Molecular Polymorphism and Mechanisms of Activation and Deactivation of the Hydrolytic Function of the Coupling Factor of Oxidative Phosphorylation†

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ABSTRACT: The 13S coupling factor of oxidative phosphorylation from *Alcaligenes faecalis* has a latent adenosine triphosphatase (ATPase) function that can be activated by heating at 55 °C for 10 min at pH 8.5 in 50% glycerol. The specific activity increases from 0.1 to 20–30 μmol min^{-1} mg^{-1}. Adenosine 5′-triphosphate (ATP) is not required for stabilization at 55 °C when glycerol is present. Activation involves displacement of the endogenous ATPase inhibitor subunit (ϵ subunit), and readdition of this subunit results in deactivation. In the deactivation process the ATPase inhibitor subunit can be replaced by other cationic proteins such as protamine, histones, or poly(lysine). Mg^{2+} and H^+ also are effective deactivators. The fact that every positively charged substance tested deactivated the enzyme suggests that the inhibitor subunit is complexed with the enzyme at a site containing a surplus of negative charges. The activated enzyme is not cold labile, but it is salt labile, having a half-life of 2–3 min in 0.1 M KI at either 25 or 0 °C. The activated ATPase is also inhibited by aurovertin, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD), and by the cross-linking agent dimethyl suberimidate. Evidence for polymorphism comes from finding that the properties of the unactivated enzyme (intrinsic ATPase) are different in many ways from the properties of activated ATPase. With respect to the coupling factor's ability to hydrolyze ATP, the data in this study suggest that there are at least four distinct functional allomorphs of this enzyme: (1) the latent enzyme, which has no kinetically measurable ATPase activity, (2) intrinsic ATPase, which is catalyzed by a small percentage of the molecular population that has been activated by some natural mechanism, (3) activated ATPase, which has properties different from those of intrinsic ATPase, and (4) aged activated ATPase, in which some of the properties (K_m for substrate, sensitivity to deactivation by Mg^{2+} and H^+) spontaneously change within 30 min.

The coupling factor of oxidative phosphorylation is a polymorphic enzyme (Moudrianakis and Adolfsen, 1975). *Structural polymorphism* of HLF[1] (and also of F_1 and CF_1) was the subject of a previous publication (Adolfsen et al., 1975). Five electrophoretically distinct species of HLF were found. Since polyacrylamide gel electrophoresis resolves proteins on the basis of their size and charge, each of these species must be slightly different in terms of its size and/or charge. Sodium dodecyl sulfate gel electrophoresis showed that each species contained four classes of subunits (α, β, γ, and ϵ). This suggested that all of the allomorphs had the same molecular weight. Therefore, the differences between them could be due to differences either in the three dimensional arrangement of subunits or in the tertiary structure of the individual subunits. Conceptually, such differences could easily give rise to species having different surface charge distributions and/or different molecular volumes. The allomorphs were also *interconvertible* by treatment with ATP, by aging, or by exposure to phosphate buffer during the isolation procedure. The interconvertibility of species is referred to as *polymorphic flux*.

HLF also exhibits *functional polymorphism* (Adolfsen and Moudrianakis, 1976). By this we mean that the enzyme population consists of a number of different allomorphs, the presence of which can be detected by some functional assay—for example, nucleotide binding. One species of HLF did not bind any nucleotide (ADP or ATP), another bound nucleotide very slowly (turnover time 1–2 h), and a third had sites available for very rapid interaction with nucleotide. This last species is responsible for the very low level of ATPase activity (about 0.1 μmol min^{-1} mg^{-1}) that is catalyzed by an enzyme preparation that hs not been subjected to any treatment that would "activate" the hydrolytic function. Since the enzyme preparations did not exhibit a rapidly equilibrating site, even during equilibrium nucleotide binding studies, we concluded that the allomorph responsible for the low ATPase activity was present in very small amounts in the enzyme population. As in the case of structural polymorphism, there was also some indication that these functionally different species were interconvertible.

The low level of ATPase activity catalyzed by the enzyme preparation was termed "intrinsic" ATPase, since it was manifest without any special treatment that would result in activation. Most investigators in this field of research prefer to study the properties of an enzyme that has been activated either by heat treatment, treatment with a reducing agent, or trypsinization. We felt that it was important to study the indigenous or intrinsic level of activity because of the possibility that the harsh treatments used to activate the enzyme might alter basic catalytic properties and give rise to kinetic anomalies (Adolfsen and Moudrianakis, 1973). The data in the present paper demonstrate that this is no longer a speculative possibility: heat activation does indeed give rise to changes in the kinetic properties of the enzyme. This means that any

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[1] Abbreviations used are: HLF, heat-labile factor, the 13S coupling factor from *Alcaligenes faecalis;* F_1, the 13S coupling factor from beef heart mitochondria; CF_1, the 13S coupling factor from spinach chloroplasts; NBD-Cl, 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole chloride; Mes, 2-(*N*-morpholino)ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; AMP, ADP, ATP, adenosine mono-, di-, and triphosphate; ATPase, adenosine triphosphatase; P_i, inorganic phosphate; $HClO_4$, perchloric acid.

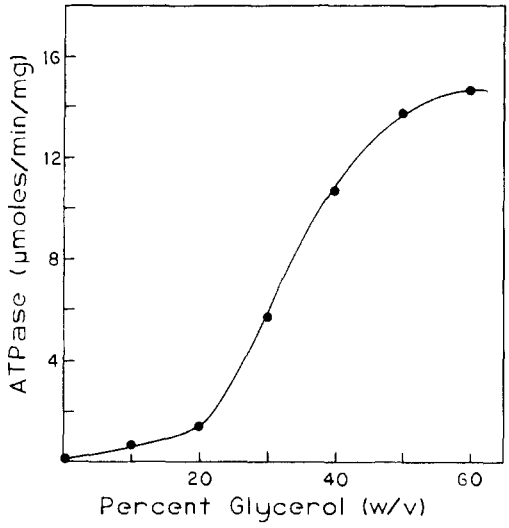

FIGURE 1: Effect of glycerol on heat activation of ATPase. HLF was incubated at 0.1 mg/ml in 10 mM Tris (pH 8.5) plus the indicated concentration of glycerol for 5 min at 60°C, after which ATPase activity was measured as described under Methods.

studies that are performed on activated ATPase are subject to restrictive interpretations, and that generalizations as to how the enzyme functions when it is membrane-bound and in vivo should not be made. The value of studies on the activated HLF is, at this point, solely to learn about the protein chemistry of the active site and the details of the chemical mechanism by which ATP is hydrolyzed. Subsequent experiments on intrinsic ATPase or the ATpase activity of the membrane-bound enzyme may yield the information necessary to deduce how ATP is utilized as a source of energy for various cellular processes in vivo.

This paper has four themes: the first is mechanisms of activation and deactivation of the hydrolytic function; the second is comparison of the properties of intrinsic ATPase and activated ATPase; the third is functional polymorphism of the activated ATPase; the fourth is comparison of the properties of HLF ATPase to those of other ATPases, especially F_1, to further demonstrate how similar these types of enzymes are.

A note on terminology: we will use the term "heat activation" or simply "activation" to refer to the effect of incubating the enzyme at high temperature prior to commencing the assay. This is distinct and separate from "kinetic activation", which refers to the essentially instantaneous activation by ions and/or other effectors that occurs in the reaction mixture in the course of the assay. The term "deactivation" will be used to describe a reduction in the ATPase function (either intrinsic or activated) that occurs when the enzyme is preincubated under specified conditions. This term will be applied only when the loss of activity occurs without dissociation of the enzye into subunits. Loss of activity due to dissociation into subunits is simply "lability." The term "inhibition" will be used to refer to a decrease in the kinetic parameters of the enzyme caused by changes in the composition of the assay medium.

Methods and Materials

HLF was isolated by the previously described procedure (Adolfsen et al., 1975), which gave type IB HLF (the most native electrophoretic species) with small amounts of type IA enzyme. Occasionally, small amounts of type IIB also were present.

The hydrolytic function was activated by incubating HLF at a concentration of 0.10 mg/ml in 10 mM Tris or triethanolamine buffer at pH 8.5, containing 50% glycerol (w/v), at 55 °C for 10 min (or 60 °C for 5 min), after which the enzyme was placed on ice.

ATPase activity was assayed by incubating 2 μg of enzyme for 1 min at 37 °C in 0.50 ml of reaction mixture containing 1 mM ATP, about 50 000 cpm of [γ-^{32}P]ATP, 0.3 mM KCl, and 10 mM Tris (pH 8.5). Substrate hydrolysis was between 5 and 10%. The reaction was terminated by adding 0.5 ml of 10% $HClO_4$, which was followed by 1 ml of 5% $HClO_4$ containing 25 mg/ml of Norit A to adsorb nucleotide. The charcoal was removed by suction filtration through a glass fiber filter, and the filtrate (containing the product [^{32}P]P_i) was collected in a scintillation vial. The reaction vessel was washed with two successive 2-ml volumes of 1% $HClO_4$ to ensure quantitative transfer of radioactivity, and Čerenkov radiation was measured in a Packard Model 2002 liquid scintillation spectrometer at 65% gain.

Intrinsic ATPase was measured by incubating 2 μg of HLF for 20 min at 37 °C in 0.50 ml of reaction mixture containing 0.10 mM ATP, about 50 000 cpm of [γ-^{32}P]ATP, 0.10 mM $MgCl_2$, 30 mM KCl, and 10 mM Tris (pH 8.5). The rest of the procedure was the same as described above.

Electrophoresis in 5% polyacrylamide in the Hepes–imidazole buffer system was carried out as previously described (Adolfsen et al., 1975). Sodium dodecyl sulfate gel electrophoresis was carried out according to Weber and Osborn (1969).

Materials. Aurovertin was isolated by Dr. John Barnes of this laboratory, using the method described by Chang and Penefsky (1974). Reagent [γ-^{32}P]ATP was synthesized by the method of Weiss et al. (1968), using [^{32}P]P_i obtained from New England Nuclear Corp. Dimethyl suberimidate was from Pierce Chemical Co. Buffers and other reagents were obtained from Sigma Chemical Co.

Results

Heat Activation of HLF ATPase. Heat activation of coupling factor ATPase is usually optimized by having a high concentration of ATP present to prevent heat denaturation. About 30 mM was optimal for CF_1 (Farron and Racker, 1970). However, this procedure brought the activity of HLF up to only about 1 μmol min^{-1} mg^{-1} (Adolfsen and Moudrianakis, 1971b). During attempts to improve the activation, we found that glycerol was an effective stabilizing agent for HLF (Figure 1) with a maximal activity evident at 50–60% (w/v). Glycerol had no effect on activity unless the enzyme was incubated at high temperatures. Since glycerol can bind to proteins (Detrich et al., 1976), and since higher concentrations of glycerol can induce conformational changes in a variety of proteins (Myers and Jakoby, 1975), it was important to determine whether the glycerol in the reaction mixture (carried over with the enzyme from the preincubation step) had any effect on initial velocity. We found that the initial velocity of ATP hydrolysis by heat-activated HLF was not affected by glycerol when the concentration of glycerol in the assay medium did not exceed 25%; higher glycerol concentrations were inhibitory. In all the assays reported in the present study the only glycerol present was that carried together with the enzyme from the heat-activation pretreatment; this amounted to a final glycerol concentration of 2–4% in the assay mixture. Thus, it is clear that the effect shown in Figure 1 is due entirely to the stabilizing effect of glycerol on the enzyme at elevated temperature and cannot be attributed to any kinetic effects of glycerol on the assay.

Time courses of activation at various temperatures are

TABLE I: Polymorphic Flux of Activated ATPase.[a]

Preincubation at Room Temp (min)	Sp Act. (μmol min^{-1} mg^{-1})	% Decrease	Apparent V_m	Apparent K_m
0	18.4	0	29	0.41
30	13.4	27	19	0.35
90	9.0	51	13	0.29

[a] The enzyme was heat activated for 5 min at 60 °C as described under Methods, after which substrate saturations were performed with 0, 30, or 90 min of preincubation at room temperature. $MgCl_2$ and ATP were varied at a constant ratio of 1:3, which was the optimal ratio. The kinetic constants were determined from double-reciprocal plots.

shown in Figure 2A. The highest level of activation was reached after 10 min at 55 °C, and there was no significant heat denaturation for at least 15 min at this temperature. Figure 2B is an Arrhenius plot of the initial velocity of heat activation. A discontinuity occurred at 50 °C. Below 50 °C the activation energy was 47 kcal, while above this temperature it decreased to 25 kcal.

Attempts to further optimize activation resulted in the following findings: maximal activation occurred between pH 8 and 9 (measured at 25 °C), and it declined sharply below pH 7.5 and above pH 9.0. Activation was greatest at 0.10 mg/ml of enzyme protein; specific activities were about 50% lower when the enzyme was 1.0 or 0.01 mg/ml. Supplementing the incubation mixture with 1 mM ATP had no effect, 1 mM ADP inhibited activation by 40%, 1 mM AMP had no effect, and 1 mM P_i inhibited activation by 40%. Inclusion of 1 mM $MgCl_2$ inhibited activation by 50%, while 1 mM EDTA gave a small and variable stimulation (usually about 10%). Increasing the ionic strength by adding KCl to 0.1 M resulted in a 40% inhibition of activation. Dithiothreitol (1 or 10 mM) had no effect, 0.1% Triton X-100 had no effect, 0.5 M urea inhibited by 50%, and 2% ethanol inhibited 100%. Polyacrylamide gel electrophoresis showed that ethanol caused a very rapid dissociation of the enzyme into subunits at elevated temperatures.

Stability of Activated ATPase. The activated enzyme exhibited a partial lability that was dependent upon the temperature to which it was exposed subsequent to its activation. When kept at 0 °C, there was no significant change in activity for at least 1 h. At 25 °C (room temperature), the activity decreased by about 30% in the first 30 min and by about 50% within 2 h. The remaining activity was stabe for at least 2 days longer at room temperature. The partial lability was accelerated by incubation at 37 °C, with about 50% loss of activity in 30 min.

This partial lability was not due simply to denaturation of the activated enzyme. If it were, the apparent V_m would decrease while the apparent K_m remained constant. The data in Table I show that the K_m decreased by 15% after 30 min and by 30% after 90 min. These data indicate that there are at least two forms of activated ATPase, which may be distinguished by their K_m values. Their interconversion is another instance of polymorphic flux. Other properties of the activated enzyme also change during aging (see below).

As part of a search for further evidence of polymorphism of activated ATPase, the kinetic parameters K_m and V_m were determined during a time course of activation at 60 °C similar

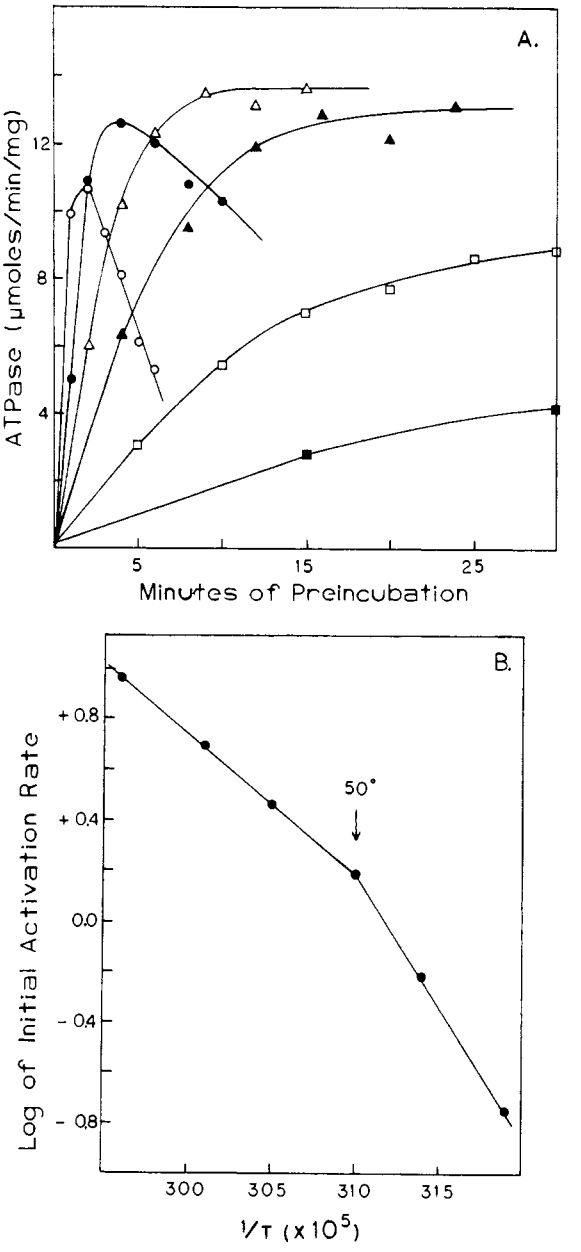

FIGURE 2: Effect of temperature on heat activation of ATPase. (A) Time courses of activation were carried out with HLF at 0.1 mg/ml in 10 mM Tris (pH 8.5) and 50% (w/v) glycerol at the indicated temperatures: (■) 40 °C; (□) 45 °C; (▲) 50 °C; (△) 55 °C; (●) 60 °C; (○) 65 °C. (B) Arrhenius plot of initial velocity of heat activation.

to the one illustrated in Figure 2A. The K_m was constant from 1 to 10 min of incubation at 60 °C, with the only variation being in the V_m. The K_m was also constant when the enzyme was activated for 30 min at 45 °C, for 20 min at 50 °C or for 10 min at 55 °C. Thus, the process of activation can be described as a simple two-state conversion—unactivated to activated.

The activated enzyme is *not* cold labile as long as the incubation medium does not contain significant amounts of salt. This may also be true of F_1 to some extent, although the importance of salts is not usually stressed. Figure 3 shows the lability of heat-activated HLF in 0.1 M KI. The half-life was 2–3 min, and temperature did not have a significant effect on it. Because of these results, we will refer to this property as "salt lability" instead of cold lability.

The mechanism of salt lability is the same as that usually described for cold lability. Polyacrylamide gel electrophoresis

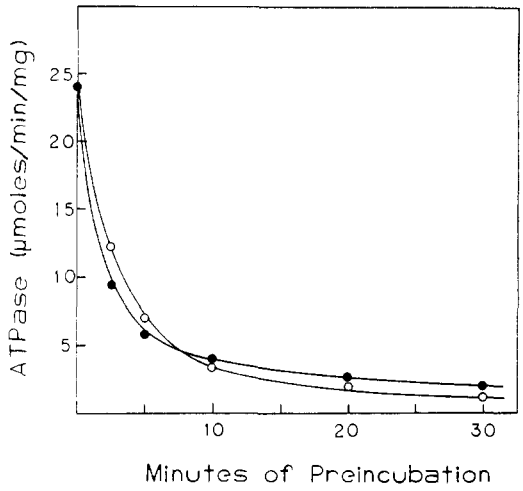

FIGURE 3: Salt lability of activated ATPase. The activated enzyme was incubated for the indicated length of time in 0.1 M KI and assayed. The protein concentration was 0.01 mg/ml and glycerol was 5%. (○) 0 °C; (●) 25 °C.

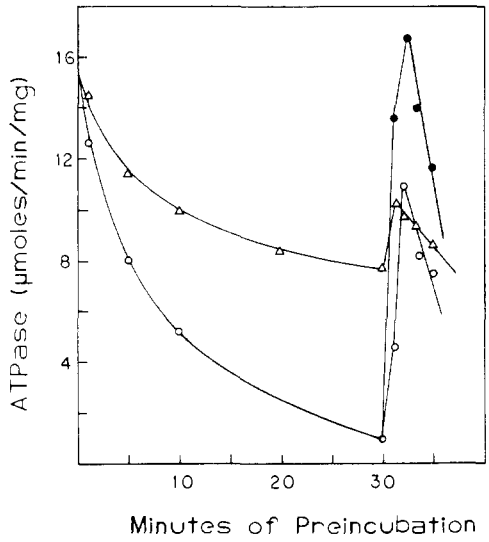

FIGURE 4: Reversibility of deactivation of ATPase by Mg^{2+}. Freshly activated enzyme was incubated at 37 °C with or without 1 mM $MgCl_2$. At 30 min, the temperature was changed to 60 °C. (△) control system without $MgCl_2$; (○) system with $MgCl_2$; (●) system with $MgCl_2$ that was made 1 mM in EDTA just before changing the incubation temperature. ATPase activity was measured as described under Methods.

showed that KI acted by promoting dissociation of the enzyme into subunits. KI also promoted dissociation of the unactivated enzyme into subunits, though at a much slower rate than for the activated enzyme.

Salt lability is also important when ATPase activity is measured in reaction mixtures containing 30 mM KCl (or higher). The half-life of activated enzyme preincubated in unlabeled reaction mixture and then sampled for subsequent assay in labeled reaction mixture was about 7 min. This is quite different from the results obtained with intrinsic ATPase, where preincubation in 30 mM KCl had no significant effect on activity and where time courses were linear for at least 20 min of incubation at 37 °C (10% hydrolysis of substrate).

In addition to its role during heat activation, glycerol also plays a role in stabilizing the activated enzyme. When the activated enzyme was diluted tenfold in 10 mM Tris (pH 8.5) the partial lability that occurred at room temperature more than doubled. However, this additional lability did not occur when the dilution was performed in buffer containing 50% glycerol. Glycerol also reduced the salt lability, increasing the half-life in 0.1 M KI from 2 min to more than 10 min.

TABLE II: ATPase Inhibitor Subunit of HLF.[a]

Preincubation (min)	% Deactivation by a Given Molar Excess of Inhibitor		
	2.5×	5×	10×
3	16	76	91
10	35	83	91
20	55	81	85

[a] Freshly activated HLF was mixed with the supernatant fraction of heat-denatured HLF (prepared as described in text) and incubated for the indicated length of time at room temperature before being assayed for ATPase activity. The percent deactivation was computed with respect to a system incubated with the same solvent that the ATPase inhibitor preparation contained. This compensated for lability of ATPase activity due to addition of some KCl to the activated ATPase along with the inhibitor. The molar excess of ATPase inhibitor was computed assuming complete release during heat treatment.

ATPase Inhibitor Subunit of HLF. The ATPase inhibitor subunit of F_1 can be released from the enzyme by heat treatment (Warshaw et al., 1968). To determine if HLF contained an inhibitor subunit, the enzyme was incubated for 2 min at 100 °C at 1 mg/ml in the presence of 0.1 M KCl. KCl promoted coagulation of the denatured large polypeptides, which facilitated their removal by centrifugation. The supernatant fraction contained less than 10% of the total enzyme protein. Sodium dodecyl sulfate gel electrophoresis showed only one polypeptide present in large amounts. This was the small one having a molecular weight of about 12 000 (ε subunit). The experiment shown in Table II demonstrates that this supernatant fraction also contained an ATPase inhibitor. If we assume complete release and recovery of the inhibitor by this procedure, then the system containing undiluted extract had a tenfold molar excess of inhibitor over activated ATPase. The results show that the more dilute inhibitor solution was effective if longer preincubation was permitted before assay. The ATPase inhibitor in the heat extract was also excluded from a Bio-Gel P-6 column, which indicated that it is a macromolecule. Taken together, these facts indicate that the small polypeptide subunit of HLF is the ATPase inhibitor subunit and that it is similar to those reported in F_1 and CF_1 (Pullman and Monroy, 1963; Horstman and Racker, 1970; Brooks and Senior, 1971; Nelson et al., 1972). An interesting question (which we cannot answer at present) is whether there is 1 or more than 1 ATPase inhibitor subunit/molecule of HLF.

Deactivation of ATPase. When F_1 is incubated with Mg^{2+}, ATPase activity is rapidly lost (Caterall and Pedersen, 1972). When HLF was incubated with 1 mM Mg^{2+} for 10 min at 37 °C, a 60% decrease in activity was observed. Similar degrees of deactivation were found with 1 mM Ca^{2+}, Mn^{2+}, Zn^{2+}, Cd^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, Ba^{2+}, or Sr^{2+}. The deactivation by Mg^{2+} was completely prevented by having equimolar amounts of ATP present to chelate the cation. Polyacrylamide gels showed that this deactivation did not involve dissociation of the enzyme into subunits.

Mg^{2+} deactivation is reversible (Figure 4). A second heat treatment step resulted in recovery of all of the lost activity. The extent of reactivation was higher when EDTA was added before heating. A control system incubated with EDTA at 37

TABLE III: Deactivation of ATPase by Polycationic Substances.[a]

System	Sp Act.	% Deactivation
Control	14.0	
Spermine	6.7	53
Spermidine	10.4	26
Histones	12.4	12
Cytochrome *c*	11.4	18
Protamine	3.9	73
Polylysine:		
mol wt 2 000	6.5	54
15 000	6.1	57
30 000	4.9	65
85 000	3.8	74

[a] HLF (100 μg/ml) was activated for 10 min at 55 °C, cooled to room temperature, and incubated with each of the indicated cationic materials (50 μg/ml) for 10 min, after which specific activity (μmoles min^{-1} mg^{-1}) was determined as described under Methods.

°C showed only a threefold increase of activity in 60 min (not shown). This result suggests that temperature is more important than EDTA with respect to reactivation. In another experiment, the free Mg^{2+} was removed by passing the enzyme through a Bio-Gel A 0.5 column. The ATPase activity did not increase significantly, but full reactivation was obtained by heating at 60 °C for 2 min. EDTA had no significant effct on this reactivation, indicating that the stimulation by EDTA in the experiment shown in Figure 4 was due to removal of free Mg^{2+} and not due to interaction of EDTA with the enzyme. This conclusion is supported by the finding (reported above) that 1 mM $MgCl_2$ inhibited heat activation of ATPase by nearly 50%.

Another method of deactivation was incubation at a lower pH. When Mes buffer at pH 6.0 was added to a final concentration of 20 mM to give a final pH of 7.0, ATPase activity decayed with a half-life of about 3 min, with about 90% deactivation being attained after 15 min of incubation at 37 °C. Polyacrylamide gel electrophoresis showed once again that the deactivation did not involve dissociation of the enzyme into subunits. Partial reactivation was obtained by raising the pH back up to 8 and heating for 2 min at 60 °C.

The three types of deactivation have different characteristics. Mg^{2+} deactivation was prevented by chelating agents such as EDTA or ATP, whereas deactivation by adding H^+ or ATPase inhibitor subunit was not affected. Also, freshly activated enzyme was almost totally deactivated by adding Mg^{2+} or H^+, but after aging for 1 h at room temperature Mg^{2+} deactivated only about 50%, and adding H^+ gave only about 40% deactivation. In contrast, aging had no effect at all on the ability of the enzyme to be deactivated by the ATPase inhibitor. This deactivation was nearly complete, even after aging for 1 day at 25 °C.

The properties of intrinsic ATPase were significantly different from those of activated ATPase. With intrinsic ATPase, incubation with 2 mM $MgCl_2$ gave about 20% deactivation. Lowering the pH to neutrality resulted in no reduction of activity at all. And the equivalent of a tenfold molar excess of ATPase inhibitor gave only 15% deactivation.

Mechanism of Deactivation. All three ways of obtaining deactivation of ATPase involve adding a positively charged material—Mg^{2+}, H^+, or the ATPase inhibitor subunit. (Knowles and Penefsky (1972) have reported that the inhibitor subunit of F_1 has an isoelectric pH of about 10.) This raised the possibility that any cationic substance could cause deactivation. The results in Table III show that all of the cationic substances tested were indeed effective in promoting deactivation. The least effective were cytochrome *c* and histones. When the length of the preincubation was increased from 10 to 60 min, the deactivation by histones increased to 42% and the deactivation by cytochrome *c* increased to 43%. Polyanions, such as poly(glutamate) and poly(ethylene sulfonate) (also at 50 μg/ml), had no effect at all when preincubated with the ATPase for up to 60 min. These results suggest that deactivation involves covering up important negative charges on the surface of the activated enzyme.

Partial reactivation of ATPase was obtained as follows: first, deactivation was effected by incubating the ATPase for 10 min at 25 °C with poly(lysine) at 50 μg/m; then poly(glutamat) was added to 50 μg/ml to remove the excess poly(lysine) by complexing with it. There was no reversal of deactivation. However, when the system was incubated for 5 min at 55 °C, the activity increased from 1.5 to 5.5 μmol min^{-1} mg^{-1}. This may be compared to the activity of the untreated control, which was 10 μmol min^{-1} mg^{-1}.

Poly(glutamate) also protected the enzyme against poly(lysine)-induced activation. There was no detectable deactivation when poly(glutamate) was added to the enzyme before the poly(lysine) or when the two polyions were mixed first and then added to the enzyme.

Aurovertin. As part of a continuing effort to determine whether intrinsic and activated ATPase are similar or different, we tested their sensitivity to a number of substances that are known to have potent effects on other coupling factor ATPases. Thus, the kinetic effect of aurovertin on both the intrinsic and activated ATPase functions of HLF was examined. Intrinsic ATPase was inhibited 80% by 1 μM aurovertin, and activated ATPase was inhibited 60%. Increasing the concentration up to 20 μM had no further effect. Even preincubating the enzyme with 10 μM aurovertin for 10 min at 25 °C gave the same amount of inhibition as that observed by simply having 1 μM aurovertin present in the assay mixture. This ruled out the possibility that the inhibition was partial because binding of aurovertin to the enzyme was slow, and it demonstrated that there is no preincubation effect of aurovertin on purified HLF.

Aurovertin was also a very potent inhibitor of ATP synthesis by the membrane-bound coupling factor. Using the previously described assay (Adolfsen and Moudrianakis, 1971a), oxidative phosphorylation was 95% inhibited by 1 μM aurovertin. Half-maximal inhibition occurred at 0.05 μM aurovertin.

NBD-Cl. Ferguson et al. (1975) reported that NBD-Cl deactivates the ATPase function of F_1 by reacting with tyrosine at the active site. Time courses of deactivation of the ATPase function of HLF were performed in 0.1 mM NBD at 25 °C. Semilog plots of the remaining activity were linear with time, indicating that the decay was first order. The half-life of intrinsic ATPase was about 2 min, while that of activated ATPase was about 3 min (not significantly different). Aging the activated ATPase for 1 h at room temperature did not affect the half-life.

It was also of interest to determine whether the unactivated enzyme was sensitive to NBD. The unactivated enzyme was incubated for 20 min at 25 °C with 0.1 mM NBD, passed through a Sephadex G-50 M column to remove excess NBD, and then heat activated. The specific activity was 0.7 μmol min^{-1} mg^{-1}, compared to 11.2 for another sample of enzyme treated in exactly the same manner but with no NBD present. Thus, the target residues are accessible to NBD even before heat activation.

Suberimidate. Bragg and Hou (1975) reported that the *E. coli* membrane ATPase is sensitive to a cross-linking agent called dithiobis(succinyl propionate). The ATPase activity of HLF is sensitive to another cross-linking agent, dimethyl suberimidate. When activated ATPase was incubated with 0.01% suberimidate in 20 mM triethanolamine buffer (pH 8.5), a first-order decay of activity was found, which had a half-life of 6 min. After aging for 1 h at 25 °C, the half-life was about 4 min (not significantly different). Under these same conditions, suberimidate had no effect on intrinsic ATPase activity. Increasing the concentration by tenfold gave 30% deactivation of intrinsic ATPase activity after 10 min at 25 °C. These results suggested that the groups that were cross-linked to result in deactivation of the activated ATPase did not have the same spatial geometry as those in the intrinsic ATPase.

It was also of interest to determine whether the unactivated enzyme could be influenced by suberimidate. The unactivated enzyme was incubated with 0.1% suberimidate for 30 min at 25 °C, passed through a Sephadex G-50 M column to remove excess reagent, and then heat activated. The specific activity was 12.3 μmol min^{-1} mg^{-1}, which was about the same as that of the untreated control. Thus, the groups in the activated ATPase that are sensitive to the cross-linking agent have a different spatial orientation in the unactivated enzyme.

Kinetic Activation by Ions. Further differences between intrinsic and activated ATPases were found in studies on effects of ionic activators. First, the enzyme hydrolyzes free ATP at a slow rate in the complete absence of any added divalent cation activators. We previously suggested that this might be due to the presence of a tightly bound metal in the enzyme—i.e., that the enzyme was a metalloprotein (Adolfsen and Moudrianakis, 1973). The specific activity of intrinsic Mg^{2+}-independent ATPase was about 0.1 μmol min^{-1} mg^{-1}. After heat activation it was about 1.0 μmol min^{-1} mg^{-1}. The K_m of both intrinsic and activated enzyme was approximately 0.1 mM. Both activities were inhibited by a variety of metal-complexing agents, such as EDTA, azide, and citrate. However, the enzyme changed its response to hydroxyquinoline as a consequence of heat activation. There was very little inhibition (about 15%) by 2 mM hydroxyquinoline before heat treatment, but after heat treatment the inhibition was 85%. A similar effect was seen with *o*-phenanthroline (also 2 mM). The enzyme behaved in the same manner toward the more hydrophobic chelator bathophenanthroline, except that 85% inhibition occurred at a concentration of 5 μM instead of 2 mM. These data suggest that the site with which the chelators interact is more accessible in the activated ATPase than it is in the intrinsic ATPase and also that it is hydrophobic.

Further differences were found with respect to activation by divalent cations. Mg^{2+} stimulated intrinsic ATPase by about 1.5-fold. Activated ATPase was stimlated more than 20-fold. Also, monovalent cations controlled the selectivity of intrinsic ATPase for the divalent cation activator. Almost any divalent cation could stimulate intrinsic ATPase if the reaction mixture did not contain monovalent cations, but only Mg^{2+} and Fe^{2+} were stimulatory when monovalent cations were present (Adolfsen and Moudrianakis, 1973). This interesting regulatory property was absent from the activated ATPase. The order of effectiveness of stimulation for activated ATPase was $Mg^{2+} > Mn^{2+} > Co^{2+} > Cd^{2+} > Zn^{2+}$, and monovalent cations did not change it.

Discussion

We propose the following hypothesis for the mechanism of activation and deactivation of ATPase: The ATPase inhibitor subunit is cationic and is electrostatically complexed with the enzyme at a region containing a number of negative charges; heat activation involves displacement of the inhibitor subunit and a conformational change in the protein; negative charges that become exposed during heat activation are instrumental in holding the protein in the proper conformational state for ATP hydrolysis; deactivation involves covering these negative charges, which permits a return to an inactive conformational state. Support for the individual aspects of this hypothesis is given below.

The presence of negative charges in the bonding domain between the enzyme and the inhibitor subunit can be inferred from the deactivation studies. Preincubating the activated ATPase with every cationic substance tested here promoted deactivation (Tables II, III, Figure 4). Preincubating the activated enzyme with anions either had no effect, as in the case of poly(glutamate) and poly(ethylene sulfonate), or it prevented deactivation by a cationic substance, as in prevention of Mg^{2+} deactivation by EDTA or ATP, or as in prevention of poly(lysine) deactivation by poly(glutamate). These effects can be explained in terms of the formation of complexes between the anionic substances and the appropriate cationic substances, which makes the cationic substances unavailable for complexing with the enzyme.

The data presented here underscore the potential for the appearance of serious artifacts during attempts to isolate an inhibitor for the ATPase function of the 13S coupling factor. All the polycationic peptides tested in Table III could erroneously qualify for this, thus raising the possibility that many more cellular basic polypeptides could fit in this category. It appears to us then, that before deciding that a certain polypeptide is indeed a specific inhibitor of the ATPase function of the 13S coupling factor, it must be demonstrated that this polypeptide normally exists in a molecular complex with the coupling factor.

The two most important conditions for obtaining high levels of activation were alkaline pH and high temperature. Since the ATPase inhibitor protein is cationic, it is reasonable to expect that negative charges in solution (OH^-) would stabilize the inhibitor and thereby promote its solubility. The temperature requirement for activation (Figure 2A) suggests that high temperature increases the rate constant for the approach to the new equilibrium. Figure 2A also shows that the maximal level of activation was higher at higher temperatures. This suggests that temperature may also affect the absolute magnitude of the equilibrium constant.

The occurrence of a conformational change during heat activation is suggested by the discontinuity in the Arrhenius plot in Figure 2B. Warshaw et al. (1968) also report a conformational change during heat activation of F_1, which they detected by optical rotatory dispersion measurements. The fact that deactivation of HLF was not reversible simply by removing the deactivation agent—by gel filtration, by increasing pH, by adding EDTA to remove Mg^{2+}, or by adding poly(glutamate) to remove poly(lysine)—but required a second exposure to high temperature, suggests that the deactivation process involves a second conformational change. The deactivation is presumably a return to a state resembling the original, unactivated enzyme. The events occurring during reactivation may be a repeat of the changes that occurred during the first heat activation. Moyle and Mitchell (1975) have also reported on reversible active/inactive state transitions in mitochondrial F_1, which may be similar to those mentioned here for HLF.

The involvement of negative charges, in general, in holding

TABLE IV: Comparison of Properties of HLF Allomorphs.

Property	E_1 (Latent)	E_2 (Intrinsic)	E_3 (Activated)	E_4 (Aged)
Salt lability	Low	Low	High	
Deactivation by $MgCl_2$		20%	90%	50%
Deactivation by lowering pH		0%	90%	40%
Deactivation by ATPase inhibitor subunit		15%	95%	95%
Aurovertin inhibition		80%	60%	
Inhibition by NBd)0.1 mM)	Sensitive	$t_{1/2}$ 2 min	$t_{1/2}$ 3 min	$t_{1/2}$ 3 min
Inhibition by suberimidate (0.01%)	Not sensitive	Not sensitive	$t_{1/2}$ 6 min	$t_{1/2}$ 4 min
Inhibition by bathophenanthroline (10 μM)		15%	85%	
Stimulation by $MgCl_2$		2X	20X	
Regulation by KCl		Yes	No	
K_m for ATP		0.1 mM	0.4 mM	0.3 mM

the protein in the activated state is suggested by the lack of specificity for the deactivating agent that was observed during the deactivation studies. Any substance that can neutralize these charges promotes deactivation. Lowering the pH may actually play a dual role. First, addition of H^+ can result in neutralization of the negative charges on the enzyme. Second, the equilibrium of the ATPase inhibitor subunit may be shifted back toward association with the enzyme due to a reduction in the number of stabilizing charges in solution. It is interesting to note that the effectiveness of the inhibitor subunit of F_1 in deactivating the ATPase function of this enzyme was found to be greater at lower pH values (Pullman and Monroy, 1963; Horstman and Racker, 1970).

The reason for the partial lability of the activated enzyme is not understood. One possibility that has been ruled out is dissociation of some of the molecules into subunits, since this would not explain changes in the properties of the enzyme (lower K_m and decreased sensitivity to Mg^{2+} and H^+). It is possible that a partial deactivation could be effected by the very dilute inhibitor subunit (5 μg/ml at most) that is still present in the solution. The inhibitor may not complex in the same way as it was originally complexed, so that deactivation is only partial and other properties also change. An alternative explanation is that the activated enzyme is metastable and spontaneously relaxes into a different conformational state that does not have inhibitor bound to it but which is sufficiently different to result in changes in some of its properties.

Differences in the properties of intrinsic, activated, and aged activated ATPase indicated that there was functional polymorphism in the enzyme preparation. This polymorphism may be explained by the simple scheme in Figure 5. The previously reported finding that the enzyme did not have a detectable rapidly equilibrating nucleotide binding site led to the conclusion that only a small portion of the molecules are responsible for the intrinsic ATPase activity of the preparation (Adolfsen and Moudrianakis, 1976). Thus, the unactivated preparation is mostly E_1 with a small amount of E_2. E_2 may be molecules that have undergone activation by some other, natural mechanism, since the preparation was not treated in any manner that would cause activation. Heat activation converts E_1 into E_3, and extended exposure to high temperature leads to denaturation (Figure 2A). The properties of E_3 are different from those of E_2; these differences are summarized in Table IV. The properties of activated ATPase are stable for at least 1 h if the enzyme is kept at 0 °C. (There is no significant lability at 0 °C unless salt is added.) However, the K_m for substrate decreases and the ability of the enzyme to be deactivated by Mg^{2+} and H^+ drops by approximately 50% when

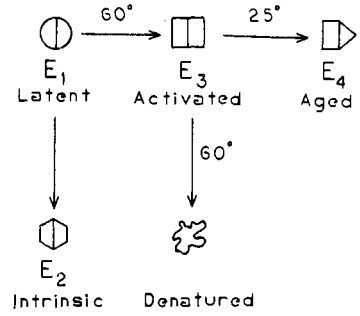

FIGURE 5: Proposed scheme to explain polymorphism of ATPase. The symbols represent different overall conformations of the enzyme functioning as an ATPase (not an ATP synthetase), and the bilateral symmetry is consistent with current views on the subunit stoichiometry of the enzyme. The arrows indicate the observed sequence of events and are not meant to imply irreversibility of conversions.

the enzyme is incubated at higher temperatures. This is a case of polymorphic flux, in which E_3 converts into E_4 with a half-time of about 30 min. It is important to prevent this from happening if careful studies are to be done on the enzyme kinetics of ATP hydrolysis. To study the properties of E_3, the enzyme is placed on ice immediately after heat activation and used within 10 min. To study E_4, the enzyme is aged for 2 h at 25 °C prior to being assayed.

The polymorphism observed in this system so far is quite extensive. There are five separate and distinct electrophoretic forms of HLF (Adolfsen et al., 1975). In addition to this structural polymorphism, we now have evidence for five functional allomorphs. There are the four described in the present paper (Figure 5) plus an additional form, which was detected in nucleotide binding studies as being incapable of binding any nucleotide (Adolfsen and Moudrianakis, 1976). This form would be a subpopulation of E_1 in the scheme in Figure 5. Thus far, we have found no evidence for any direct or one-to-one correspondence between the structural and functional allomorphs. If each functional allomorph can exist in each of the five structural states (or vice versa), then there may be a total of 25 different states in which this enzyme may exist in vitro. This is a theoretical maximum. If, on the other hand, a direct one-to-one correlation could be established between each structural allomorph and one and only one functional allomorph, then the theoretical minimum of five states could be reached. However, we have not yet been able to demonstrate such one-to-one correspondence, and our experimental obervations suggest that this number could be closer to ten. The question arises as to whether these different states

are all just in vitro artifacts or whether they have some significance in vivo. Undoubtedly, some of them exist only under special in vitro conditions. However, previous studies showed that the native slow and fast electrophoretic species (types IA and IB) were present on the membrane and could undergo interconversions while bound to the membrane. If we also concede that the enzyme can exist in either a latent or an activated state on the membrane, there are a minimum of four species which may have in vivo significance. One may ask what is the use of having all of these different species on the membrane. The answer to this question may be that the 13S coupling factor is a multienzyme system that in the cell is required to perform a number of different functions (not only ATP synthesis, but also several other energy-linked functions, including various forms of transport), and plasticity of structure is advantageous for adapting the multienzyme system to these various functions. In short, the enzyme may need to be polymorphic simply because it is polyfunctional.

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