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Vicinal Dithiol in Pig Heart Mitochondrial F_1-ATPase related to Thermal or ATP-Dependent Conformational Changes

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Abstract

Active F_1-ATPase prepared from pig heart mitochondria can react with about 2 mol of DTNB (5,5′-dithiobis-2-nitrobenzoic acid) or CPDS (6,6′-dithiodinicotinic acid). The reactivity of these thiol reagents decreases if ATP is absent or if F_1-ATPase has been submitted to thermal treatment that increases the specific activity without eliminating any contaminating protein. Affinity chromatography on a Sepharose-DTNB column has shown that the thermal treatment of F_1-ATPase induces a conformational change of the enzyme that completely prevents it from being retained on the column while the normal active enzyme can be specifically bound to the Sepharose-DTNB column.

A comparative study of the thiols of F_1-ATPase reacting with CPDS measured by spectrophotometric estimation of the thione released from CPDS and by [^{14}C]CPDS binding to F_1-ATPase suggests involvement of a vicinal dithiol in active F_1-ATPase. After CPDS reaction, this vicinal dithiol may become an internal disulfide bridge.

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Introduction

For a long time, it has been known that reagents of thiols inhibit ATP synthesis and energy recovery while they stimulate ATPase activity in transducing membranes (cf. Gautheron [1, 2]). In previous work, we have implicated very reactive thiols in the process of oxidative phosphorylation in pig heart mitochondria [3, 4]. The problem is to find out whether changes in titrable –SH reflect conformational aspects of the components of the ATP–synthase complex or neighboring units in the membrane, or if thiols are directly involved in the ATP synthetic or hydrolytic sites. Experimental data appear to rule out the presence of essential –SH groups at the ATP hydrolytic sites since isolated F_1- or CF_1-ATPase can be treated with inhibitors (*N*-ethylmaleimide, *p*-chloromercuribenzoate, DTNB [5,5′-dithiobis-(2-nitrobenzoic acid)], iodoacetate, etc.) without any change in ATPase activity [5–8]. However, this does not exclude the implication of thiol groups in the ATP synthesis site.

As early as 1960, Fluharty and Sanadi [9] suggested a role for a vicinal dithiol in the coupling mechanism of ATP–synthase, comparable to that of lipoic acid in oxidative decarboxylation of α-ketoacids. Experimental data confirm the direct participation of thiols in ATP synthesis both by oxidative [4] and by photosynthetic phosphorylation [10, 11] in intact membranes. The combined use of isotopically labelled CPDS (carboxypyridine disulfide or 6,6′-dithiodinicotinic acid) and spectrophotometric studies allowed us to detect vicinal dithiols involved in the coupling mechanism of pig heart mitochondria [12]. Among proteins implicated in the coupling mechanism, the ATP–synthase complex plays a central role. In the present work, we demonstrate that isolated active F_1-ATPase contains a vicinal dithiol whose reactivity with thiol reagents depends on the conformation of the enzyme.

Materials and Methods

Enzyme Preparation

Pig heart mitochondria were obtained at 0–4°C as previously described [13]. F_1-ATPase was prepared according to Senior and Brooks [14] as modified earlier [15]. The final heating step was omitted except when specified. Protein contents were estimated using the Lowry procedure [16]. Purity was determined by electrophoresis in sodium dodecyl sulfate with 10% polyacrylamide gels (Fig. 1) according to Catterall et al. [17]. ATPase activity was determined either by a spectrophotometric method, using pyruvate kinase and lactate dehydrogenase as auxiliary enzymes and measuring the rate of NADH disappearance according to Pullman et al.

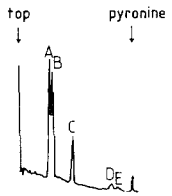

Figure 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of pig heart F_1-ATPase. The conditions were as described by Catterall et al. [17]; 47 µg F_1-ATPase was applied on top of the gels.

[18], or by a colorimetric method measuring inorganic phosphate release as previously described [15].

Thiol groups were estimated spectrophotometrically at 412 nm using DTNB [5,5′-dithiobis-(2-nitrobenzoic) acid] according to Ellman [19] or at 344 nm using CPDS following the procedure described by Merishi and Grassetti [20]. CPDS can react with thiols in two different ways (Fig. 2) [20]: If CPDS reacts with a single thiol (type A), a mixed disulfide is formed between this thiol and one CPDS moiety while one thione is released (6-mercaptonicotinic acid absorbing at 344 nm); if CPDS reacts with two vicinal thiols (type B), a mixed disulfide is first formed, as previously, but the second thiol may attack the mixed disulfide to release a second thione and form a new disulfide bridge between the two vicinal thiols in the protein. The number of thiones released indicates in both cases (types A and B) the number of reacting –SH, and can be measured spectrophotometrically. However, it should be noted that in type A mechanism, 1 mol of thione only is formed per mole of CPDS split, whereas in type B, the splitting of 1 mol of CPDS in the presence of a vicinal dithiol leads to 2 mol of free thione. We obtained exactly the latter stoichiometry with dithioerythritol. The same types of mechanisms have been proposed for analogs of CPDS such as 2,2′-dithiobis-[5-nitropyridine] [10] and 5,5′-dithiobis-[2-nitrobenzoate] [21]. If CPDS reacts through type A mechanism, it remains bound to the protein. In type B mechanism, on the contrary, no bound CPDS is found; [^{14}C]CPDS was therefore especially synthesized by the French CEA to detect the presence of vicinal dithiol. It was established that radioactive CPDS had the same reactivity and effects as unlabeled CPDS. To measure the binding of [^{14}C]CPDS, 0.5 mg F_1-ATPase was incubated in 0.2 ml TEA buffer (40 mM Tris-SO_4, 1 mM EDTA, 2 mM ATP, pH 7.5) for 15 min at 30°C in the presence of 1 mM [^{14}C]CPDS, 10 mCi/mmol. Then F_1-ATPase was separated from free CPDS by passage through a Sephadex G-25 column (0.8 × 30 cm) equilibrated with TEA buffer. Fractions were analyzed for radioactivity, protein content, and ATPase activity. It was confirmed that the specific activity of F_1-ATPase was

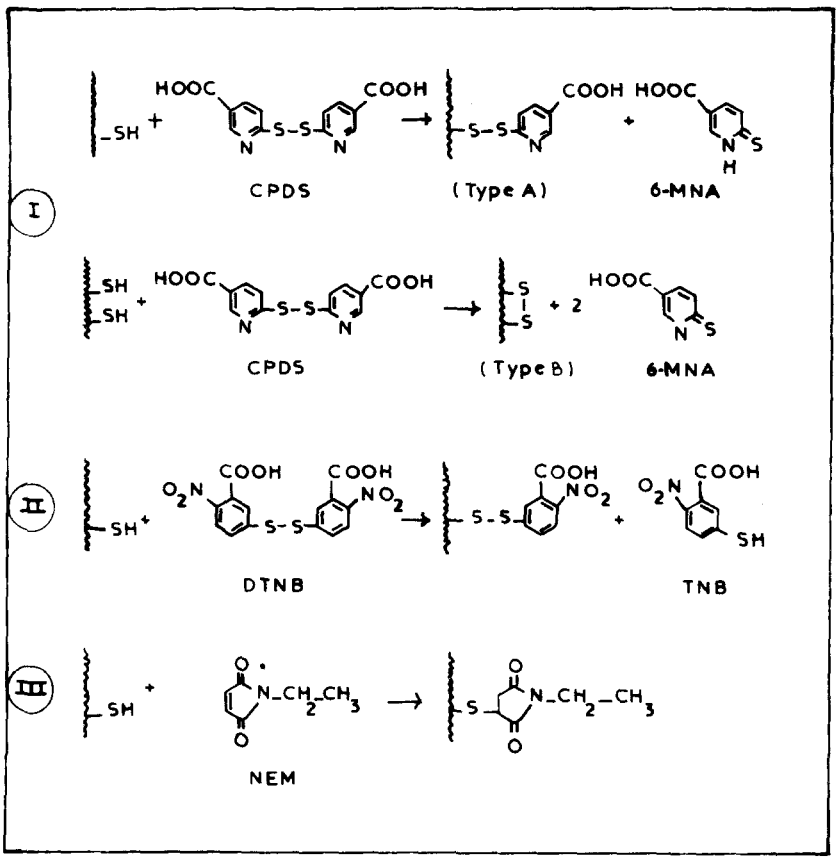

Figure 2. Reaction mechanisms of thiol reagents. CPDS=carboxypyridine disulfide; 6-MNA=6-mercaptonicotinic acid=thione; DTNB=5,5′-dithiobis-(2-nitrobenzoic) acid; TNB=5-thio-2-nitrobenzoic acid; NEM=*N*-ethylmaleimide.

not modified during the process. Specific radioactivity was determined directly in the fractions containing ATPase activity.

Binding of *N*-[^{3}H]ethylmaleimide to F_1-ATPase was measured by incubating the enzyme with TEA buffer containing 1 mM *N*-[^{3}H]ethylmaleimide (180–230 mCi/mmol) for 30 min at 30°C. Free *N*-ethylmaleimide was eliminated either by passage through a Sephadex G-25 column or by precipitating F_1-ATPase with 2.2 M $(NH_4)_2SO_4$, dissolving the pellet in TEA buffer, and repeating this precipitation twice. Addition of an excess of cysteine at the end of the incubation did not modify the number of titrated –SH groups. The radioactivity incorporated in F_1-ATPase was measured by liquid scintillation in Instagel or Bray solution.

Affinity Chromatography on Sepharose-DTNB Column

A Sepharose-DTNB column was prepared according to the procedure described by Lin and Foster [22]: Sepharose 4B was first coupled to 1,6-diaminohexane by the cyanogen bromide method modified by Cuatrecasas [23]. DTNB was then added to the Sepharose-1,6-diaminohexane suspension in the presence of a water-soluble carbodiimide: 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide HCl at pH 5.5. After extensive washing with 0.05 M phosphate buffer, pH 8.0, followed by molar KCl, the Sepharose-DTNB gel was packed in a column (8 ml packed gel in a 10-ml disposable plastic syringe) and equilibrated with TEA buffer. Just before use, the column was washed with TEA buffer containing 0.2 M KCl to eliminate any loosely bound DTNB or TNB until no material absorbing at 280 or 412 nm could be detected. The column was again equilibrated with TEA buffer. F_1-ATPase was applied to the gel and 2-ml fractions were collected. The enzyme was retained by the column. The enzyme not bound specifically by reaction with DTNB was eluted by TEA buffer containing 0.2 M KCl; specifically bound enzyme was recovered quantitatively by elution with TEA containing 0.2 M KCl and 1 mM β-mercaptoethanol. After use, the column was washed with TEA containing molar KCl and regenerated by elution with 0.04 M DTNB in TEA buffer and extensive washing with molar KCl-TEA. The regenerated column could be used in a manner similar to the original one.

Results

Titration of Thiol Groups in Pig Heart Mitochondrial F_1-ATPase

Kinetics. Figure 3 shows the kinetics of reaction of F_1-ATPase with CPDS or DTNB as measured spectrophotometrically; ATP is present in all assays. Whether the enzyme is fully active or inactivated by SDS treatment, it reacts quickly with both reagents. A plateau is reached in 2 min except when CPDS is added to the active enzyme, in which case the absorbance increase proceeds for about 5 min. In any case, after 10 min, no absorbance change can be detected. Therefore all subsequent determinations have been made 15 min after CPDS or DTNB addition.

Titration by DTNB. DTNB titrates one thiol per mole of normal active enzyme ("native"); the addition of ATP reveals one more thiol. After the thermal treatment, which stimulates by 30% the ATP hydrolytic activity (Table I), only 0.15 –SH is titrable by DTNB; ATP increases this value to 0.5. After dissociation by sodium dodecyl sulfate, which results in a loss of activity, no significant effect of ATP addition or thermal treatment can be detected, and about nine –SH per mole of F_1-ATPase (mol. wt. 391,000

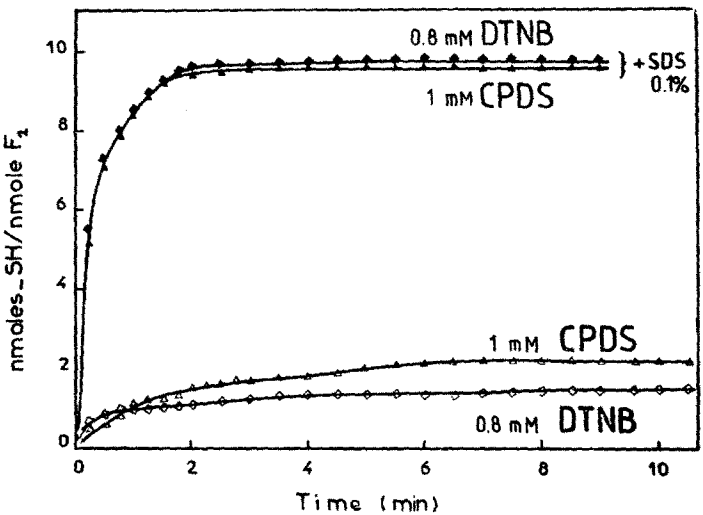

Figure 3. Kinetics of –SH titration of F_1-ATPase by CPDS or DTNB. F_1-ATPase, 0.22 mg, was incubated at 30°C in 0.5 ml 40 mM Tris-SO_4, 1 mM EDTA, 2 mM ATP, pH 7.5. At zero time, 100 µl 4 mM DTNB or 25 µl 20 mM CPDS was added and absorbance measured at 412 or 344 nm, respectively, as a function of time. Total –SH groups of inactive dissociated enzyme were determined after addition of 5 µl 10% sodium dodecyl sulfate (0.1% final concentration) by DTNB or CPDS titration. In the reference cuvette everything was identical but F_1-ATPase was missing. Moles of –SH reacting were calculated using 13.6×10^3 as molar extinction coefficient of released TNB at 412 nm and 10×10^3 as molar extinction coefficient of released thione at 344 nm. The molecular weight of pig heart F_1-ATPase was assumed to be 391,000 [15].

[15]) are measured by DTNB. Before studying the ATP effect, the enzyme was treated with charcoal to remove free nucleotides from the medium. No special attempt was made to extract the tightly bound nucleotides [24]. Charcoal treatment could be omitted without change in the –SH titration, if the assays were made in the presence of ATP. It must be remembered that reaction of active F_1 with thiol reagents did not modify ATPase activity.

Titration by CPDS. CPDS reacts in a way similar to that of DTNB with pig heart mitochondrial F_1-ATPase, active or dissociated by sodium dodecyl sulfate (Table II). However, in the presence of ATP the number of moles of CPDS reacting with the active enzyme is slightly greater than the number of moles of DTNB. This difference is greater after thermal treatment of F_1-ATPase: F_1-ATPase reacts then with 0.3 mol of CPDS in the absence of ATP and 1 mol of CPDS in the presence of ATP.

Binding of CPDS to F_1-ATPase was measured by passing F_1-ATPase after reaction with [^{14}C]CPDS through a Sephadex G-25 column and

TABLE I. Titration of F_1-ATPase thiol groups by DTNB [a]

Enzyme treatment	Moles –SH/mol F_1-ATPase			
	Active enzyme		SDS-dissociated enzyme inactive	
	No ATP	+2 mM ATP	No ATP	+2 mM ATP
No heating 92 units/mg protein	1.04 ± 0.14 (4)	1.97 ± 0.27 (11)	9.7 ± 0.7 (3)	9 ± 1.9 (3)
Thermal treatment 125 units/mg protein	0.14 ± 0.05 (3)	0.49 ± 0.04 (5)	8.1 ± 0.2 (3)	9.3 ± 1.6 (4)

[a] Means ± SEM; number of experiments in parentheses; molecular weight of F_1-ATPase 391,000 [15]. Heating F_1-ATPase was performed just before use as described in Fig. 5. Before titration of the enzyme, free nucleotides were removed by charcoal treatment; after centrifugation of the F_1 ammonium sulfate suspension, the protein precipitate (2–4 mg) was dissolved in 0.2–0.4 ml of 0.2 M phosphate (K) buffer, 1 mM EDTA, pH 7.5, and precipitated again by adding 0.45–0.9 ml saturated ammonium sulfate solution containing 1 mM EDTA, pH 7.5. After centrifugation, the pellets were dissolved in phosphate buffer (0.2–0.4 ml); 1–2 mg activated charcoal was mixed. The charcoal was removed by centrifugation and F_1 was again precipitated with ammonium sulfate and the pellet dissolved in 0.25–0.50 ml of 40 mM Tris-SO_4, 1 mM EDTA, ±2 mM ATP, pH 7.5. Charcoal treatment could be omitted without change in assays made in the presence of ATP. SDS-denatured enzyme was obtained by adding sodium dodecyl sulfate (0.1%) to the enzyme. Thiol groups were determined as in Fig. 3, 15 min after DTNB addition. SDS = sodium dodecyl sulfate.

counting the radioactivity associated with the protein. Results presented in Table II indicate that almost no [^{14}C]CPDS remained bound to active F_1-ATPase in the presence of ATP, before or after thermal treatment. No more [^{14}C]CPDS was bound in the absence of ATP. This result indicates that CPDS reacts with F_1-ATPase according to the type B mechanism, forming a disulfide bridge between two vicinal thiols.

Titration by Radioactive N-*[^{3}H]Ethylmaleimide.* *N*-Ethylmaleimide reacts with –SH groups to give an addition product linked by a covalent bond (Fig. 2). Contrary to DTNB or CPDS, this binding is not reversible. Table III shows that only around 0.3 mol of *N*-[^{3}H]ethylmaleimide binds to active pig heart mitochondrial F_1-ATPase whether or not a thermal treatment has been applied and in the presence or the absence of ATP or phosphate. Only a preliminary treatment with 1 mM CPDS + 2 mM ATP was able to lower this figure by about 40%. All attempts made to detect a whole number of moles of *N*-ethylmaleimide bound to F_1-ATPase either

TABLE II. Titration of F_1-ATPase thiol groups by CPDS [a]

	Moles –SH/mol F_1-ATPase				
	Native enzyme			SDS-dissociated enzyme	
	Reacting CPDS		Bound [^{14}C]-CPDS	Reacting CPDS	
Treatment	No ATP	+2 mM ATP	+2 mM ATP	No ATP	+2 mM ATP
No heating	0.97 ± 0.12 (7)	2.33 ± 0.19 (6)	0.12 ± 0.02 (3)	9 ± 0.3 (7)	10.3 ± 1.2 (3)
Thermal treatment	0.32 ± 0.11 (3)	0.98 ± 0.03 (3)	0.13 ± 0.04 (3)	8.9 ± 0.14 (4)	10.6 ± 0.8 (3)

[a] Reacting CPDS was determined by spectrophotometry of thione release at 344 nm (cf. experimental conditions in Fig. 3). Bound [^{14}C]CPDS was estimated as described in the Materials and Methods section. SDS=sodium dodecyl sulfate. Means ± SEM; the number of experiments is in parentheses; molecular weight of F_1-ATPase 391,000.

TABLE III. Binding of *N*-ethylmaleimide to F_1-ATPase [a]

Enzyme treatment	Mol *N*-[^{3}H]ethylmaleimide bound/mol F_1-ATPase
Thermal treatment +2 mM ATP	0.28 ± 0.03 (2)
No heating	
No ATP, no P_i	0.28 ± 0.02 (2)
+2 mM ATP	0.32 ± 0.04 (3)
+0.2 M P_i	0.31 ± 0.03 (2)
+2 mM ATP +1 mM CPDS	0.18 ± 0.02 (3)

[a] Binding of *N*-[^{3}H]ethylmaleimide to F_1-ATPase was measured as reported in the Materials and Methods section. The heated enzyme prepared as in Fig. 5. Enzyme with or without ATP treated as in Table I. Enzyme containing 0.2 M P_i was charcoal treated as described for enzyme without ATP but the final pellet was dissolved in 0.2 M phosphate (K) buffer, 5 mM EDTA, pH 7.5. Enzyme with CPDS and ATP was first reacted with 1 mM CPDS, 2 mM ATP for 15 min as in Table II; afterward, *N*-[^{3}H]ethylmaleimide was added and its binding determined. Means ± SEM; the number of experiments is in parentheses; molecular weight of F_1-ATPase 391,000.

by lengthening the time of reaction from 30 min to 3 h or by increasing *N*-ethylmaleimide concentration were unsuccessful as long as F_1-ATPase activity was maintained. However, sodium dodecyl sulfate-denatured enzyme bound 8 to 9 mol *N*-[^{3}H]ethylmaleimide/mol as observed with CPDS or DTNB.

If the active enzyme was first treated by 1 mM *N*-ethylmaleimide for half an hour, the number of moles of CPDS reacting (determined spectrophotometrically) was diminished by about 25%. Therefore, on one hand, reaction of CPDS diminishes the accessibility of SH groups to *N*-ethylmaleimide, and on the other, binding of *N*-ethylmaleimide lowers the reaction with CPDS. Treatment of sodium dodecyl sulfate-denatured enzyme with CPDS almost completely prevents subsequent binding of *N*-[^{3}H]ethylmaleimide: Only 23.4 cpm *N*-[^{3}H]ethylmaleimide was incorporated per microgram of denatured F_1-ATPase pretreated with CPDS whereas 1520 cpm/μg F_1-ATPase was counted when the SDS-denatured enzyme was directly labeled by *N*-[^{3}H]ethylmaleimide. This indicates that, when all –SH groups are exposed by unfolding of the peptide chains, CPDS and NEM react similarly.

Retention of F_1-ATPase by Affinity Chromatography on a Sepharose-DTNB Column

When F_1-ATPase has been heat-treated, DTNB reacts with less than one –SH per mole of F_1-ATPase (Table I). One could then wonder if the thermal treatment could not induce a conformational change of only a part of the enzyme. In other words, we proposed the hypothesis that F_1 could exist in at least two forms, one of them exhibiting –SH accessible to DTNB, the other having buried –SH or being in a more oxidized state. To test this hypothesis, an affinity column was prepared with DTNB bound to Sepharose 4B bearing hexamethylenediamine as a spacer arm. The results are shown in Figs. 4 and 5.

If the active unheated F_1-ATPase is applied to the Sepharose-DTNB column, it is completely retained by the column (Fig. 4). The small amount of material absorbing at 280 nm not retained upon addition of F_1-ATPase or eluted by further addition of 0.2 M KCl had no ATPase activity, and corresponds to TNB released from the Sepharose-DTNB beads. Indeed, reaction of F_1-ATPase with DTNB on the column induces the splitting of DTNB, and TNB is released in the medium. TNB is partly retained by the column probably by ion exchange and can be eluted by increasing ionic strength. However, F_1-ATPase is specifically bound to the column after its reaction with DTNB; it can only be eluted in the presence of β-mercaptoethanol which reduces the disulfide bridge formed between the enzyme and the column. Therefore the elution profile of material absorbing at

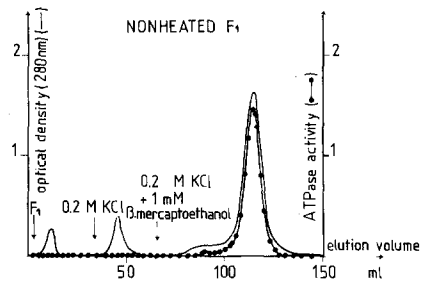

Figure 4. Chromatography on Sepharose-DTNB column of F_1-ATPase. Eight milliliters of packed Sepharose-DTNB column gel was prepared according to the procedure described in the Materials and Methods section, washed just before use with 0.2 M KCl to eliminate DTNB or TNB not covalently bound to the column, and then equilibrated with TEA buffer: 40 mM Tris-SO_4, 1 mM EDTA, 2 mM ATP, pH 7.5. F_1-ATPase (2.3 mg) dissolved in TEA buffer was applied on the column. After washing with 0.2 M KCl in TEA, F_1-ATPase was eluted by 0.2 M KCl, 1 mM β-mercaptoethanol in TEA. ATPase activity and optical density at 280 nm were followed in the eluate. About 95% of ATPase activity is finally recovered after β-mercaptoethanol addition (right peak).

280 nm corresponds exactly to that of ATPase activity. It was determined that F_1-ATPase could not be detached from the column by 1 M KCl in the absence of β-mercaptoethanol and that 0.2 M KCl was necessary to elute quickly the enzyme in the presence of β-mercaptoethanol. It was concluded that the column has ion-exchange capacity besides its affinity property. The ion-exchange capacity becomes insignificant for F_1-ATPase in the presence of 0.2 M KCl.

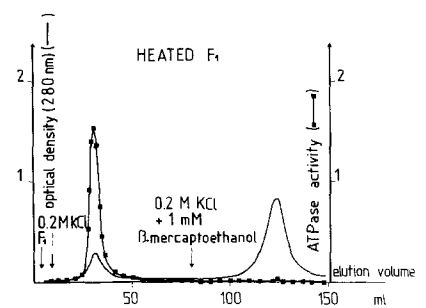

Figure 5. Chromatography on Sepharose-DTNB column of F_1-ATPase after thermal treatment. Conditions of chromatography are the same as in Fig. 4, except that F_1-ATPase was heat-treated before the chromatography: F_1-ATPase (10 mg protein/ml 40 mM Tris-SO_4, 1 mM EDTA, 4 mM ATP pH 7.4) was heated 2 min at 65°C in a water bath. After centrifugation (12,000 *g* for 4 min) at room temperature, ammonium sulfate was added to the soluble fraction to give a final concentration of 2.1 M. The ammonium sulfate precipitate was kept at 0–4°C until use; 1.5 mg heated F_1-ATPase was applied on the column. About 92% of activity is recovered after 0.2 M KCl elution (left peak).

Figure 5 shows the elution profile of F_1-ATPase applied to the column after thermal treatment. In contrast to unheated F_1-ATPase (Fig. 4) the enzyme was not specifically retained by its –SH groups since 0.2 M KCl completely removed the enzyme from the column. Further addition of β-mercaptoethanol does not elute any ATPase activity. Therefore, after thermal treatment, there are no additional –SH groups located on the surface of the enzyme. Those –SH that were reachable before this treatment have been completely buried or oxidized. There is no equilibrium detectable between the two forms of enzyme but the enzyme exists in one or the other form. In both cases the enzymatic activity was completely recovered.

One could argue that the thermal treatment removes a protein bound to F_1-ATPase which contains a very reactive –SH group. However, the sodium dodecyl sulfate gel electrophoresis pattern obtained after thermal treatment of F_1-ATPase was identical to that of the normal unheated enzyme (Fig. 1).

Discussion—Conclusion

Reactivity of Cysteine Residues of Pig Heart Mitochondrial F_1-ATPase toward Thiol Reagents

After dissociation of pig heart mitochondrial F_1-ATPase by sodium dodecyl sulfate, CPDS, DTNB, or *N*-ethylmaleimide detects about nine cysteine residues per mole of inactive enzyme, assuming a molecular weight of 391,000 [15]. The presence of ATP or the thermal treatment of the enzyme does not change this figure significantly. Senior [5] found eight free –SH groups and two disulfide bonds per mole of beef heart F_1-ATPase using 360,000 as molecular weight [26, 27]. Recalculation of the total –SH groups per mole of pig heart mitochondrial F_1-ATPase on the basis of molecular weight 360,000 would give 8.6 instead of 9.2 (average of the 30 determinations made under all the various conditions). Our figure is therefore slightly higher than the value of Senior. This difference could come either from the origin or from the method of preparation of the enzyme, although Senior's preparative procedure is similar to ours. The pig heart F_1-ATPase studied may be in a more reduced state than the beef heart one.

In the active or "native" enzyme (unheated and not treated by sodium dodecyl sulfate) two sulfhydryl groups per mole were freely accessible for reaction with DTNB on beef heart F_1-ATPase as noticed by Senior [5]. The striking difference, however, is that we titrate these two –SH only in the presence of ATP. When ATP is absent, only one –SH is accessible to DTNB

or CPDS, whereas Senior did not observe any effect of ATP on the DTNB-titrable –SH. It should be remembered that to observe this unmasking by ATP of one more –SH, a preliminary charcoal treatment was applied to pig heart F_1-ATPase to eliminate any loosely bound nucleotides. The observed ATP effect on the titrable –SH can be related either to a conformational change or to a change in the redox equilibrium of thiols.

The thermal treatment widely used to increase hydrolytic activity of F_1-ATPase markedly decreases the thiols available for DTNB or CPDS estimation, 0.14 and 0.32 per mole of F_1, respectively, in the absence of ATP; however, the ATP-induced increase in titrable –SH is partially maintained since the levels rise to 0.49 in the presence of DTNB and to 0.92 with CPDS. It could be invoked that F_1-ATPase exists in two conformations and that the thermal treatment or the addition of ATP could modify the distribution between these forms in one direction or the other. The two forms could differ in the affinity or accessibility of their –SH groups to thiol reagents, or by the degree of oxidation of their thiols.

When F_1-ATPase was passed over a column of DTNB linked through a hexamethylene spacer arm to a Sepharose matrix, no such interconversion of the two forms could be detected: The Sepharose-DTNB retained the "native" unheated enzyme only and not the heat-treated one at all. This can be easily explained by the estimation experiments (Table I) which indicate that at least one –SH is no longer available for DTNB, in F_1-ATPase, after thermal treatment. This thiol must be located in a polar region of native F_1-ATPase readily accessible to DTNB, and is exposed by ATP.

It is clear that the DTNB column recognizes only this thiol in F_1-ATPase, but this does not exclude the existence of some microheterogeneity similar to that detected by Adolfsen et al. [28] in CF_1-ATPase. The fact that ATP exposes more than one thiol titrable by CPDS in "native" unheated F_1, and the fact that CPDS, DTNB, or *N*-ethylmaleimide can titrate fractional numbers of thiols, especially in heat-treated enzyme, are in favor of this microheterogeneity. This microheterogeneity must involve –SH different from that responsible for the retention of F_1-ATPase on the Sepharose-DTNB column.

The differences in the reactivity of F_1-ATPase toward various –SH reagents yield further information: *N*-ethylmaleimide that readily reaches nonpolar regions, binds very poorly to the enzyme, and never titrates more than 0.3 –SH per mole, whether or not ATP is present. CPDS, which is strongly negatively charged and should preferentially remain in polar regions, reacts with the maximum number of –SH and detects the ATP-sensitive thiols, even after thermal treatment of the enzyme. Finally, DTNB is attracted to polar regions through its carboxyl but to a lesser extent than

CPDS since the *o*-NO_2 affects the dissociation of the carboxyl, and moreover brings both additional steric hindrance and attraction to nonpolar groups. The fact that ATP-sensitive –SH groups are easily and better reached by CPDS than by DTNB but cannot react with *N*-ethylmaleimide (which usually readily reacts with reactive thiols) suggests for these –SH a location in a buried polar region of pig heart F_1-ATPase.

The lack of –SH reactivity observed after thermal treatment might be correlated with the loss of cooperativity between adenine nucleotide sites previously observed [6].

Intramolecular Vicinal Dithiol in Pig Heart Mitochondrial F_1-ATPase

The results presented in Table II have shown that, although CPDS is the most reactive thiol reagent that we have tried, [^{14}C]CPDS binding to pig heart mitochondrial F_1-ATPase is barely detectable. This can be easily understood by examining the mechanisms by which CPDS can react with protein –SH groups (Fig. 2). In the type B mechanism, for which a vicinal dithiol must be present in the protein, CPDS induces the formation of an intramolecular disulfide bridge from this vicinal dithiol. We can therefore conclude that a vicinal dithiol was present in pig heart mitochondrial F_1-ATPase. In the absence of ATP, no additional radioactive CPDS was bound to the enzyme. This indicates that the disulfide bridge was also formed in the absence of ATP. Since the total number of –SH groups reacting with CPDS was higher in the presence of ATP, this increased value means that ATP either renders these –SH groups more readily accessible to CPDS or shifts the equilibrium between S–S bridges and –SH groups in the protein on the more reduced side.

One can wonder what role this vicinal dithiol plays in the mechanism of oxidative phosphorylation. Experiments are in progress to determine it. But it is tempting to correlate this result with the recent demonstration of the presence of vicinal dithiols in a strategic position for the coupling mechanism of oxidative phosphorylation [12] and the phosphate transport linked to ATP synthesis [29] and with the recent findings of Griffiths on the possible role of lipoic acid in oxidative phosphorylation [30].

It has been shown that the molecular properties of ATPases purified from mitochondria or chloroplasts are very similar [31, 32]. Although differences exist between the thiol reactivity of chloroplast CF_1-ATPase and mitochondrial F_1-ATPase, CF_1 also contains eight free sulfhydryl groups and two disulfide bridges [8]; also, Vallejos and Andreo [10, 33] have proved that an analog of CPDS, 2,2′-dithiobis(5-nitropyridine), can oxidize a vicinal dithiol exposed by a light-induced conformational change of membrane-bound CF_1 and at the same time inhibits ATP synthesis by

chloroplasts. The authors suggest that the inhibition by this thiol reagent, the mechanism of which is similar to that of CPDS, is related to the oxidation of chloroplast vicinal dithiols probably exposed by a light-induced conformational change.

If the mechanism of ATP synthesis in chloroplasts and mitochondria is of the same type, as suggested by more and more evidence, it is very likely that the sulfhydryl groups involved in photophosphorylation are of the same nature as those involved in oxidative phosphorylation.

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