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THE "ENERGIZED STATE" OF MITOCHONDRIA: LIFETIME AND ATP EQUIVALENCE

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SUMMARY

1. Aequorin, a bioluminescent jellyfish protein, can measure the concentration of free Ca^{2+} in a rapid, sensitive and specific manner, in biological systems.

2. The movements of the nanomolar concentration of mitochondrial "endogenous" Ca^{2+} have been studied, and found to be a reversible indicator of the energization of mitochondria.

3. The comparison between the kinetics of cytochromes, changes on addition of respiratory inhibitors, and of the release of endogenous mitochondrial Ca^{2+} has brought to the conclusion that the lifetime of the energized state is 7 ± 2 sec in rat-liver mitochondria at 25°.

4. The energy content of the energized state was also determined, by comparing the kinetics of Ca^{2+} efflux from mitochondria in the presence and absence of oligomycin and measuring the content of endogenous ATP. It was found to be 0.3 μmole ATP equivalents per g mitochondrial protein.

5. The endogenous mitochondrial Ca^{2+} has been found to be in a highly dynamic state. Therefore the proton movements observed on activation of respiration can be considered not primary, but associated with ion movements.

INTRODUCTION

The mechanisms of oxidative phosphorylation in mitochondria that have been so far proposed require the existence of intermediates between the oxidation reactions of the respiratory carriers and the synthesis of ATP. The intermediates of the energy transfer chain have been tentatively assumed to be specific chemical compounds[1], gradients of electrochemical activity[2], or changes in the structure of the mitochondrial membrane[3–6].

Attempts have been made to identify these intermediates in terms of their physical nature, dimensions, and lifetime. The isolation of chemical intermediates of oxidative phosphorylation has not yet been successful[7] and the direct determination of the membrane potential[8] has given values that do not support a chemiosmotic mechanism of energy coupling. Similarly, the identification of different conformational

Abbreviation: FCCP, carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone.

states in different metabolic conditions has not, in most cases, progressed beyond a simple correlation with the gross morphology and functional states of the mitochondrial membranes[4]. Thus it appears that structural changes at the molecular level as detected by fluorescence indicators[6] may bear a more direct and close relationship to the energy conservation system.

ATP-jump studies of the content of high energy intermediates in rat-liver mitochondria have given a value of 0.8 nmole of ATP per mg mitochondrial protein[9]. The same type of experiment has instead lead VAN DAM[10] to conclude that the concentration of high energy intermediates must be at least one order of magnitude smaller than that of the endogenous NAD^+. The values that can be calculated on the basis of a transmembrane potential of 270 mV, or a pH gradient of 2 units across the coupling membrane, according to the chemiosmotic hypothesis, are, respectively, 0.24 or 12 nmoles/mg protein[2].

The lifetime of the energized state varies equally widely according to the parameter measured. In one of the first measurements of the energized state, cytochrome b_{555} compounds were spectroscopically detectable for 16 min at 25° in sulfide-treated pigeon-heart mitochondria[11]. A similar duration has recently been determined in rat-liver mitochondria, employing Ca^{2+} binding in the absence of an energy supply as indicative of the energized state [12]. The electron micrographic data[13] for the "condensed-to-orthodox" transition indicate that mitochondria freshly prepared from rat liver remain in the energized state for approx. 20 min at 0° in the absence of O_2; the data for beef-heart mitochondria are of the same type and order of magnitude[14].

According to the chemiosmotic hypothesis of oxidative phosphorylation[2], approx. 6 min are required for proton equilibration across the membrane and the decay of the energized state in intact rat-liver mitochondria; the half-time for this process is 90 sec. The transmembrane potential equilibrates in 7 sec.

In the studies reported here, the movement of nanomolar amounts of Ca^{2+} has been taken as indicative of the energy state of the mitochondria, and the luminescence of the jellyfish protein, aequorin[15], has been used to measure the concentration of Ca^{2+} appearing external to the mitochondria.

Although the detailed biochemical mechanisms are very different, the sensitivity of the aequorin luminescence to free Ca^{2+} may be compared with the sensitivity of luminous bacteria to very low concentrations of O_2, and affords another example of the many applications of bioluminescence to analytical biochemistry. The bacteria, which were found to be without parallel in the analysis of the kinetics of O_2 utilization by mitochondria[16], here find their counterpart in aequorin, which provides a detailed analysis of the time course of Ca^{2+} utilization by the mitochondria.

By using this new approach, evidence is provided that the energy content of rat-liver mitochondria in the absence of an energy supply from respiration or ATP is as low as 0.4 μmole of ATP per g mitochondrial protein, and that the lifetime of the energized state is 7 sec at room temperature for rat-liver mitochondria.

MATERIALS AND METHODS

Rat-liver mitochondria were prepared as described previously[17]. 1 mM EDTA was always included in the preparation media, except for the last two washing steps.

Cytochrome absorbance was recorded by means of a double-beam spectro-

photometer[18], or a triple-beam spectrophotometer[19]. Aequorin bioluminescence was measured by an EMI 9524B phototube connected to an electrometer. When no other optical measurements were carried out together with aequorin bioluminescence, aequorin light emission was not filtered at any particular wavelength. A Corning glass blue filter (CS 5-59) was used instead to avoid interference during simultaneous cytochrome measurements.

The reaction medium always contained 150 mM KCl and 20 mM Tris-HCl (pH 7.4). 200 μM $MgCl_2$ was also included in order to saturate the chelating capacity of the EDTA present in the aequorin solution (5 μM final concentration). Aequorin was kindly donated by Drs. F. H. Johnson and O. Shimomura.

RESULTS

The reaction of Ca^{2+} with aequorin

Aequorin, extracted and purified for the first time by SHIMOMURA *et al.*[15], has been shown to react with Ca^{2+} in a highly specific and sensitive manner. Aequorin has also been employed by RIDGEWAY *et al.*[20], as an indicator of Ca^{2+} movements associated with muscle contraction and relaxation. The pseudo-first-order velocity constant for the reaction of Ca^{2+} with aequorin has been found to be 2600 sec^{-1} (unpublished observations in collaboration with F. Blinks).

Fig. 1A shows a typical record of the response of 5 nM aequorin to the addition of 10 μM Ca^{2+}. The intensity of the aequorin luminescence increases rapidly after mixing, reaches a maximum, and then decays with an exponential time course. The height of the peak of the light emission and the velocity constant for the decay are proportional to the Ca^{2+} concentration. The relationship between Ca^{2+} concentration and light emission was found to be linear, and a log–log plot gives a slope of 2.0. On the other hand, the integral of the light intensity is proportional to the aequorin concentration.

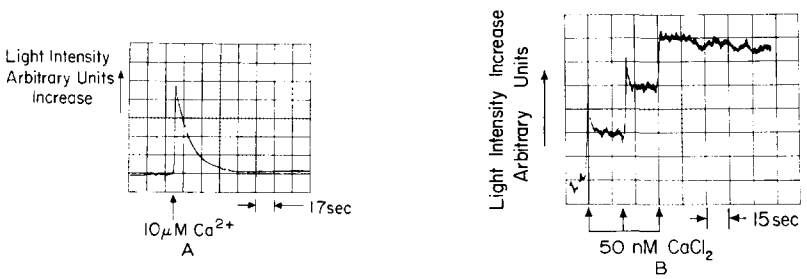

Fig. 1. The reaction of Ca^{2+} with aequorin. The reaction medium in (A) was: 250 mM sucrose, 10 mM Tris–succinate (pH 7.4), 5 nM aequorin; in (B) 200 μM $MgCl_2$, 3 nM aequorin. (AMA-86,89.)

Fig. 1B is a record of the reaction of aequorin with a very small concentration of Ca^{2+}. The addition of 50 nM $CaCl_2$ to a solution containing 5 nM aequorin results in a rapid light emission, followed by a slow decay phase. The signal-to-noise ratio of this record (as high as 10) and the reproducibility of the reaction induced by subsequent additions of Ca^{2+} are the most interesting features of this record. These characteristics combine with the slow decay of light intensity at low Ca^{2+} concen-

trations to make aequorin an extremely useful indicator for the continuous rapid measurement of very low Ca^{2+} concentrations.

The quantitative study of Ca^{2+} release from mitochondria requires that the response of the indicator be immediate and proportional to the increase in free Ca^{2+} concentration. These two kinetic characteristics of aequorin were examined by injecting $CaCl_2$ at a constant rate of 0.4 $\mu M \cdot sec^{-1}$ by means of an infusion pump into a 1.5 nM solution of the indicator (Fig. 2A). The light emission starts simultaneously with the Ca^{2+} infusion and increases linearly until aequorin becomes first limiting, and then exhausted. These two stages are represented by the decrease in the light emission to zero. In Figs. 2B and 2C are recorded for comparison the effects of adding

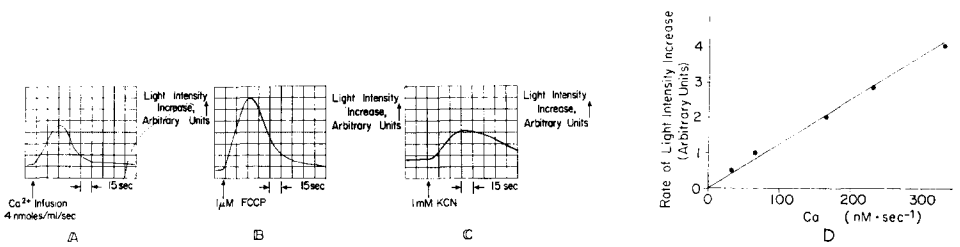

Fig. 2. The reaction of Ca^{2+} with aequorin: The linearity in time and Ca^{2+} concentration. The reaction medium contained 150 mM KCl, 20 mM Tris–HCl (pH 7.4), 10 mM succinate, 200 μM $MgCl_2$ and 1.3 nM aequorin. (AMA-87,82.) Washed rat-liver mitochondria concentration in B en C, 1,5 mg protein per ml.

carbonyl cyanide *p*-trifluoromethoxyphenyl hydrazone (FCCP) and KCN to a suspension of rat-liver mitochondria under the same experimental conditions of Fig. 2A. Since the characteristics of the process of Ca^{2+} release from mitochondria will be discussed in detail in the later paragraphs, the interest of these records is focussed upon the similarity between the simulated infusion (A) and the release of endogenous Ca^{2+} from mitochondria (B and C), and in the different slopes of the rising part of the curves, that is proportional to $\Delta Ca^{2+}/\Delta t$ (see Fig. 2D). In fact, a plot of the rate of change of light intensity *vs.* the rate of Ca^{2+} infusion (Fig. 2D) is linear between 0 and 0.3 $\mu M \cdot sec^{-1}$ Ca^{2+}. The method is, therefore, adequate for the kinetic measurement of the release and uptake of Ca^{2+} in mitochondria on the order of $\mu M \cdot min^{-1}$.

Oxidation–reduction changes of cytochromes and energy conservation

In coupled mitochondria, electron transport in the respiratory chain is associated with energy conservation. The consequences of this energy conservation are ATP synthesis, ion transport, and other reactions that utilize energy to form high energy bonds, ion concentration gradients, and oxidation–reduction or membrane potentials.

The unequal distribution of the Ca^{2+} on the two sides of the membrane that is normally associated with mitochondria has been chosen as an index of the energized state for the following reasons: first, the use of a normal constituent, such as the endogenous Ca^{2+}, of the mitochondria for detecting the state of the membrane does not perturb the energetic equilibrium of the system; and second, the aequorin indicator is the most sensitive and specific indicator for the fast and continuous readout of an energy-linked reaction.

In Fig. 3, the redox state of cytochrome *b* and the rate of Ca^{2+} efflux from rat-liver mitochondria are measured simultaneously. The addition of CN^- brings about a reduction of cytochrome *b* that is complete in about 16 sec. The luminescence trace during this time interval shows that Ca^{2+} is released very slowly after CN^- addition. The addition of oligomycin to block energy-coupled reactions results in a several-fold stimulation of the rate of Ca^{2+} efflux, suggesting that even after complete reduction of the respiratory chain, sufficient energy from endogenous ATP is still available to retain Ca^{2+} bound to the membrane. In the following experiments, therefore, oligomycin is always included when the characteristics of the non-ATP energy "pool" are studied.

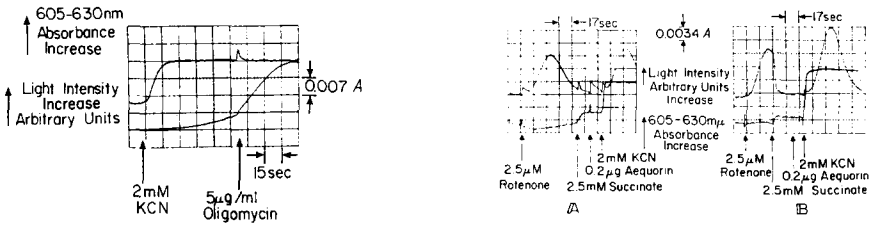

Fig. 3. Ca^{2+} movements and the redox state of cytochrome *b*. The reaction medium was 150 mM KCl, 10 mM Tris–HCl (pH 7.4), 200 μM $MgCl_2$, 10 nM aequorin. Pigeon-heart mitochondria, 5 mg protein per ml. (AMA-90.)

Fig. 4. Reversibility of Ca^{2+} movements in State 1 to 3 transitions. The reaction medium was as in Fig. 3 except that 10 μg oligomycin were also present and washed rat-liver mitochondria were used instead, at a concentration of 6.2 mg protein per ml. (AMA-45.)

The reversibility of the process of Ca^{2+} release from mitochondria

The postulate that free Ca^{2+} is a reversible indicator of the energy state of the mitochondrial membrane requires evidence that the Ca^{2+} released in the absence of energy supply can be taken up again when energy is again provided. This phenomenon is shown in the two experiments of Figs. 4 A and 4 B, where Ca^{2+} uptake and release from mitochondria are followed, together with oxidation–reduction changes of cytochrome oxidase. In Fig. 4A, the addition of rotenone to block the utilization of most endogenous substrates, induces an oxidation of cytochrome *a* and the release of endogenous Ca^{2+}. The addition of succinate activates electron transport and reduces cytochrome oxidase; aequorin, added after succinate, does not respond, indicating that the released Ca^{2+} had already been taken up by the mitochondria. The addition of CN^- at this point reduces the oxidase completely and stimulates a second release of Ca^{2+}, as shown by the sharp rise in the aequorin trace.

Fig. 4B is very similar to Fig. 4A, the only difference being that the addition of succinate when there is still some unreacted aequorin in the medium induces a decrease of light intensity that is much more rapid than the spontaneous decay of Fig. 4A. This observation is consistent with a rapid accumulation of Ca^{2+} induced by the activation of electron transfer and associated energy coupling on adding succinate.

It appears that the endogenous Ca^{2+} of mitochondria is in a highly dynamic state, being released when the electron transport is stopped or an uncoupler added, and taken up when the system is provided with an energy supply. The release or reten-

tion of endogenous Ca^{2+} affords, therefore, a true indicator of the energy state of the membrane.

Fig. 5 provides a record of the State 5→3 transition. The mitochondria are allowed to become anaerobic in the regenerative flow apparatus in the presence of succinate as an oxidizable substrate, and of aequorin as an extramitochondrial Ca^{2+} indicator. Ca^{2+} efflux starts when anaerobiosis is reached and is complete in several seconds. At this point, O_2 is added at a final concentration of 15 μM. The O_2 response of cytochromes *a* and *b* is shown in Fig. 5A on a time scale of 50 msec per division. The rapid oxidation of the cytochromes (half-time, 0.5 msec for cytochrome *a* and 50 msec for cytochrome *b*) may be compared with the time course of the decrease in light intensity of aequorin to a zero level in Fig. 5B (top line) on a 1-sec time scale (for experimental details, see the figure legend). The half-time for Ca^{2+} transport appears to be about 20-fold longer than cytochrome *b* oxidation and 2000-fold longer than for cytochrome *a* oxidation.

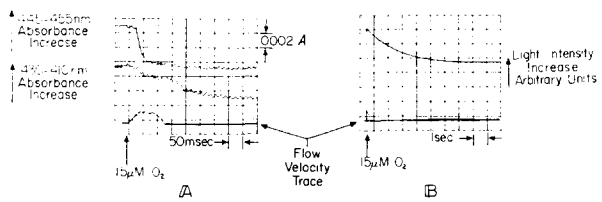

Fig. 5. The reversibility of Ca^{2+} movements during State 5 to 3 transitions. The reaction medium was as in Fig. 2. Washed rat-liver mitochondria were used at a concentration of 2 mg protein per ml. (AMA-91.)

The effect of mitochondrial inhibitors on Ca^{2+} release from mitochondria: The lifetime of the energized state

The experiments of Figs. 4A and 4B not only have established a correlation between Ca^{2+} transport and energized state in mitochondria, but also permit to give a value to the lifetime of the energized state. The addition of an inhibitor such as KCN to oligomycin-supplemented State 4 rat-liver mitochondria, induces a fast reduction of cytochrome *a* (=1 sec). When the electron transport is inhibited, Ca^{2+} starts to be released from the mitochondrial membrane, but reaches its maximum efflux rate only after 6.5 sec from the complete reduction of cytochrome *a*. During this induction period energy appears thus to be available for Ca^{2+} retention in the mitochondrial membrane, and only when these energy reserves are exhausted Ca^{2+} is free to move out of the mitochondrial membrane. The lifetime of these energy reserves in KCN–oligomycin-treated mitochondria appeared to be in eight different experiments 72 ±sec.

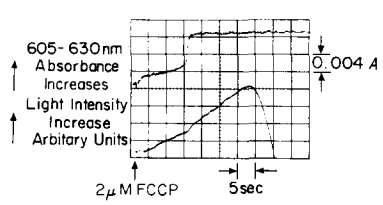

Fig. 6. Effect of FCCP on the rate of Ca^{2+} release from mitochondria. The conditions were equal to Fig. 4. Washed rat-liver mitochondria concentration, 7.4 mg protein per ml. (AMA-115.)

The effect of FCCP on the release of endogenous mitochondrial Ca^{2+} is reported in the experiments of Fig. 6. On addition of FCCP (2 μM) to rat-liver mitochondria, Ca^{2+} efflux begins in less than 1 sec from the moment when cytochrome *a* has reached its new, more reduced, steady state. After 10 sec of active respiration, cytochrome *a* becomes suddenly maximally reduced and a slight increase in the rate of Ca^{2+} efflux is also observed, indicating that the suspension has become anaerobic.

The effect of temperature on Ca^{2+} release

By lowering the temperature to 7°, a clearer picture of the relationship between cytochrome changes and Ca^{2+} release from mitochondria may be obtained. In Fig. 7, the addition of CN^- induces a reduction of cytochrome *a* that is complete in approx. 8 sec. At this point the Ca^{2+} efflux starts, and the maximal rate is attained after 50 sec. This 50-sec lifetime of the energized state at 7° may be compared with the 7-sec lifetime at 22°.

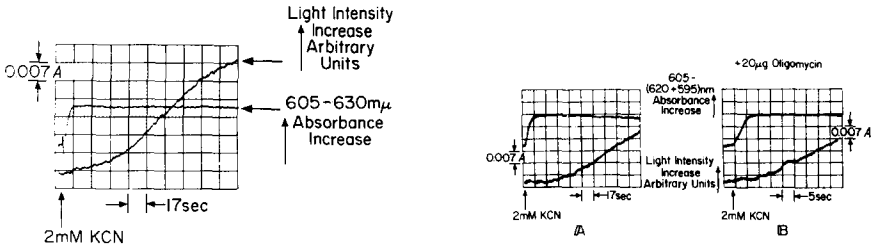

Fig. 7. The effect of temperature on the release of Ca^{2+} from mitochondria. Experimental conditions were equal to Fig. 2. 10 mM glutamate was also present and washed pigeon-heart mitochondria, at a concentration of 3.4 mg protein per ml. Temp., 7°. (2435 A.)

Fig. 8. A comparison between Ca^{2+} release from mitochondria in the presence and absence of oligomycin. Conditions as in Fig. 2. 2 μM rotenone was also present. This experiment was carried out in the triple-beam spectrophotometer. (AMA-114.)

The energy content of the energized state

In Figs. 8A and 8B, the addition of CN^- causes reduction of the oxidase and Ca^{2+} release. In Fig. 8A, in the absence of oligomycin, the induction period of 75 sec between the addition of CN^- and the release of Ca^{2+} is attributed to the utilization of endogenous ATP as an energy source for maintaining Ca^{2+} in the mitochondria (also see Fig. 3). However, in the presence of oligomycin, the release of Ca^{2+} starts within 5 sec of CN^- addition. This experiment indicates that in the presence of oligomycin, only 1/15 of the energy available in the absence of oligomycin can be utilized for the retention of Ca^{2+}. The ATP content of the mitochondria used in the experiment of Fig. 8B was 5 μmoles/g protein in State 4 and 0.07 μmole/g protein in State 5 (+KCN). Therefore, assuming that ATP is hydrolyzed linearly with time[20], 1/15 of 5 μmoles is 0.33 μmole/g protein that is the energy stored at 25° in the presence of oligomycin and KCN.

DISCUSSION

The suitability of aequorin for measurements of Ca^{2+} movements

Various properties of the reaction of aequorin with Ca^{2+} render this luminescent protein most useful for the precise and rapid measurement of microquantities of Ca^{2+}.

First, the luminescence of aequorin is highly specific for Ca^{2+}, and is not induced by any other cation, except Sr^{2+} at much higher concentrations.

Secondly, aequorin is more sensitive than other indicators to very small amounts of Ca^{2+}, responding to Ca^{2+} concentrations as low as 50 nM with a signal-to-noise ratio of 10, or, at the limit, 5nM. This sensitivity may be compared with that of murexide[22] (in the region of 5 μM) or the Ca^{2+} microelectrode[23] (also in the region of 5 μM, but with a low specificity and a slow response time). The atomic absorption technique, while sensitive to nanomolar amounts of Ca^{2+}, is not suitable for continuous measurement.

Thirdly, the reaction of aequorin with Ca^{2+}, while not approaching the 5-μsec half-time of that of murexide, is very rapid: the 270-μsec response of aequorin is much faster than the 3.5-sec half-time for Ca^{2+} release upon addition of uncoupler as measured by the murexide technique. The Ca^{2+} infusion experiment of Fig. 2A, on the time scale of 15 sec per division, shows no induction period when free Ca^{2+} is added to an aequorin solution. This observation, combined with the fact that the rate of change of Ca^{2+} concentration is proportional to the rate of increase of aequorin luminescence intensity over a large range of Ca^{2+} infusion values (from 0.4 to 6 $\mu M \cdot sec^{-1}$), renders this indicator most appropriate for the kinetic study of Ca^{2+} movements in mitochondrial suspensions.

The localization of the reaction of aequorin with Ca^{2+} in mitochondrial suspensions

The high molecular weight of aequorin of approx. 21000 (ref. 24) makes it unlikely that the indicator is capable of penetrating the mitochondrial membrane.

Furthermore, aequorin has been seen to record the presence of Ca^{2+} only under conditions where the energy supply to the membrane is dissipated by respiratory inhibitors in the presence of oligomycin (Fig. 2) or by uncouplers (Fig. 6). When an energy supply is provided by endogenous ATP (Fig. 3) or by energy coupled reactions in the absence of inhibitors (Figs. 4A, 4B and 5) the aequorin luminescence disappears. Since it is known that Ca^{2+} is retained within the mitochondria in the energized state[25], it may be concluded that aequorin reacts only with free Ca^{2+} outside the mitochondrial membranes. It is thus highly specific for measuring the time course and extent of Ca^{2+} release during the transition from an energized to a non-energized state.

Ca^{2+} efflux from mitochondria: the lifetime of the energized state

Further evidence that the distribution of endogenous Ca^{2+} between the extra- and intramitochondrial spaces is controlled by the energy state of the mitochondria, rather than by the oxidation–reduction state of respiratory carriers *per se*, is provided by the time lag between the reduction of the carriers and the appearance of aequorin luminescence in the State 5→3 transition of Fig. 5 at room temperature, and in more detail in the CN^- inhibition of Fig. 7 at 7°. In the latter case, 8 sec are required for cytochrome *a* to become fully reduced, and there is a total time lag of 25 sec before the maximal rate of Ca^{2+} efflux is reached. At room temperature, the induction period is 7 sec.

The results of Fig. 6, in which an induction period of less than 1 sec is observed between the addition of FCCP to the oligomycin-treated mitochondria and the initiation of Ca^{2+} release, are inconsistent with the suggestion that the 7-sec time lag phase at 25° represents the time required for Ca^{2+} to cross the mitochondrial membrane. However, the inclusion of 200 μM $MgCl_2$ and 150 mM KCl in the incubation

medium excludes the possibility of energy-independent surface binding of Ca^{2+} (ref. 26). These ions compete strongly for the phospholipid sites of the membrane to which Ca^{2+} binds in the absence of an energy supply. In fact, in the absence of added Mg^{2+} and K^+, the lag period between the depletion of the energy reserves of the mitochondria and the appearance of free Ca^{2+} in the external medium might be attributed to the necessity of saturating such phospholipid binding sites.

The 17-sec lifetime of the energized state at 7° indicated in Fig. 7 is considerably shorter than the 20-min duration found in the electron microscopic studies of HACKENBROCK[13] and GREEN *et al.*[14]. The hypothesis that the movement of Ca^{2+} across the mitochondrial membrane in response to changes in the energy state of the mitochondria might reflect only a part of the energy supply of the mitochondria, unrelated to a second portion responsible for configurational changes of the type observed by electron microscopy, is denied by evidence from competition studies that all energy-requiring reactions in mitochondria are linked to the same energy pool[27,28]. On the other hand, it may be proposed that the electron microscopically observed conformation changes having been produced by the expenditure of energy, persists for several minutes after the energy level has reached zero.

Reactions that may be responsible for the dissipation of the energy store in terminally inhibited mitochondria even in the presence of oligomycin include movements of very small quantities of ions across the membrane, the reversed electron transport that starts immediately after the forward transport is inhibited, or the transhydrogenase reaction.

The calculation of the lifetime of the energized state does not, however, afford a means of distinguishing between the chemical and chemiosmotic hypotheses of energy conservation. A rapid decay of the energized state may be explained in the chemical hypothesis, in terms of the energy-dissipating reactions above, and in the chemiosmotic hypothesis in terms of the decay of the transmembrane potential to which only Ca^{2+} movements are sensitive. In both cases, the duration of the energized state is similar, and in the order of a few seconds.

The ATP equivalence of the energized state in mitochondria

The comparison between the times required for the release of endogenous Ca^{2+} from freshly prepared mitochondria in the presence and absence of oligomycin provides evidence that the energy storage in the terminally inhibited mitochondria in the presence of oligomycin is as small as 0.33 μmole of ATP equivalents per g protein. This calculation is based upon the assumption that the ATP hydrolysis in inhibited mitochondria is linear with time[21].

A similar conclusion can be derived by studies with aequorin in rotenone-supplemented and oligomycin-supplemented mitochondria in which Ca^{2+}, taken up upon addition of succinate, is released at a detectable rate within 1 sec of the oxidation of cytochrome *a* on addition of CN^-. Here, the energy store was calculated to be no greater than 0.3 μmole of ATP per mg protein, based on a State 4 rate of Ca^{2+} release of 20 ATP equivalents per mg protein per min[31].

The rapid formation of 0.8 nmole of ATP-jump experiments[9] has been interpreted as a direct measurement of the store of high energy intermediates of oxidative phosphorylation. However, VAN DAM[10] has suggested that the rapid ADP phosphorylation in a State 4 to State 3 transition is not related to conversion of high energy

intermediates to ATP, but to the rapid oxidation of NADH in such a transition. The concentration of high energy intermediates would be therefore equal or lower than 0.2 μmole ATP equivalents per g protein. The insensitivity of the ATP jump to uncouplers has led also to the postulate[9] that the intermediates of oxidative phosphorylation, once formed, are insensitive to uncoupling agents. While the magnitude of the ATP jump is consistent with the findings of this paper, the insensitivity to uncouplers is not supported by our results. Ca^{2+} efflux is, in fact, stimulated several-fold by FCCP (see Figs. 2B and 2C), and the induction period between reduction of the cytochromes and the release of Ca^{2+} is also decreased (*cf.* Fig. 6).

The ATP equivalence of the energized state, like its lifetime, may be equally well explained by the chemical and chemiosmotic hypotheses. The energy store of 0.24 nmole of ATP per mg protein resulting from a transmembrane potential of 270 mV in State 4 respiration[2] corresponds to the values of 0.4 nmole of ATP equivalents found by the aequorin method of this paper, and these are also consistent with the chemical hypothesis assuming that the high energy intermediates are stoichiometric with the cytochrome content of the respiratory chain[32].

Movements of the endogenous Ca^{2+} of mitochondria during metabolic transitions

A primary extrusion of H^+ upon addition of O_2 to an anaerobic suspension of coupled liver mitochondria has been considered by MITCHELL AND MOYLE[33] to be the consequence of alternate hydrogen atom and electron flow along the respiratory chain, and the cause of the transmembrane potential of 270 mV. The possibility that these H^+ movements could, instead, arise from concomitant movements of cations, particularly Ca^{2+}, as been proposed by CHANCE[34] and CHAPPELL AND HAARHOFF[35], who base this suggestion upon the decrease of H^+ movements following an O_2 pulse when ethyleneglycol-bis-(aminoethyl)-tetraacetic acid (EGTA) is included in the experimental medium. MITCHELL AND MOYLE[33], on the other hand, have instead questioned the mobility of Ca^{2+} associated with the mitochondrial membrane on the basis of the presence of Ca^{2+} in mitochondria prepared in an EDTA medium.

The results of this paper lead to the conclusion that the small calcium content of EDTA-prepared mitochondria behaves in the same way as free, added Ca^{2+} during metabolic transitions (*cf.* Fig. 3) and must, therefore, be regarded as highly mobile. As a consequence of this mobility of endogenous Ca^{2+}, the movements of H^+ associated with the transport of electrons in the respiratory chain of coupled mitochondria cannot be considered to be independent of the observed Ca^{2+} movements, and the primacy of H^+ movements in the events of energy conservation is thus questionable.

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