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Thermal inactivation of electron-transport functions and F_0F_1-ATPase activities

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Bovine heart submitochondrial particles in suspension were heated at a designated temperature for 3 min, then cooled for biochemical assays at 30 °C. By enzyme activity measurements and polarographic assay of oxygen consumption, it is shown that the thermal denaturation of the respiratory chain takes place in at least four stages and each stage is irreversible. The first stage occurs at 51.0 ± 1.0 °C, with the inactivation of NADH-linked respiration, ATP-driven reverse electron transport, F_0F_1 catalyzed ATP/P_i exchange, NADH and succinate-driven ATP synthesis. The second stage occurs at 56.0 ± 1.0 °C, with the inactivation of succinate-linked proton pumping and respiration. The third stage occurs at 59.0 ± 1.0 °C, with the inactivation of electron transfer from cytochrome *c* to cytochrome oxidase and ATP-dependent proton pumping. The ATP hydrolysis activity of F_0F_1 persists to 61.0 ± 1.0 °C. An additional transition, detectable by differential scanning calorimetry, occurring around 70.0 ± 2.0 °C, is probably associated with thermal denaturation of cytochrome *c* and other stable membrane proteins. In the presence of either mitochondrial matrix fluid or 2 mM mercaptoethanol, all five stages give rise to endothermic effects, with the absorption of approx. 25 J/g protein. Under aerobic conditions, however, the first four transitions become strongly exothermic, and release a total of approx. 105 J/g protein. Solubilized and reconstituted F_0F_1 vesicles also exhibit different inactivation temperatures for the ATP/P_i exchange, proton pumping and ATP hydrolysis activities. The first two activities are abolished at 49.0 ± 1.0 °C, but the latter at 58.0 ± 2.0 °C. Differential scanning calorimetry also detects biphasic transitions of F_0F_1, with similar temperatures of denaturation (49.0 and 54.0 °C). From these and other results presented in this communication, the following is concluded. (1) A selective inactivation, by the temperature treatment, of various functions of the electron-transport chain and of the F_0F_1 complex can be done. (2) The ATP synthesis activity of the F_0F_1 complex involves either a catalytic or a regulation subunit(s) which is not essential for ATP hydrolysis and the proton translocation. This subunit is 10 °C less stable than the hydrolytic site. Micromolar ADP stabilizes it from thermal denaturation by 4–5 °C, although ADP up to millimolar concentration does not protect the hydrolytic site and the proton-translocation site. (3) Protein complexes of the mitochondrial energy-transducing membrane are metastable, i.e., they are in a high-energy state under aerobic environments. These complexes are susceptible to oxidation (consuming 250 nmol O_2 per mg protein) and as a consequence dissipate heat. Whether this exothermic energy is relevant to the in vivo ATP synthesis remains unclear.

Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)-ethyl]glycine; P_i, inorganic phosphate; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; DSC, differential scanning microcalorimetry; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

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Introduction

The generally accepted theory for the coupling of electron transport and ATP synthesis in mitochondria is the chemiosmotic hypothesis [1–4]. While this theory postulates a proton electrochemical gradient as the key intermediate for ATP synthesis, little is known of how such a gradient can be coupled to the ATP synthesis reaction. The mechanism proposed by Boyer and his coworkers [1,5] postulates that on binding of ADP and P_i to the enzyme ATP is formed at the enzyme surface, with a free energy of the conversion close to zero. The function of the proton electrochemical gradient is to provide an electrochemical potential energy that triggers the release of the tightly bound ATP. In fact, measurements of the rate constants of nucleotide binding to isolated F_1-ATPase support the view that the free energy of conversion of ADP and P_i to ATP on the enzyme surface is near zero [6–8]. Other views, although different in details, all revolve around the idea of altering the affinity of enzyme for ATP [7,9–12]. It is suspected that changes in the affinity for ATP could result from electrochemical potential-induced structural or conformational changes of the F_0F_1-ATPase.

It is in this last context that we have been investigating the role of membrane potential in ATP synthesis [13–17]. It is known that the peptide unit of a protein is an electric dipole of 3.5 debye, and the secondary structures of proteins are electric dipoles of considerable strength [18,19]. Most proteins are also charged at neutral pH. A molecule with net charges and electric dipoles in its structure is capable of responding to an electric field, especially when the electric field is amplified on a membrane surface [15,16]. In the case of membrane-bound ATP synthetases, this field-induced conformational shift may provide free energy required for the release of tightly bound ATP or for an ATP synthesis through electroconformational coupling [13–17]. Such a rationale has proved fruitful and electric-field driven ATP synthesis has now been demonstrated in spinach chloroplasts, lettuce chloroplasts, *Escherichia coli,* PS3 thermophilic bacterium, rat liver submitochondria, bovine heart submitochondria, bovine heart F_0F_1-ATPase reconstituted lipid vesicles and chloroplast CF_0F_1-ATPase reconstituted lipid vesicles [13,14,20–23]. These results pose new challenges for understanding the energy-transducing process, and at the same time, they may furnish useful information for bridging the gap between the conformational coupling hypothesis and the chemiosmotic hypothesis [1–5, 15,16,24].

Several considerations prompted us to undertake the present study. Firstly, in experiments using electric fields to induce ATP synthesis, considerable Joule heating is associated with the application of each electric pulse. Effects of temperature on various functions of the electron-transport chain need to be studied in greater detail. ATP formed by pulsed electric field may include contributions from yet unidentified sources which are not relevant to the in vivo mechanism of oxidative phosphorylation (Chauvin, F., Astumian, R.D. and Tsong, T.Y., unpublished results). Secondly, there are several reports on heat sensitivity of F_1 and F_0 subunits of the enzyme and other protein components of the electron-transport chain [49–51,25,26]; yet, no systematic efforts have been invested to study heat inactivation and its implication to enzyme functions, in vivo. Thirdly, although the conformational coupling hypothesis is considered a viable mechanism, experimental evidence of conformational changes in the coupling factor is still lacking [14,15]. Many accessible conformational states of an enzyme are detectable to DSC or spectrophotometric methods, and there is a high likelihood that we might be able to detect these conformational transitions. Fourthly, the question of whether ATP synthesis is a direct reverse of the hydrolysis reaction has often been asked. Information on differential inactivation of the several functions of the F_0F_1 complex may provide some insight concerning the relationship between these functions of the enzyme. Partial inactivation by temperature of different activities of the respiratory chains may also allow us to distinguish mechanisms of ATP synthesis with various forms of energy source. In this communication, we report a stepwise temperature inactivation of several functions of the electron-transport chain and the different activities of lipid reconstituted bovine heart F_0F_1-ATPase.

Materials and Methods

Submitochondrial particles and ATPase reconstitution. Mixed layer bovine heart mitochondria were prepared according to the large scale procedure of Blair [27]. Submitochondrial particles were prepared by sonicating mitochondria for 30 s at 0 °C, with a Branson S-200 3/8-inch step-horn tip, in the presence of 0.25 M sucrose, 1 mM succinate, 1 mM ATP, 5 mM $MgCl_2$, 10 mM $MnCl_2$ and 10 mM sodium-Hepes (pH 7.4), and separating from other fractions by differential centrifugation [28]. A more drastic sonication procedure was also employed. Mitochondria were suspended in 0.25 M sucrose, 10 mM Tris-HCl (pH 7.4) and sonicated for 4 min. F_0F_1-ATPase was prepared by cholate extraction of submitochondrial particles and subsequent $(NH_4)_2SO_4$ fractionation [29]. This preparation is about 80% pure as judged by SDS-polyacrylamide gel electrophoresis. Reconstitution of the isolated F_0F_1-ATPase into asolectin vesicles followed the procedures of Kagawa and Racker [29] with a slight modification. Briefly, F_0F_1-ATPase at 10 mg/ml was added to a sonicated mixture of 20 mg/ml of cholate and 80 mg/ml of partially purified soybean phospholipids (Sigma Chem.) in 50 mM Tricine (pH 7.8). The mixture was dialyzed against 300 volumes of medium containing 10% (v/v) methanol, 0.2 mM sodium-ATP, 0.5 mM EDTA, 0.4 mM dithiothreitol and 10 mM Tricine (pH 8.0). The buffer was changed once after 2 h, and dialysis was continued overnight. The solution was centrifuged at 45000 r.p.m. in a 50 rotor (Beckman Instruments) and the pellet was resuspended to 20 mg/ml in 20 mM Tricine (pH 8.0).

Thermal inactivation. Submitochondria (10 mg/ml) or reconstituted F_0F_1 vesicles (1 mg/ml) were suspended in a medium containing 0.25 M sucrose and 10 mM Tris-HCl (pH 7.8). 50 µl was placed into a glass tube and heated at indicated temperatures for 3 min. The suspension was cooled to room temperature and a 10 µl aliquot was drawn and immediately assayed at 30 °C for electron-transport chain activities. Duplicates were performed for each temperature, as well as a control sample which was kept at room temperature for an equal length of time before assay. When effects of reagents on thermal stability needed to be examined, the reagent was added to the heating medium at indicated concentrations. 3-min heat treatment was found to be adequate. Other slow and time-dependent processes were a few percent and would have no effect on the analysis of result.

Biochemical assays. ATP hydrolysis was measured by a coupled enzyme assay, using lactate dehydrogenase and pyruvate kinase [30]. Succinate driven oxidative phosphorylation was assayed by a coupled enzyme assay using hexokinase and glucose-6-P dehydrogenase [31]. NADH driven ATP synthesis was assayed by radioactivity incorporation of $^{32}P_i$. Reverse electron transport (using cyanide-treated particles) was assayed with succinate as the electron donor, NAD^+ the electron acceptor and ATP as the energy source [32]. ATP and succinate-dependent proton translocation was monitored by the 9-amino-6-chloro-2-methoxyacridine fluorescence quenching method using an Aminco spectrofluorometer [33]. ATP/P_i exchange was measured in the presence of 20 mM [^{32}P]P_i 10 mM and MgATP. The amount of esterified ^{32}P was determined after extraction with molybdate and isobutanol [34]. Oxygen consumption was measured in a vessel (1.5 ml volume) which is temperature controlled and tightly sealed against air at indicated temperature using a Clark oxygen electrode. The solubility of oxygen in the medium used was estimated to be 220 nmol O_2/ml at 30 °C. Oxygen consumption was initiated by addition of 0.3 mM NADH or 10 mM succinate. Cytochrome *c* reductase activity was monitored polarographically as described [35]. Electrophoresis was carried out in the presence of SDS and 8 M urea according to Laemmli [36]. Protein was determined by a modified Lowry procedure [37].

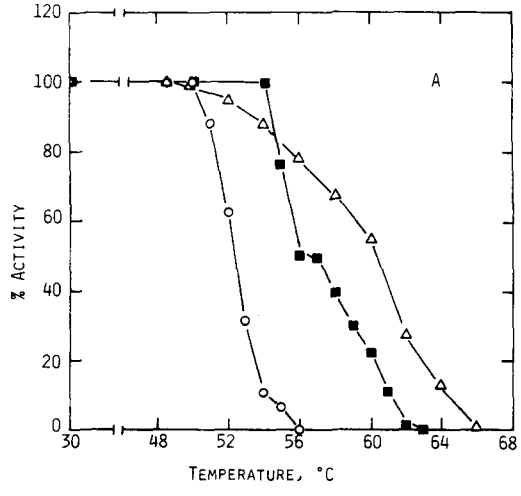
Differential scanning microcalorimetry. MCl microcalorimeter (MicroCal, Inc.) was used to measure excess heat uptake or heat liberation of mitochondria, submitochondria, and solubilized and reconstituted F_0F_1-ATPase upon thermal denaturation. Membranes or proteins were suspended in the concentration range between 0.5 and 10 mg protein per ml in 0.25 M sucrose, 10 mM Tris/Cl (pH 7.8) for measurements. The heating rate ranged from 5 to 25 °C per hour. The heat of transitions was determined by a planimeter, or by cutting out the transition peaks and weighing the paper [38]. Deconvolution using a

nonlinear least-squares program was also done for selected DSC runs. The results are consistent with our present interpretation. Samples used for calorimetry analysis were bovine heart mitochondria [27], 4 min sonicated submitochondrial particles [28], and mildly treated and 30 s sonicated submitochondrial particles.

Results

Thermal inactivation of enzyme activities in submitochondrial particles

Several activities of the electron-transport chain of mitochondria were monitored for their thermal stability. Fig. 1A shows typical transition curves for O_2 consumption rate of submitochondrial particles plotted against temperature of incubation, driven by three different substrates. The maximum rate of NADH-driven oxygen consumption in our samples was approx. 350 nmol O_2/min per mg at 30 °C. This rate was not affected for samples pretreated below 50 °C. However, a dramatic reduction in rate occurred for samples preincubated above 50 °C. The midpoint of inactivation (T_m) for this activity was 52.0 °C. When the respiration was driven by succinate, the midpoint of inactivation was significantly higher, with a T_m of 56.0 °C. The thermal inactivation curve was frequently found to be biphasic. Typically, the starting submitochondrial particles consumed 180 nmol O_2/min per mg in this case. Cytochrome-*c*-driven respiration persisted to higher temperatures, with the T_m of inactivation at 59 °C. In this case, the inactivation curve also appeared to be biphasic and much broader than that of NADH-linked respiration.



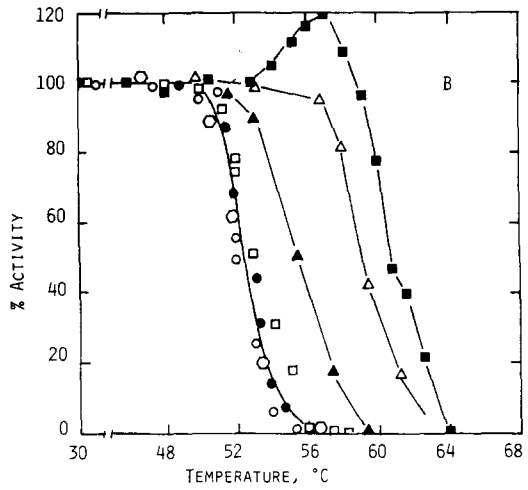


Fig. 1. Thermal inactivation of various functions of electron-transport chain. (A) Oxygen consumption was measured polarographically, at 30 °C, on submitochondrial particles (1 mg of proteins) that had been incubated at the indicated temperature for 3 min. The substrates were NADH (○), succinate (■), and reduced cytochrome *c* (△). The maximal respiration rates of submitochondrial particles, not heat treated, were 330, 180 and 430 nmol O_2 per min per mg, respectively, for substrates NADH, succinate and cytochrome *c*. (B) Thermal inactivation curves of ATP hydrolysis without FCCP (■), ATP-driven proton pumping (△), succinate-driven proton pumping (▲), succinate-driven ATP synthesis (●), NADH-driven ATP synthesis (○), ATP-driven reverse electron transport from succinate to NAD^+ (○), and ATP/P_i exchange (□) are given. The proton-pumping activity was measured by 9-amino-6-chloro-2-methoxyacridine fluorescence change, as described in Materials and Methods, and was given in relative activity. The maximal activity of ATP hydrolysis was 2.0 μmol/min per mg; of NADH-driven ATP synthesis, 120 nmol/min per mg; of succinate-driven ATP synthesis, 170 nmol/min per mg; of ATP/P_i exchange, 140 nmol/min per mg; and of reverse electron transport, 50 nmol/min per mg.

The thermal stability of the F_0F_1-ATPase was also determined by a variety of assays. Fig. 1B shows the thermal inactivation curves for ATP hydrolysis (■), ATP- (△) and succinate- (△) driven proton pumpings, ATP/P_i exchange (□), NADH- (○) and succinate- (●) driven ATP synthesis, and reverse electron transport (○). ATP hydrolysis was the most stable, usually showing an activation

around 56 °C folowed by an inactivation around 60 °C. ATP/P_i exchange was the least stable. In Fig. 2A several typical spectrofluorometric tracings of succinate-driven proton pumping using the 9-amino-6-chloro-2-methoxyacridine method are shown. Similar tracings of ATP-driven proton pumping are shown in Fig. 2B. In both cases, the rate of proton pumping can be measured either by the maximum slope of the fluorescence quenching curves or by the final level of the quenching curves. We have used the latter to express the relative level of proton pumping using samples not treated with heat as the reference. The T_m values for these measurements varied from 54 to 59 °C. Table I summarizes the thermal inactivation results using submitochondrial particles. Mean values and standard deviations are given.

Several facts of these experiments are noted. First, there appears to be a stepwise inactivation of various functions associated with different enzyme complexes of the respiratory chain. NADH-ubiquinone reductase of Complex I apaprently was inactivated at 51.0 ± 1.0 °C, but Complex II associated with succinate-ubiquinone reductase was inactivated at 56.0 ± 1.0 °C. Both Complex III and Complex IV were more resistant to thermal inactivation, and they continued to function up to around 59.0 ± 1.0 °C. Surprisingly ATP/P_i exchange and ATP synthesis activity, either driven by succinate, or by NADH, were all denatured at 51.0 ± 1.0 °C despite the fact that succinate and ATP-driven proton pumping continued to function at or close to 100% efficiency, i.e., there was no indication of membrane leakage and inactivation of F_0F_1 complex up to this temperature.

Second point of interest is that the ATP synthesis and ATP hydrolysis activity of F_0F_1-ATPase seemed to denature quite independently. For nearly a dozen experiments done on different preparations of submitochondrial particles ATP synthesis driven by different energy sources, namely, succinate, NADH, or ATP in the case of ATP/P_i exchange, all inactivated around 51–53 °C, but ATP hydrolysis all persisted up to 60–62 °C. ATP-driven proton pumping denatured around 54–59 °C. For several preparations of sub-

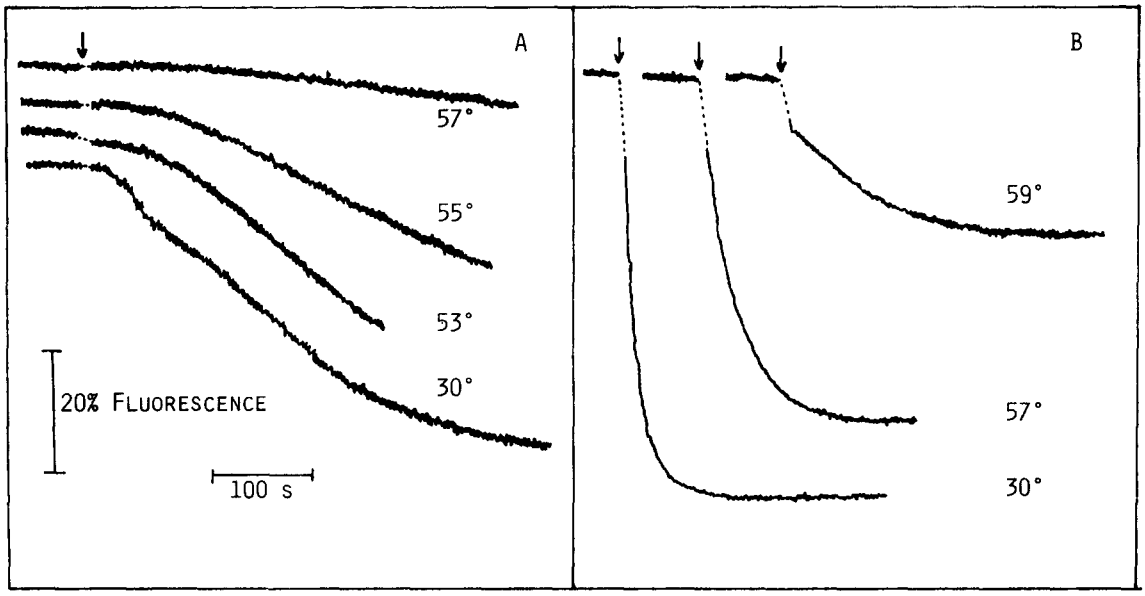


Fig. 2. Relative proton-translocating activity of heat-treated submitochondrial particles as measured by quenching of 9-amino-6-chloro-2-methoxyacridine fluorescence. (A) Succinate-driven proton pumping was monitored for samples treated at indicated temperatures for 3 min prior to the 9-amino-6-chloro-2-methoxyacridine assay at 30 °C. Fluorescence intensity is expressed in an arbitrary unit. The dashed line indicates where 20 mM succinate was added. 2 ml solution contained 0.5 mg proteins. (B) ATP-driven proton pumping was followed by the same 9-amino-6-chloro-2-methoxyacridine assay. 2 ml solution contained 0.1 mg proteins. The dashed line indicates where 1 mM ATP was added.

TABLE I

THERMAL INACTIVATION OF ELECTRON TRANSPORT FUNCTIONS OF BOVINE HEART SUBMITOCHONDRIA

Activity measured	Maximum activity	Added ADP	T_m (reproducibility) [d]	Comments
Submitochondria				
NADH-linked respiration	330 nmol O_2/min per mg	no [a]	51.0 (1.0)	inactivation of Complex I
Succinate-linked respiration	180 nmol O_2/min per mg	no	56.0 (1.0)	inactivation of Complex II
Cytochrome *c*-linked respiration	430 nmol O_2/min per mg	no	59.0 (1.0)	inactivation of Complex VI
NADH-linked ATP synthesis	120 nmol/min per mg	no	51.0 (1.0)	inactivation of Complex I
NADH-linked ATP synthesis	120 nmol/min per mg	5.0 mM	51.0 (1.0)	inactivation of Complex I
Succinate-linked ATP synthesis	170 nmol/min per mg	no	52.0 (1.0)	inactivation of ATP synthetic site of F_0F_1
Succinate-linked ATP synthesis	170 nmol/min per mg	5.0 mM	56.0 (1.0)	inactivation of ATP synthetic site of F_0F_1
ATP/P_i exchange	140 nmol/min per mg	no	51.5 (1.0)	inactivation of ATP synthetic site of F_0F_1
ATP/P_i exchange	140 nmol/min per mg	5.0 mM	56.5 (1.0)	inactivation of ATP synthetic site of F_0F_1
Reverse electron [b] transport	50 nmol/min per mg	no	51.5 (1.0)	inactivation of Complex I
Succinate-driven proton pumping	relative activity	no	56.5 (2.0)	inactivation of complex II
ATP-driven proton pumping	relative activity	no	59.0 (2.0)	inactivation of F_0
ATP hydrolysis	2.0 μmol/min per mg	no	62.0 (1.0)	inactivation of ATP hydrolytic site of F_0F_1
ATP hydrolysis	20 μmol/min per mg	5.0 mM	62.0 (1.0)	inactivation of ATP hydrolytic site of F_0F_1
F_0F_1-ATPase reconstituted into lipid vesicles [c]				
ATP hydrolysis	1.2 μmol/min per mg	no	60.0 (1.0)	inactivation of ATP hydrolytic site
ATP dependent proton pumping	relative activity	no	49.0 (1.0)	inactivation of F_0
ATP/P_i exchange	10 nmol/min per mg	no	49.0 (1.0)	inactivation of ATP synthetic site

[a] [ADP] $< 1 \cdot 10^{-8}$ M.

[b] ATP-driven electron backtransport from succinate to NAD^+.

[c] Reconstituted ATPase activity was 91% sensitive to 1 μg oligomycin per mg protein.

[d] Number of measurements for each value listed below ranges from 2 to 20.

mitochondrial particles, differential inactivation of the ATP/P_i exchange and succinate-driven ATP synthesis activity was also noted [39]. When this happened, the ATP synthesis activity usually persisted to a higher temperature, ranging from 0.5 to 3.0 °C above the denaturation of the ATP/P_i exchange, which was consistently measured at 51–53 °C. This observation is puzzling in view of the fact that ATP/P_i exchange is believed to be an essential step in the ATP synthetic pathway, and is usually used to measure the ATP synthesis activity of the F_0F_1-ATPase in solubilized form. At this point we have no satisfactory explanation to offer, except to report these observations.

No release of F_1 or its subunits upon heat denaturation

At least two possibilities must be considered for the finding that upon the thermal inactivation of ATP synthesis activity there was a concomitant increase in the ATP hydrolysis activity of the F_0F_1 complex. These are membrane leakage to proton and release of F_1 or its subunits from the membrane. The first possibility was ruled out by the fact that the ATP-dependent proton translocation activity of the enzyme was preserved at this temperature, i.e., 56° C. Although this fact also argued against the release of F_1 from the membrane, it is likely that certain subunits of F_1 essential for ATP synthesis but not required for ATP hydrolysis might be released to the solution at this temperature. The experiment described in Fig. 3 shows that this was not the case. The ATPase activity of supernatant after spinning down the submitochondrial particles remained at less than 5% of the total activity for temperatures between 30° and 65° C. An antibody (polyclonal) against F_1 detected an α subunit in the supernatant, but the level of the α subunit released was well within 5% of the total concentration of this subunit (data not shown), and the effect of the temperature was gradual; there was no sudden release in the range of temperature studied here.

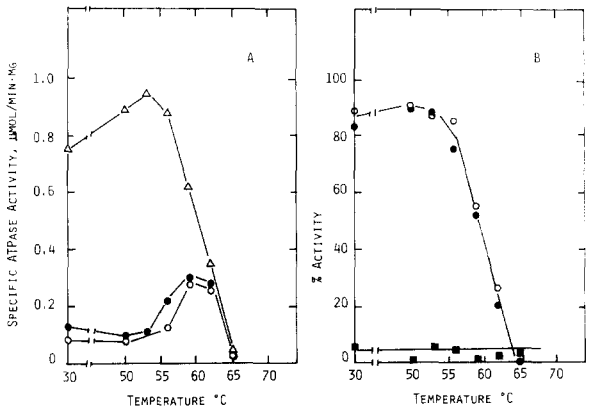


Fig. 3. F_1-ATPase activity of heat-treated submitochondrial particles. (A) Oligomycin and DCCD sensitivities of the ATP hydrolytic activity of F_1 were examined for heat-treated submitochondrial particles. The ATP hydrolysis in the absence of inhibitors (△), in the presence of 1.25 μg oligomycin/mg proteins (●), and in the presence of 48 nmol DCCD/mg proteins (○) are plotted against temperatures in which submitochondrial particles samples were incubated for 3 min before assay at 30° C. No loss of oligomycin and DCCD sensitivities were detected before 55° C. Initial rate of ATP hydrolysis activity was measured, without FCCP. (B) The percentage oligomycin sensitive (●) and DCCD sensitive (○) ATP hydrolytic activity of F_1 is plotted against temperature of inactivation. F_1 activity in the supernatant is also shown (■). Data indicate that the loss of oligomycin and DCCD sensitivities was not due to release of F_1 particle from the submitochondrial particle membranes.

Thermal inactivation of reconstituted F_0F_1-ATPase

The fact that different functions of F_0F_1 could be independently inactivated was of some significance, but the results do not rule out the possibility that there are other protein components within the electron-transport membrane which may be required for various functions of the F_0F_1 complex, and they were inactivated at different temperatures. This possibility can be ruled out by performing similar experiment for lipid-reconstituted F_0F_1-ATPase. The result shown in Fig. 4 indicates that purified F_0F_1 ATPase also exhibited

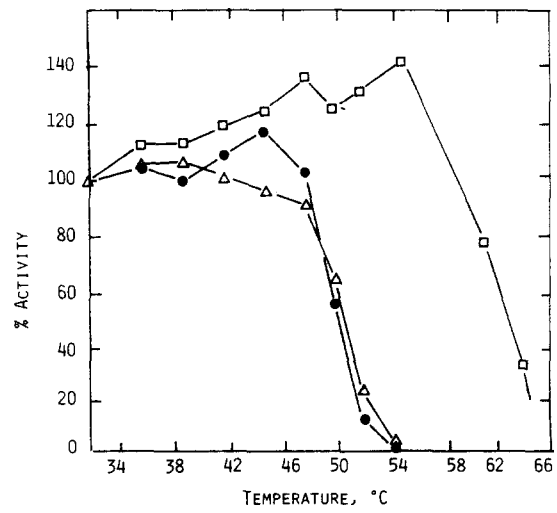
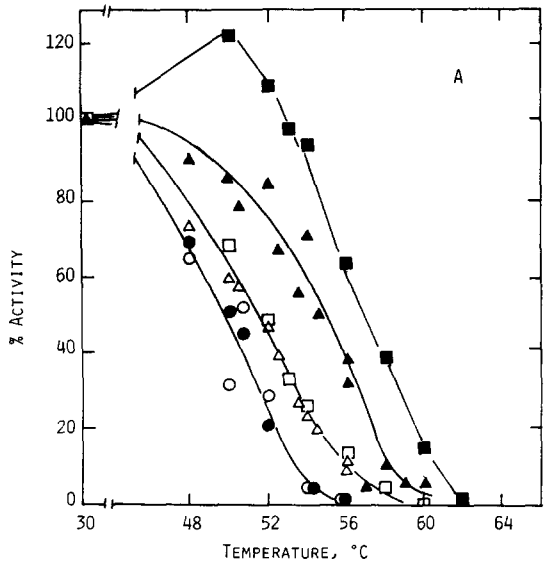


Fig. 4. Thermal inactivation of various activities of the soybean phospholipid-reconstituted F_0F_1-ATPase. In 2 ml solution 62 μg of F_0F_1-ATPase and 1–2 mg of soybean phospholipids were present. ATP hydrolysis activity of the reconstituted proteoliposomes was 1.2 μmol/min per mg, which was 91% sensitive to 1 μg of oligomycin. The ATP/P_i exchange activity of the sample not treated with heat was 10 nmol/min per mg. Proton pumping activity was measured by the 9-amino-6-chloro-2-methoxyacridine fluorescence quenching method, and is given in arbitrary unit. ATP hydrolysis (□), ATP/P_i exchange (●), and proton-pumping (△) activities are shown.

differential thermal stability for its various functions, though the stability towards thermal inactivation was not fully compatible with that of the enzyme in its native membrane environment. Inactivation of the ATP/P_i exchange and the ATP hydrolysis were similar in proteoliposomes as in submitochondrial particles (about 2°C less stable in proteoliposomes). However, the ATP-dependent proton-pumping activity was denatured at 51°C in proteoliposomes compared to 59.0°C in submitochondrial particles, suggesting that there was some modification of the supramolecular structure of the enzyme complex in the reconstituted form. A sudden proton leak of the lipid bilayer is unlikely, since there are no known phase transitions of soybean phospholipid at 51°C.

Effects of ADP on the thermal stability

Substrate ADP seemed to protect F_0F_1 complex from thermal inactivation. Data in Fig. 5A show that 5 mM ADP raised the temperature of inactivation of succinate-driven ATP synthesis and ATP/P_i exchange by 3.5 and 5.5°C, respectively. NADH-driven ATP synthesis was not affected, however. Two observations suggest that NADH dehydrogenase activity was denatured at 51.0°C. Firstly, ADP did not protect NADH-driven ATP synthesis. Secondly, while succinate-driven proton pumping persisted to 56.0°C NADH-linked proton pumping diminished at 51.0°C (Fig. 5B).



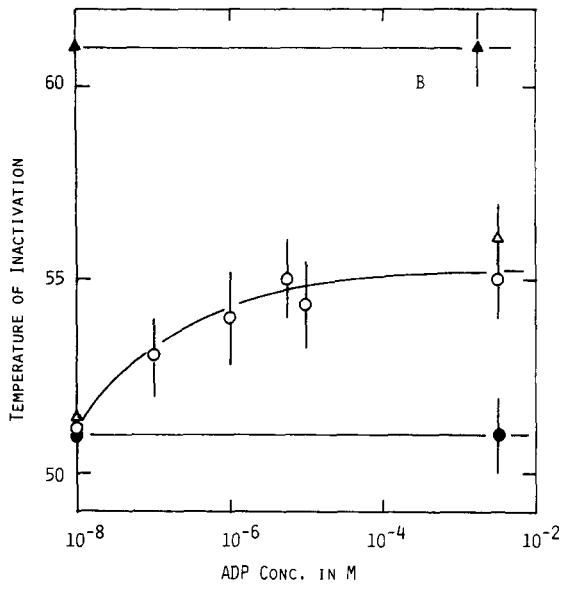


Fig. 5. Effect of ADP on the thermal stability of F_0F_1-ATPase. (A) 5 mM ADP was added to submitochondrial samples during the heat incubation period (3 min), and thereafter enzyme assays were done in an identical condition for the control samples that were heat treated in the absence of ADP. Data points with ADP are given in the filed symbols, and that without ADP are given in the open symbols. Thermal inactivations of NADH-driven ATP synthesis (○, ●), succinate-driven ATP synthesis (△, ▲), and ATP/P_i exchange (□, ■) were examined. Each point represents the average of 2–4 separate experiments. (B) The thermal inactivation temperature (temperature for 50% inhibition) was plotted against ADP concentration in the medium during the heat incubation period. ATP hydrolysis (▲) and NADH-driven ATP synthesis (●) were not affected, while the succinate driven ATP synthesis (○) and ATP/P_i exchange (△) were stabilized by 4–5°C. The effect of ADP reached a plateau around 10 μM.

The ADP protection against the thermal denaturation reached a plateau value around 10 μM ADP (Fig. 5B). On the other hand, oligomycin-sensitive ATP hydrolysis activity was not affected by ADP (Fig. 5B), although total ATP hydrolysis activity increased dramatically from 1.60 μmol/mg per min to nearly 4.00 μmol/mg per min by a temperature treatment around 58°C in the presence of 1.25 mM ADP (Fig. 6). In the absence of ADP in the medium, such an effect was not observed (Fig. 6). Surprisingly, ATP hydrolysis activity of State III submitochondrial particles was not affected by the millimolar concentration of ADP (T_m stayed the same, at 62°C). Since the affected activity might be due to an altered K_m of

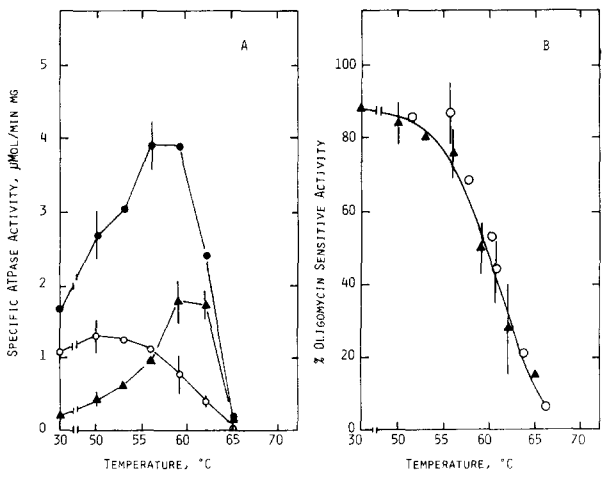


Fig. 6. Effect of ADP on the thermal inactivation of F_1-ATPase. (A) Incubation with 1.25 mM ADP during the heat treatment of submitochondrial particles enhanced both the oligomycin-sensitive and -insensitive ATP hydrolytic activities of F_1. Data are for samples without ADP (○), with 1.25 mM ADP (●), and with 1.25 mM ADP plus 2 μg/mg oligomycin during the heat treatment (▲). (B) The percentage oligomycin-sensitive ATP hydrolysis activity of samples-with (▲) with and without (○) 1.25 mM ADP during heat treatment appear to be similar in their thermal stability.

enzyme for the substrate, these values were determined for submitohconcrial particles, heat treated at 52 and 59 °C, in the presence and absence of ADP. Table II summarizes the result. It appears that both heat treatment and incubation with ADP reduced the affinity of enzyme toward ADP, but not toward P_i and ATP.

TABLE II

APPARENT K_m OF HEAT-TREATED SUBMITOCHONDRIAL PARTICLES

Temperature of treatment (°C)	[ADP] during heat treatment	K_m for ADP 9μM)	K_m for P_i (mM)	K_m for ATP (μM)
30 [a]	not added [b]	35.0	0.58	137
30 [a]	3 mM	69.0	0.56	114
52	not added	64.5	0.55	n.d. [c]
52	5 mM	114	0.40	n.d.
59	not added	n.a. [d]	n.a.	102
59	1.25 mM	n.a.	n.a.	97

[a] Not heat-treated sample. Assay temperature is given.
[b] [ADP] $< 10^{-8}$ M.
[c] n.d. means not determined.
[d] n.a. means not applicable. ATP synthesis activity of these samples was inactivated.
[e] Each value is the average of two determinations.

Calorimetric study

Fig. 7 gives thermal denaturation curves for 9.0 mg bovine heart mitochondria (A), 6.4 mg bovine heart submitochondria in the presence of 2 mM mercaptoethanol (B), 0.9 mg bovine heart submitochondria in the absence of mercaptoethanol (C), and 0.9 mg rat liver submitochondria in the absence of mercaptoethanol (D). From curve A, one can identify thermal transitions corresponding to inactivation of different activities of the electron-transport chain. There are 5 stages of inactivation at 51, 56, 59, 61 and 70 °C as indicated by the arrows. The same number of transitions is also discernible in the submitochondrial particle sample. The first four transitions correspond to the four stages of enzyme inactivation summarized in Table I. The fifth transition (70 °C) possibly detected thermal denaturation of cytochrome *c* and other thermally stable proteins. All transitions were endothermic, in the presence of mitochondrial matrix fluid or mercaptoethanol, and absorbed a total of roughly 25 J/g protein. The most dramatic observation we made was that the first four transitions became exothermic in the absence of matrix fluid or a reducing reagent, such as mercaptoethanol or dithiothreitol. The heat evolved in total more than 105 J/g protein. It was suspected that this exothermic heat might reside in the supramolecular structure of the electron-transport proteins and might be relevant for ATP synthesis. However, as will be discussed elsewhere (Tsong, T.Y. and Knox, B.E., unpublished results), associated with the exothermic processes, the samples consumed about 250 nmol/mg of molecular oxygen, indicating that there was oxidation of protein complexes, presumably the heme- and iron-sulfur-containing proteins. Curves C and D of Fig. 7 indicate that both bovine heart and rat-liver submitochondrial particles gave rise to similar exothermic transitions when sufficient oxygen was available in suspensions. However, this exothermic effect was completely absent in a liver submitochondrial particle sample of an infant who inherited a fatal congenital lactic acidosis [40]. Mitochondria from this infant were shown to be deficient in the iron-sulfur clusters of the Complex

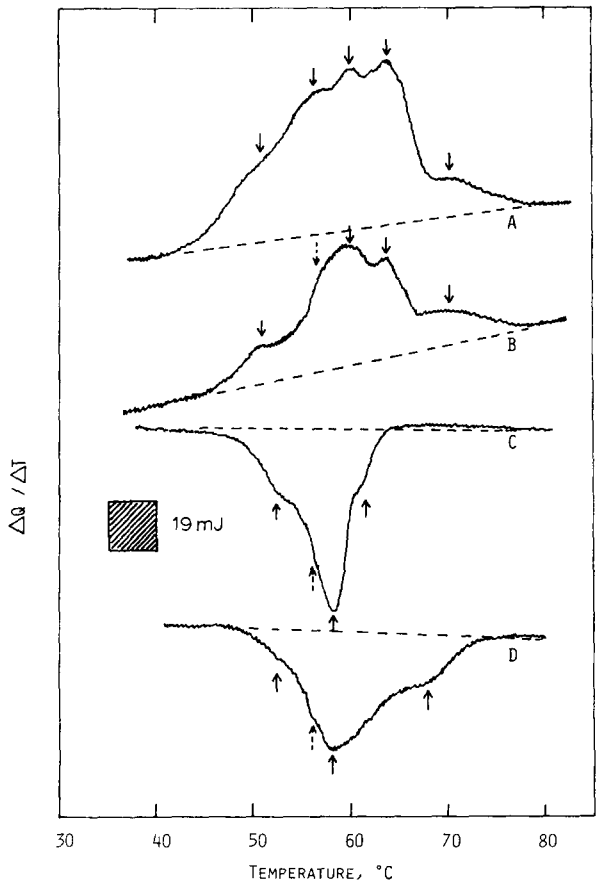


Fig. 7. Differential scanning microcalorimetric study of mitochondria and submitochondrial particles. Curves A, B, C and D contained, respectively, 9.0 mg bovine heart mitochondria, 6.4 mg bovine heart submitochondrial particles in the presence of 2 mM mercaptoethanol, 0.9 mg bovine heart submitochondrial without adding mercaptoethanol, and 0.9 mg rat liver submitochondrial without mercaptoethanol, each suspended in 0.9 ml isotonic sucrose plus 10 mM Tris/HCl at pH 7.4. The arrows in curve A point at peaks or shoulders that can be identified as separate transitions, at 51.0, 56.0, 59.5, 63.5 and 71 °C. Identical transitions are seen with curve B, except that the shoulder at 56.5 °C is barely discernible. Arrows for curve C point at 52.0, 56.0, 58.5 and 62.0 °C, while for curve D at 52.0, 56.0, 58.5 and 68.0 °C. The dashed lines indicate baselines. Areas above the baselines detected endothermic effects and areas under the baselines detected exothermic effects. A 19 mJ calibration is shown with the shaded square. See text for details. The curves A and B each gave rise to approx. 25–29 J/mg of endothermic effect, while the curves C and D each gave rise to aprox. 105–117 J/mg of exothermic effect. See text for details.

I by Moreadith et al. [40]. Resolution of these composite transition curves and detailed study of the exothermic effect will be presented elsewhere.

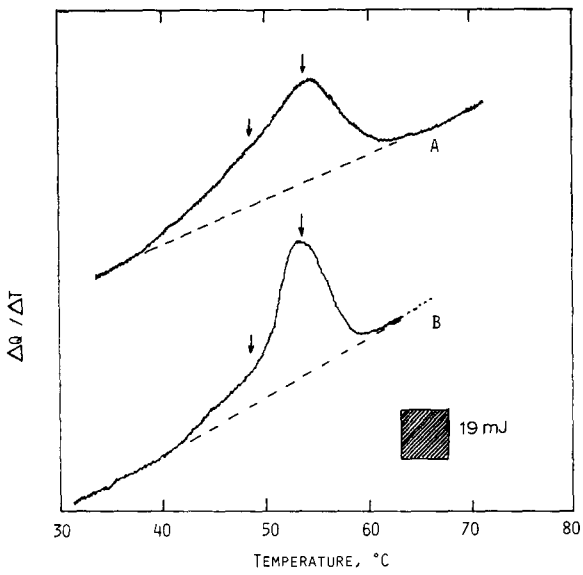


Fig. 8. Differential scanning calorimetry of F_0F_1-ATPase. Curve A was obtained with a sample containing 10.8 mg of solubilized F_0F_1 complex, suspended in 0.9 ml of 50 mM sucrose, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.5 mM dithiothreitol, and 10 mM Tris/SO_4 at pH 7.8. Curve B was obtained with a 0.9 ml of sample containing 10.8 mg F_0F_1 reconstituted into 90 mg soybean phospholipids, 25 mM Tris/SO_4, 1 mM $MgCl_2$, 0.5 mM dithiothreitol at pH 7.8. The arrows in curve A point at 49.0, 54.0 and 59.0 °C, while in curve B, at 49.0 and 54.0 °C. The total heat absorbed was approx. 3 MJ/mol based on the molecular mass of 380 kDa, or an endothermic heat of 8 J/g, in each case. This is roughly one-fifth of the heat of unfolding for small proteins such as ribonuclease A [41]. Detailed deconvolution of individual transitions and analysis will be presented elsewhere.

DSC measurements were also done with solubilized F_0F_1-ATPase in detergent (curve A) and in the soybean phospholipid reconstituted form (curve B), as shown in Fig. 8. In both cases, the transitions were biphasic, and can be resolved into two transitions, one at 49.0 °C and the other at 54.0 °C. The ΔH of transitions were 3 MJ/mol, or 8 J/g, which is approx. one-fifth of the heat of denaturation of ribonuclease A on a weight basis [41]. The thermal transition of the F_0F_1 complex was endothermic and was not sensitive to the presence of molecular oxygen.

Discussion

Different activities of F_0F_1 complex

In all of our experiments with heart sub-

mitochondrial particles and reconstituted F_0F_1-ATPase, various activities of the electron-transport chain were inactivated in the temperature range 45–60°C. These results are consistent with the earlier observations of Penefsky et al. and Kagawa and coworkers and Sone and Nicholls [49–51]. However, the thermal stability of other energy-transducing membranes could be quite different. For example, TF_1 from the thermophilic bacterium PS3 has a much higher thermal stability because of its amino acid compositions which have several unique features [52,53]. Among these features are the high propensities of secondary structures and abundant charge interactions and reverse turns [52,53].

Our results consistently show that ATP synthetic capabilities of the enzyme were more sensitive to temperature than the proton-pumping ability and ATP hydrolysis activity. For example, at 54°C, the synthesis activity, either by the substrates NADH, succinate or by ATP (for ATP/P_i exchange) was completely demolished, yet the hydrolysis activity of the enzyme was either unchanged or slightly enhanced. At this temperature F_0 was still intact, and there was no leakage of proton through the membrane (Fig. 1B). Neither was there significant release of enzyme subunits from the membrane-associated complex (Fig. 3B). Most investigators agree that a slight leakage of the energy-transducing membrane could lead to uncoupling of protonmotive force and the coupling factor leading to a complete abolition of ATP synthetic ability without much effect on hydrolysis and proton-pumping activities of the ATPase. Our result on $^{86}Rb^+$ uptake by heat-treated submitochondrial particles indicates that there was no change of membrane permeability up to 58°C (unpublished result of Knox and Tsong), consistent with proton pumping and oligomycin-sensitivity activity measurements. These results are difficult to reconcile with current notions that the ATP synthetic and ATP hydrolytic mechanisms are the reverse of each other [1]. However, there are some experimental evidences bearing on the possibility of separate catalytic pathways. It has been shown that removal of 'tightly bound' adenine nucleotides can inhibit selectively energy-coupled reactions, but not ATP hydrolysis [32]. Modified F_1 when reconstituted with F_1-depleted membranes retain approx. 50% of the synthetic capability, while ATP hydrolysis is nearly completely inhibited [42]. Effects of various inhibitors on the two activities of the F_0F_1 complex have been found to be at variant with a single active-site model [43]. Finally, results of trypsin treatment of F_1 compared to F_0F_1-ATPase suggest two pathways for ATP synthesis and hydrolysis [44]. A review bearing on this topic is available [7].

Two proposals concerning the organization of F_0F_1-ATPase could explain our result on thermal inactivation. The first one envisions two separate proton channels, one for synthesis (μ_{H^+} channel) and the other for hydrolysis (pumping channel). The opening/closing of each channel is regulated by the enzyme in accordance with its function. The thermal inactivation could denature the 'synthesis channel' before denaturing the 'pumping channel'. Alternatively, there can be a single proton channel (F_0), with a crossing point from two control mechanisms within the F_1, one control for synthesis and the other for pumping, or hydrolysis. The thermal inactivation results, in this case, would indicate denaturation of the synthesis control subunits before denaturation of the channel. The hydrolytic function, apparently, remained intact until 62°C. There is no evidence which favors either one of the two mechanisms, although we feel that the latter is a more likely path.

The nature of the change caused by heating is of interest because it could lead to a further understanding of the conformational requirements for energy coupling. Mapping of subunits affected at different temperatures and their functional consequences should provide useful information for the understanding of mechanisms of ATP synthesis. It is unlikely that the thermally induced conformational changes reported here are directly coupled to energy transduction, since these transitions were irreversible. However, reversible conformational changes could be induced by other means, e.g., protonation and deprotonation of a charged group, cation binding, ligand binding, charge migration [45], or by an electric field associated with a transmembrane potential. When these occur, the conformational changes are equivalent to thermally induced transitions because the effect of electric field, proton, cation and ligand bindings is to alter the thermal stability of proteins. It

appears that the voltage, or $\Delta\mu_{H^+}$, sensing region of the F_0F_1 complex, or the region for transduction of the electric field energy, extends beyond F_0 and it was rendered inoperative 6–8 degrees below the temperature in which F_0 was denatured. Data show that this same region is also required for the substrate-driven synthesis and it was stabilized against temperature inactiviation by a tight ADP binding.

Most of the electron transport process can now be traced back to the action of enzymes, redox or transport proteins. Thus, energy coupling must be governed by the same set of thermodynamic and kinetic principle that governs the chemistry of proteins. Conformational coupling hypothesis [5,9–12,24] aptly considers these facts. Recent experimental observations that membrane-bound ATPases are capable of absorbing free energy from an oscillating or a pulsed electric field [13–17,20–23] would suggest that conformational changes of these proteins could be induced by a transmembrane electric field. In these experiments a proton gradient was not present initially. Since redox potential of the electron-transport chain can establish, or is equivalent to, a transmembrane electric field, F_0F_1-ATPase should be able to respond, directly, to the electron flow, which inevitably must conduct through the prosthetic groups or iron-sulfur clusters of the protein complexes, and capture from it free energy for ATP synthesis [15]. Our recent experiments (Tomita, M. and Tsong, T.Y., unpublished data) indicate that thermal inactivation of the pulsed-electric-field-induced ATP synthesis follows the same inactivation curve of the succinate-driven ATP synthesis, suggesting that both syntheses are by the same mechanism. These results have been successfully interpreted by proposing an electroconformational coupling of the ATPase with the applied electric field [15–17,46–48]. Furthermore, we have observed that pulsed-electric-field-induced ATP synthesis was greatly enhanced by dithiothreitol, suggesting that certain –SH groups are essential for voltage sensing [54]. Whether the low thermal stability region (52°C) involves denaturation of the hypothetical voltage-sensing region remains to be established.

References

1 Boyer, P.D., Chance, B., Ernster, L., Mitchell, P., Racker, E. and Slater, E.C. (1977) Annu. Rev. Biochem. 45, 955–1026
2 Mitchell, P. (1979) Science 206, 1148–1159
3 Nicholls, D.G. (1982) Bioenergetics, Academic Press, London
4 Williams, R.J.P. (1982) FEBS Lett. 150, 1–3
5 Boyer, P.D. (1977) Trends Biochem. Sci. 2, 38–41
6 Penefsky, H.S., Cross, R.L. and Grumeyer, C. (1982) Abstracts of the Second European Bioenergetics Conference, Lyon, France, pp. 17–18
7 Cross, R.L. (1981) Annu. Rev. Biochem. 50, 681–714
8 Amzel, M. and Pedersen, P.L. (1983) Annu. Rev. Biochem. 52, 801–824
9 Kozlov, I.A. and Skulachev, V.P. (1977) Biochim. Biophys. Acta 463, 29–89
10 Boyer, P.D. (1980) in Membrane Bioenergetics (Lee, C.P., Schatz, G. and Ernster, L., eds.), pp. 461–479, Addison-Wesley, Palo Alto
11 Mitchell, P. (1981) in Mitochondria and Microsomes (Lee, C.P. and Schatz, G., eds.), pp. 427–457, Addison-Wesley, Palo Alto
12 Boyer, P.D., Kohlbrenner, W.E., McIntosh, D.B., Smith, L.T. and O'Neal, C.C. (1982) Ann. NY Acad. Sci. 402, 65–83
13 Teissie, J., Knox, B.E., Tsong, T.Y. and Wehrle, J. (1981) Proc. Natl. Acad. Sci. USA 78, 7473–7477
14 Knox, B.E. and Tsong, T.Y. (1984) J. Biol. Chem. 259, 4757–4763
15 Tsong, T.Y. and Astumian, R.D. (1985) Bioelectrochem. Bioenerg. 15, 457–476
16 Tsong, T.Y. (1983) Biosci. Rep. 3, 487–505
17 Westerhoff, H.V., Tsong, T.Y., Chock, P.B., Chen, Y.-D. and Astumian, R.D. (1986) Proc. Natl. Acad. Sci. USA 83, 4734–4738
18 Wada, A. (1976) Adv. Biophys. 9, 1–63
19 Hol, W.G.J., Van Duijnen, P.T. and Berendsen, H.J.C. (1978) Nature (London) 273, 443–446
20 Witt, H., Schlodder, E. and Gräber, P. (1976) FEBS Lett. 69, 272–276
21 Schlodder, E. and Witt, H.T. (1981) Biochim. Biophys. Acta 461, 426–440
22 Rogner, M., Ohno, K., Hamamoto, T., Sone, N. and Kagawa, Y. (1979) Biochem. Biophys. Res. Commun. 91, 362–367
23 Teissie, J. (1986) Biochemistry 25, 368–373
24 Slater, E.C. (1953) Nature (London) 172, 975–976
25 Penefsky, H.S. and Warner, R.C. (1965) J. Biol. Chem. 240, 4694–4702
26 Williams, N., Hullihen, J.M. and Pedersen, P.L. (1984) Biochemistry 23, 780–785
27 Blair, P.V. (1967) Methods Enzymol. 10, 78–81
28 Hansen, M. and Smith, A.L. (1964) Biochim. Biophys. Acta 81, 214–222
29 Kagawa, Y. and Racker, E. (1971) J. Biol. Chem. 246, 5477–5487

30 Pullman, M., Penefsky, H., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3322–3329
31 Penefsky, H.S. (1974) J. Biol. Chem. 249, 3579–3585
32 Leimgruber, R.M. and Senior, A.E. (1976) J. Biol. Chem. 251, 7103–7113
33 Dufour, J.-P., Goffeau, A. and Tsong, T.Y. (1982) J. Biol. Chem. 257, 9365–9371
34 Nielsen, S.O. and A.L. Lehninger, A.L. (1955) J. Biol. Chem. 246, 5477–5487
35 Errede, B., Kamen, M.D. and Hatefi, Y. (1978) Methods Enzymol. 53, 40–47
36 Laemmli, U.K. (1970) Nature (London) 227, 680–685
37 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
38 Strutevant, J.M. (1974) Annu. Rev. Biophys. Bioenerg. 3, 35–51
39 Knox, B.E. and Tsong, T.Y. (1984) Biophys. J. 45, 296a
40 Moreadith, R.W., Batshaw, M.L., Ohnishi, T., Kerr, D., Knox, B.E., Jackson, D., Hruban, R., Olson, J., Reynafaje, B. and Lehninger, A.L. (1984) J. Clin. Invest. 74, 685–697
41 Tsong, T.Y., Hearn, R.P., Wrathall, D.P. and Sturtevant, J.M. (1970) Biochemistry 9, 2666–2677
42 Steinmer, R.C. and Wang, J.H. (1979) Biochemistry 18, 11–18
43 Matsuno-Yagi, A. and Hatefi, Y. (1984) Biochemistry 23, 3508–3515
44 Pedersen, P.L., Hullihen, J. and Wehrle, J. (1981) J. Biol. Chem. 256, 1362–1369
45 Boyer, P.D. (1984) in H^+-ATPase: Structure, Function, Biogenesis (Papa, S., Altendorf, K., Ernster, L. and Packer, L., eds.), pp. 329–338, Adriatica Editrice, Bari
46 Astumian, R.D., Chock, P.B., Tsong, T.Y., Chen, Y.-D. and Westerhoff, H.V. (1986) Proc. Natl. Acad. Sci. USA 84, 434–438
47 Tsong, T.Y. and Astumian, R.D. (1987) Prog. Biophys. Mol. Biol., in the press
48 Tsong, T.Y. and Astumian, R.D. (1988) Annu. Rev. Physiol. 50, in the press
49 Penefsky, H.S., Pullman, M.E., Datta, A. and Racker, E. (1960) J. Biol. Chem. 235, 3330–3336
50 Kagawa, Y. and Racker, E. (1966) J. Biol. Chem. 241, 2461–2466
51 Sone, N. and Nicholls, P. (1984) Biochemistry 23, 6550–6554
52 Kagawa, Y., Ishizuka, M., Saishu, T. and Nakao, S. (1986) J. Biochem. 100, 923–934
53 Saishu, T., Nojima, H. and Kagawa, Y. (1986) Biochim. Biophys. Acta 867, 97–106
54 Chauvin, F., Astumian, R.D. and Tsong, T.Y. (1987) Biophys. J. 51, 243a