3}H]ATP, and subsequently, the mixture was filtered through Sephadex centrifuge columns. Analysis of the eluate showed that with and without 100 mM GdnHCl, the respective contents of ^{3}H-labeled adenine nucleotide were 1.6 ± 0.02 and 2.0 ± 0.3 per F1 (three experiments). Therefore, the inhibiting effect of GdnHCl on multisite ATP hydrolysis and related reactions would not seem to be due to hindrances in ATP binding.

Effect of GdnHCl on the Structure of F1 and Unisite ATP Hydrolysis. As shown in Table 1, GdnHCl at concentrations of <10 mM induces an important inhibition of multisite ATP hydrolysis. On the other hand, unisite ATP hydrolysis is hardly affected by 100 mM. Therefore, it was relevant to explore if inhibition of multisite or unisite hydrolysis (see

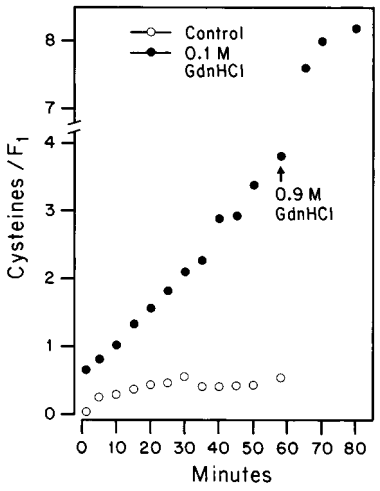

FIGURE 4: Effect of GdnHCl on the reactivity of the cysteines of F1 to DTNB. The experiment was carried out as described in Materials and Methods in mixtures with (●) and without (○) 100 mM GdnHCl. From the absorbance of the mixtures at 412 nm, the number of cysteines derivatized by DTNB was calculated. At the arrow, the concentration of GdnHCl was increased to 1 M.

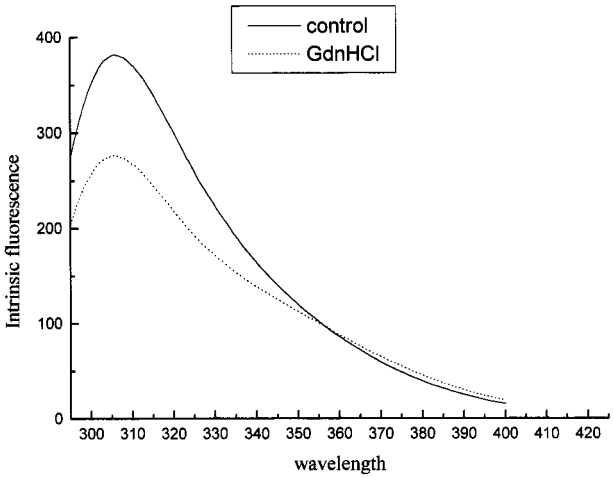

FIGURE 5: Effect of 6.0 M GdnHCl on the intrinsic fluorescence emission spectra of F1. Samples (3 mL) that contained 50 μg of F1/mL of a mixture that contained 40 mM Bis-tris, 2 mM phosphate, and 10 mM $MgCl_2$ without and with 6.0 M GdnHCl were excited at 280 nm, and their emission fluorescence spectra were recorded.

below) is accompanied by structural alterations of F1. In a first approach, we determined if GdnHCl concentrations that inhibit multisite hydrolysis by approximately 60% bring about changes in the exposure to one or more of the cysteines of F1. To this end, we incubated F1 with 0, 2.5, 5, 7.5, and 10 mM GdnHCl and DTNB. The absorbancies of the mixtures were monitored at 412 nm, to determine the number of cysteines accessible to DTNB. The results showed that in this range of GdnHCl concentrations, the denaturant did not increase the level of exposure of cysteines. For example, in five experiments, the control and the sample of F1 incubated without and with 10 mM GdnHCl yielded an average of 0.4 ± 0.15 and 0.42 ± 0.2 cysteine per F1, respectively, after incubation for 5 min. Thus, the inhibition of ATP hydrolysis by GdnHCl concentrations of <10 mM is not accompanied by an increase in the level of exposure of the eight cysteines of F1.

Using the same methodology, we studied the accessibility of the cysteines of F1 to DTNB in a medium with 100 mM GdnHCl, a concentration at which unisite ATP hydrolysis readily takes place. It has been previously reported that relatively high GdnHCl concentrations increase the level of exposure of cysteines to DTNB (*27*). Indeed, we found that 100 mM GdnHCl brought about a rapid increase in absorbance at 412 nm that amounted to 0.7 cysteine per F1 (Figure 4); this was followed by a slower rate of cysteine derivatization, and in 30 min, nearly 2 cysteines per F1 were derivatized by DTNB. The addition of 1 M GdnHCl to the mixture induced the exposure of the cysteines of F1. As evidenced by an increased reactivity of the cysteines of F1 to DTNB, 100 mM GdnHCl causes some structural alterations of F1; however, these are not large enough to induce loss of unisite catalysis.

To further explore if there is a relationship between structural changes and loss of multisite or unisite ATP hydrolysis, we also measured the intrinsic fluorescence of F1 at various GdnHCl concentrations. The fluorescence emission spectra of F1 were recorded at an excitation wavelength of 280 nm. Thus, changes in the environment of one or more of the 30 tyrosines, or of the single tryptophan of F1, were probed. The peak of maximal emission of native F1 was at 306 nm. With 6 M GdnHCl, the peak of maximal emission did not change, but there was an important decrease in fluorescence intensity (Figure 5).

At GdnHCl concentrations of 10 mM, we observed slight changes in the intrinsic fluorescence spectra of F1; in three experiments, with 10 mM GdnHCl the intrinsic fluorescence of F1 was 106 ± 2% of that observed in the absence of GdnHCl. With 50 and 100 mM GdnHCl, the results were more clear and reproducible; in five different preparations, the average increase was 11 ± 4 and 17 ± 6% with 50 and 100 mM GdnHCl, respectively. Maximal enhancement of F1 fluorescence was observed with 250 mM GdnHCl; however, in this case the magnitude of fluorescence enhancement was far more variable. In five different F1 preparations, the increase in fluorescence varied from 18 to 50% with an average of 32 ± 17%. We have no explanation for this behavior of F1, but the overall data show that in the range of 50−250 mM, GdnHCl increases the fluorescence of F1 (Figure 6A). At GdnHCl concentrations of >250 mM, there is a progressive decrease in fluorescence that reflects the exposure of the fluorophores to the solvent. This phase of the titration curve is very reproducible. The effect of different concentrations of GdnHCl on the intrinsic fluorescence of F1 is shown in Figure 6A, in which the difference between the intrinsic fluorescence of native F1 minus the fluorescence of F1 denatured by 6.0 M GdnHCl is 100%. It is relevant to point out that with 1.0 M GdnHCl, the intrinsic fluorescence of F1 is higher than with 6.0 M GdnHCl.

The effect of GdnHCl, in the concentration range used in the fluorescence experiments, on unisite [γ-^{32}P]ATP hydrolysis was determined. In these experiments, the amount of [γ-^{32}P]ATP hydrolyzed after incubation for 30 s and 5 min was measured (Figure 6B). The former values are an index of the binding and splitting of [γ-^{32}P]ATP at the high-affinity catalytic site, whereas the amount of [γ-^{32}P]ATP hydrolyzed in the interval between 30 s and 5 min indicates hydrolysis of bound [γ-^{32}P]ATP. Up to a concentration of 500 mM, GdnHCl did not affect the initial phase of [γ-^{32}P]-ATP hydrolysis; however, there was a diminution in the

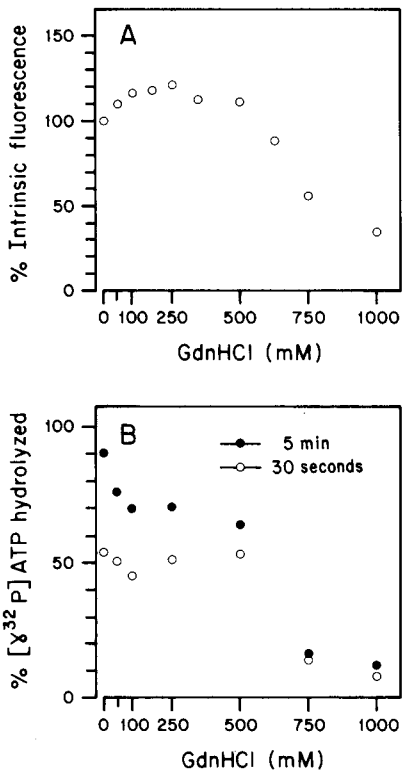

FIGURE 6: Effect of various concentrations of GdnHCl on the intrinsic fluorescence of F1 (A) and on unisite [γ-^{32}P]ATP hydrolysis (B). (A) F1 was incubated with the indicated concentrations of GdnHCl, and immediately after mixing (~30 s), the emission fluorescence spectra at an excitation wavelength of 280 nm were recorded. In the figure, 100% is the difference in fluorescence intensity of native F1 and that of F1 denatured by 6.0 M GdnHCl (see Figure 5). (B) F1 was incubated under conditions for unisite [γ-^{32}P]ATP hydrolysis in a medium that contained the indicated concentrations of GdnHCl. The amount of [γ-^{32}P]ATP hydrolyzed in 30 s (○) and 5 min (●) was determined.

amount of bound [γ-^{32}P]ATP hydrolyzed. At concentrations of >0.5 M, there was an important inhibition of both, the initial and subsequent hydrolytic phases. It is important to note that the decreases in intrinsic fluorescence accompanied loss of unisite ATPase activity (compare in panels A and B of Figure 6). This suggests that relatively large structural alterations are required to abolish unisite catalysis.

DISCUSSION

The H-ATPase/ATP synthase and its soluble component F1 carry out catalysis through some of the most complex mechanisms described. Experimentally, however, the catalytic properties of F1 may be studied at two levels of complexity by introducing either saturating or substoichiometric ATP concentrations. We took advantage of these characteristics of F1 to explore how different concentrations of GdnHCl affect these two expressions of catalysis and the structure of F1.

Sequence of Events in the Denaturation of F1 by GdnHCl. A salient feature of the data presented here is that in definite ranges of GdnHCl concentrations the enzyme acquires distinct functional and structural features. Between 0 and 10 mM GdnHCl, there is a progressive decrease in the level of multisite catalysis (Table 1). At these low concentrations and after relatively short times of incubation, GdnHCl did not affect the exposure of cysteines to the solvent and did not induce changes in the intrinsic fluorescence of F1. Thus, strong inhibition of multisite catalysis does not require gross structural alterations of F1. With 100 mM GdnHCl, the binding of adenine nucleotides to F1 is not perturbed and unisite catalysis is largely unaffected. However, at this GdnHCl concentration, there was exposure of the cysteines of F1 and discrete changes in the intrinsic fluorescence of F1.

At higher GdnHCl concentrations (0.5−1 M), aromatic residues become solvent-exposed, and this is accompanied by inhibition of unisite catalysis. However, it is noteworthy that at this stage, the changes in the environment of aromatic residues are not maximal. Thus, up to 1 M GdnHCl, the protein is not fully denatured; complete unfolding requires higher denaturant concentrations.

These observations therefore indicate that with increasing GdnHCl concentrations, F1 undergoes the following alterations: native F1 → loss of multisite catalysis due to hindrances in communication between catalytic sites → exposure of cysteines, perturbation of the environment of aromatic residues, and loss of unisite catalysis → unfolding.

Please note that these events do not include subunit dissociation, a very likely effect of GdnHCl on the structure of the oligomeric enzyme (*30*, *31*). This possible action of GdnHCl was not explored here.

Attempts To Determine the Number of GdnHCl Molecules That Bind to F1 at Low Denaturant Concentrations. An important point of the data described here is that concentrations of GdnHCl of <10 mM bring about strong inhibition of multisite ATP hydrolysis. The mechanism of action of denaturants has been the subject of numerous studies, and rather recently, calorimetric methods have been used to determine if there is a correlation between structural alterations and the number of bound denaturant molecules (*32*). Likewise, Wu and Wang (*33*) described formulations that allow an estimation of the number of urea molecules that bind to an enzyme from k_{cat}/K_m values of enzyme activity at different activities of denaturant. Following their rational and using the data of Table 1, we obtained a value of 6−7 bound denaturant molecules. Although the theoretical considerations of Wu and Wang need confirmation, it would appear that inhibition of multisite catalysis is due to the binding of a few denaturant molecules.

ATP Hydrolysis by F1 in a Medium with GdnHCl. With regard to the mechanisms that operate during multisite hydrolysis, it is relevant that at GdnHCl concentrations (<100 mM) that inhibit multisite catalysis, adenine nucleotide binding still takes place. These findings suggest that the primary effect of GdnHCl is on a locus (or loci) that is essential in the cooperative mechanisms involved in multisite catalysis. In fact, abolition of catalysis carried out by a single site requires GdnHCl concentrations much higher than 100 mM.

Agents that inhibit multisite, but not unisite, catalysis have been previously described. Azide is a well-known inhibitor of hydrolysis at high ATP concentrations, but it does not affect unisite catalysis (*34*−*36*). Dicyclohexylcarbodimide exerts a similar effect (*37*). In addition, several mutants of *E. coli* F1 have the ability to catalyze unisite, but not multisite, ATP hydrolysis (*38*−*41*). More recently, Le et al.

(*42*) studied a mutant F1 from *E. coli* in which Arg 376 of the α subunit was replaced with Ala or Lys. The rate of hydrolysis of the mutant F1 at high ATP concentrations was 3 orders of magnitude lower than that of the wild type, but unisite hydrolysis was largely unaffected. Moreover, in the mutant enzyme, the addition of high ATP concentrations did not induce the rapid breakdown of ATP that was at the high-affinity catalytic site; albeit, the mutant F1 bound adenine nucleotides. According to the authors, the mutation caused hindrances in the rotation that occurs during multisite ATP hydrolysis. Thus, it seems somewhat remarkable that the characteristics of the mutant F1 and that of F1 at low GdnHCl concentrations are nearly identical, even though they are the result of entirely different approaches.

According to the binding change mechanism, at a given time only one of the three sites catalyzes ATP synthesis or hydrolysis; the other two are involved in either substrate binding or product release. In this context, it has been suggested that only the site in its high-affinity state is catalytically active (*2*, *43*). Here we found that in the presence of GdnHCl only the high-affinity catalytic site is active; albeit, two other sites were capable of binding ATP. Accordingly, it would seem that the three sites of F1 that have been considered catalytic become truly catalytic only when they acquire the conformation of the high-affinity catalytic site.

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