ACTION OF FLUORESCEIN MERCURIC ACETATE UPON MITOCHONDRIAL ENERGIZED PROCESSES

Martin J. Lee, Robert A. Harris, and David E. Green, Institute for Enzyme Research, University of Wisconsin, Madison, Wisconsin 53706.

Received July 22, 1969

SUMMARY

Fluorescein mercuric acetate (FMA*) has been shown to be a highly selective reagent for suppressing the energized to energized-twisted configurational transition of heavy beef heart mitochondria as determined by electron microscopy and by measurement of light scattering changes. At the concentrations required for this selective suppression, FMA does not affect the primary conformational changes of energy coupling as measured by the proton shift reaction. Furthermore, it has been shown that FMA prevents the binding of inorganic phosphate by beef heart mitochondria in the energized state. The mechanism of action of FMA on oxidative phosphorylation and energized translocation of Ca^{++} can be rationalized in terms of an energy cycle involving two successive conformational changes - a primary energy-conserving conformational change (FMA-insensitive) resulting in proton release and cation uptake, and a secondary conformational change (FMA-sensitive) involving binding of inorganic phosphate.

Two sequential configurational transitions of the cristal membrane have been shown to be intrinsic to energized processes in beef heart mitochondria: the substrate- or ATP-induced transition from the nonenergized to the energized configuration, and the phosphate-induced transition from the energized to the energized-twisted configuration (1). The ultrastructural characteristic of the first transition is the expansion of the junction between two apposed, distended cristal membranes so that the matrix space between the two membranes increases. The ultrastructural characteristic of the second transition is the invagination of the engaged membranes to form helical tubes. We have shown recently (2) that an osmotic counterforce (provided by sucrose media between 0.5 and 1.0 M) suppresses the second, but not the first, configurational transition.

---

*Abbreviations used in this paper are: m-ClCCP, carbonyl cyanide m-chlorophenyl hydrazone; FMA, fluorescein mercuric acetate; HBHM, heavy beef heart mitochondria; ETP_H, phosphorylating submitochondrial particles; DNP, dinitrophenol; p-CMPS, p-chloromercuriphenylsulfonate; Pi, inorganic phosphate.

The ultrastructural dissection of the energizing cycle into two separable configurational transitions has facilitated the search for reagents which specifically suppress one of the two configurational transitions (Figure 1). The present communication deals with the properties of a highly specific reagent, fluorescein mercuric acetate (FMA). This reagent prevents the phosphate-induced transition from the energized to the energized-twisted configuration, in contrast to the uncoupler m-ClCCP which prevents the substrate- or ATP-induced transition from the nonenergized to the energized configuration.

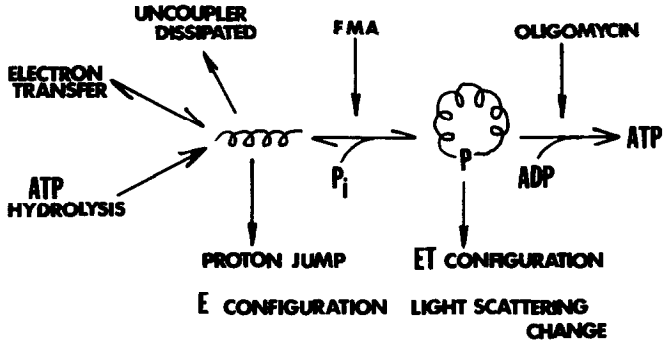

Figure 1. Postulated mechanism of energy transduction by the cristal membrane of the mitochondrion. The symbol stands for an energy conserving conformational change of the macromolecular repeating units of the cristal membrane.

MATERIALS AND METHODS

Many of the procedures and methods used in this study have been described in detail previously (1-4). These include the preparation of HBHM, HBHM (EGTA), and ETP_H, assay of ATP-^{32}Pi exchange, measurement of the rate of respiration, assay of ATPase activity, measurement of 90° light scattering changes, and analysis of the configurational state of the cristal membrane by electron microscopy. The proton jump of HBHM was monitored in a closed vessel equipped with a magnetic stirrer, oxygen electrode, and combination pH electrode in a circuit with a Beckman expandomatic pH meter and recorder. The centrifugation method used in this study to estimate phosphate binding by mitochondria was similar

in principle to that described by Hansford and Chappell (5) and also by Hunter and Brierley (6).

Fluorescein mercuric acetate (No. F-7375) was obtained from Sigma Chemical Company, St. Louis, Missouri. Stock solutions of the mercurial at a final concentration of 1 mM were prepared fresh daily. FMA was routinely dissolved in 0.1 N KOH to effect solubilization and adjusted to pH 7.4 with 0.1 N HCl.

RESULTS AND DISCUSSION

Uncoupling action of FMA - At a concentration of 10 mμmoles per mg of mitochondrial protein, FMA suppresses both the energized translocation of Ca^{++} and the ATP-^{32}Pi exchange activity of HBHM to the extent of about 90%. The electron transfer process is hardly affected (only 15% inhibited) by FMA at the concentrations which lead to suppression of these coupled processes. On the other hand, FMA markedly stimulates the ATPase activity of HBHM at these concentrations. We will document these results further in a subsequent communication (7) and show that FMA also inhibits oxidative phosphorylation in submitochondrial particles, such as ETP_H, as well as in mitochondria.

Suppression of the transition from the energized to the energized-twisted configuration - When beef heart mitochondria are exposed to substrate in the presence of inorganic phosphate, they undergo a rapid transition from the non-energized to the energized-twisted configuration (Figure 2A) via the energized configuration. The characteristic helical tubes of the energized-twisted membranes are unmistakable. When the same experiment is carried out in the presence of FMA (10 mμmoles per mg protein) (Figure 2B), the formation of the energized-twisted configuration is completely suppressed. The mitochondria shown in Figure 2B are predominantly in the energized configuration, as evidenced by the width of the engaged membrane.

Essentially the same demonstration of the action of FMA in suppressing the second configurational transition can be made by 90° light scattering measurements. The addition of succinate followed by inorganic phosphate (Figure 3A) leads to a major change in light scattering - the hallmark of the

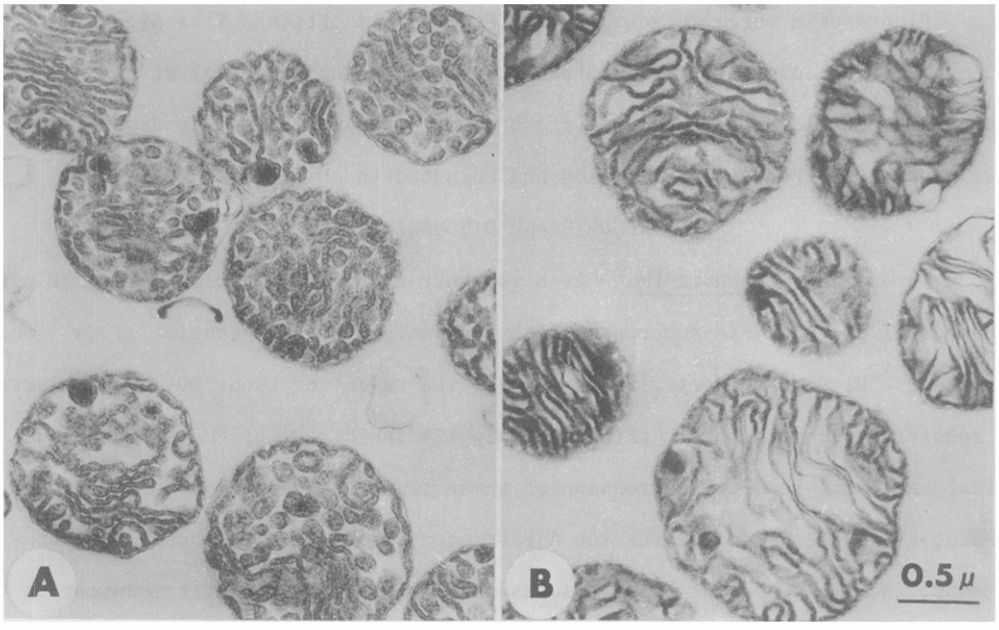

Figure 2. Effect of FMA upon generation of the energized-twisted configuration of the cristal membrane. The basic incubation medium was 0.25 M in sucrose and 5 mM in TrisCl, pH 7.4; to this was added rotenone (2 μg/mg protein) and mitochondria (1 mg protein/ml); A, energized-twisted configuration obtaining in the presence of inorganic phosphate (10 mM, pH 7.4) and succinate (5 mM); B, energized configuration (aggregated) obtaining in the presence of inorganic phosphate (10 mM, pH 7.4), succinate (5 mM), and FMA (10 μM).

phosphate-induced transition of the cristal membrane of the mitochondrion from the energized to the energized-twisted configuration (3,4). This transition is reversed by addition of m-ClCCP. When the same experiment is carried out in presence of FMA, the phosphate-induced light scattering change is completely abolished. Thus, two independent methods establish that FMA, at the appropriate concentration level, prevents the second configurational transition.

Suppression of the energy-dependent binding of inorganic phosphate - When beef heart mitochondria are energized by succinate in the presence of inorganic

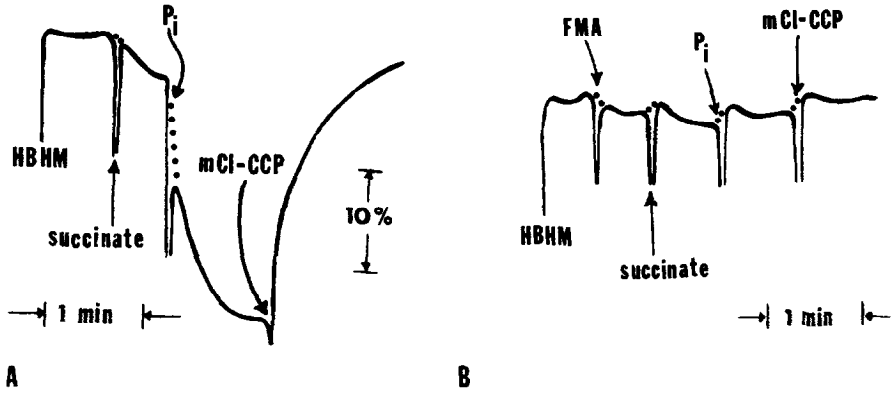

Figure 3. Effect of FMA upon the configurational changes of beef heart mitochondria as revealed by light scattering analysis. The basic incubation medium was as described in the legend to Figure 2. The additions specified in the figure made the medium 5 mM in succinate; 10 mM in Pi, pH 7.4; and 2 x 10^{-6} M in m-ClCCP. FMA was added in experiment B to a final concentration of 10 μM. The temperature was maintained at 30°. The vertical lines in the traces indicate when the light scattering instrument was opened briefly for making additions and then stirring the contents.

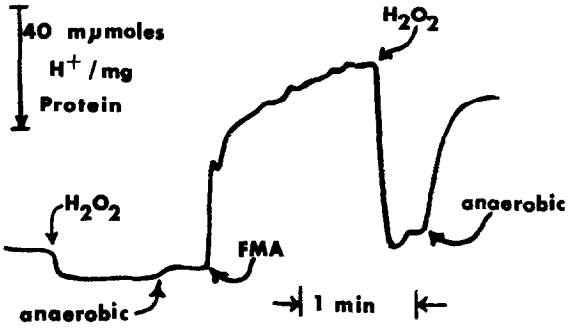

Figure 4. Effect of FMA upon the proton transfer reaction of HBHM. The incubation medium was 0.25 M in sucrose and 8 mM in tetramethylammonium succinate, and contained rotenone (2 μg/mg mitochondrial protein), catalase (40 μg/ml), and HBHM (3 mg of protein per ml). Aerobic-anaerobic transitions were induced by the injection of small quantities of H_2O_2 into the closed reaction vessel. FMA neutralized with tetramethylammonium hydroxide was added where indicated to a final concentration of 30 μM. Standardized HCl was used to measure the extent of the proton shift.

---

phosphate, some 50 mμmoles of additional inorganic phosphate per mg protein is bound by the mitochondria (Table I). This uptake is completely suppressed by both FMA and m-ClCCP; however, the mechanism of suppression is clearly different in the two cases. The uncoupler, m-ClCCP, prevents generation of the ener-

TABLE I

Effect of FMA upon the Binding of Pi by Energized Mitochondria

Additions[a]	Pi bound[b] mμmoles/mg protein
None	18
Succinate	69
Succinate; m-ClCCP	16
Succinate; FMA	15

[a]The basic incubation medium was 0.25 M in Ca^{++}-free sucrose, 5 mM in TrisCl, pH 7.4, and 10 mM in Pi, pH 7.4; it was supplemented with rotenone (2 μg/mg protein), rutamycin (2 μg/mg protein), catalase (30 μg/ml), and ^3H_2O (5 μc/ml). Succinate was added to a final concentration of 8 mM; m-ClCCP to 4×10^{-6} M, and FMA to 10 μM. Mitochondria (Ca^{++}-free) were present at a final concentration of 1 mg per ml. The samples were incubated for 45 sec at 25°. H_2O_2 was added to a final concentration of 8 mM to insure aerobic conditions. The mitochondria were sedimented with a high speed Misco centrifuge capable of reaching full speed (22,000 rpm) in less than 10 sec and of effecting complete sedimentation of mitochondria in 60 sec. The centrifuge tubes were drained of the incubation medium and wiped dry of adhering droplets. The pellets were extracted with 0.5 M perchloric acid. Total pellet water was determined from the tritium content. Total pellet phosphate was determined by the method of Chen et al. (16). The quantity of bound phosphate was calculated from the equation:

$$\text{Bound Pi (mμmoles/mg protein)} = \frac{\text{total pellet Pi (mμmoles)} - [\text{total pellet } H_2O \text{ (μl)} \times \text{medium Pi conc. (mM)}]}{\text{mg mitochondrial protein per pellet}}$$

[b]Averages of closely agreeing triplicates.

gized state of the membrane during which binding sites for inorganic phosphate are created, whereas FMA probably acts to inhibit phosphate binding and/or accumulation by reacting with sulfhydryl groups at or near the phosphate binding sites. We must distinguish between the interaction of inorganic phosphate with the energized membrane that leads eventually to ATP synthesis (the specific binding) and the interaction with binding sites generated by the conformational changes in the repeating units (the general binding). The specific binding

would amount to no more than 2 mμmoles of Pi per mg of mitochondrial protein.** Thus, FMA may suppress both the specific and the general binding of inorganic phosphate to sites exposed by generation of the energized state of the cristal membrane. Alternatively, FMA may prevent transmembrane phosphate movement. Experiments are in progress which should define this point more clearly.

Enhancement of the proton jump - When mitochondria are energized by substrate, a characteristic release of protons can be measured (Figure 4). This proton jump is an expression of the transition from the nonenergized to the energized state of the membrane providing two conditions are fulfilled: (a) a counter ion (e.g., Ca^{++}; K^{+}) is available for exchange with the protons and (b) the membrane is permeable to the ions involved. Reagents, such as valinomycin or gramicidin, can catalyze exchange of monovalent ions for protons and thus satisfy these requirements. The proton jump of energized mitochondria is enormously enhanced by FMA (Figure 4). The enhancement phenomenon is of considerable interest per se, but the important point we wish to make here is that FMA, far from interfering with the proton jump, actually increases it. We interpret this to be a token that FMA does not interfere with generation of the energized state (see Figure 1).

Reversal of uncoupling by DTT and other thiols - The uncoupling action of FMA on oxidative phosphorylation or on ATP-^{32}Pi exchange can be completely reversed by addition of dithiothreitol (5 mM). The thiol reagent can be added before or after the mercurial has interacted with the mitochondrial suspension. Substantial regeneration of coupling activity by addition of thiols has also been observed with m-ClCCP as uncoupler (up to 75% recovery with 5 mM dithiothreitol) but regeneration is marginal with 2,4-dinitrophenol (5% recovery).

Relative efficiencies of uncoupling agents and mercurials - The uncoupling

---

**This estimated number of specific phosphate binding sites is based upon the assumption that there probably are no more than 2 binding sites for inorganic phosphate per repeating unit of the cristal membrane and upon the calculation that there is approximately 1 mμmole of repeating units in the cristal membrane per mg of HBHM protein.

efficiencies of FMA, m-ClCCP, 2,4-dinitrophenol and p-chloromercuriphenylsulfonate were compared by their effects on the ATP-^{32}Pi exchange reaction of ETP_H. To achieve 50% inhibition of the exchange reaction with ETP_H at a final protein concentration of 1 mg per ml, the following concentrations of the above reagents were required: m-ClCCP, 0.042 μM; FMA, 4.4 μM; dinitrophenol, 16 μM; and p-chloromercuriphenylsulfonate, 47 μM. FMA, thus, lies in potency between one of the most powerful of uncouplers, m-ClCCP, and the classical uncoupling agent, 2,4-dinitrophenol.

Interaction of mercurials with sulfhydryl groups - Many organic mercurials have the capability for interacting with exposed sulfhydryl groups and such groups are intrinsic to almost all mitochondrial functions, be they electron transfer, coupled phosphorylation, or citric cycle oxidations. FMA shows somewhat higher sensitivity as well as selectivity over other mercurials in the sense that at the minimal concentration required for uncoupling, FMA appears to react preferentially with one or more sulfhydryl groups that have a key role in the second configurational transition. At higher concentrations, FMA can inhibit electron transfer as well and thus no longer acts as a selective reagent. The anthracene nucleus very likely makes an important contribution to the sensitivity of the mitochondrion to FMA, probably by enhancing its ability to penetrate the mitochondrial membranes and interact with sulfhydryl groups in the hydrophobic domains of the cristal membrane.

Reports from a number of laboratories have implicated sulfhydryl groups with an important but undefined role in energy conservation (8-15). Inhibition of various energized processes of the mitochondrion by sulfhydryl reagents has been repeatedly demonstrated. These include Cd^{++} (8), arsenite plus BAL (10), p-chloromercuriphenylsulfonate (11), p-hydroxymercuribenzoate (12), mersalyl (11,12) and Ellman's reagent (13). Tyler (12), Fonyo (14), and Haugaard et al. (15) have all provided evidence that sulfhydryl reagents make phosphate unavailable for participation in mitochondrial processes. The availability of a highly specific mercurial, namely FMA, now makes it possible to explore more systemat-

ically the mechanism of involvement of sulfhydryl groups in coupled phosphorylation. Since the binding of inorganic phosphate by mitochondria is energy dependent, it follows that the binding sites must be exposed or generated during the first configurational transition of the membrane. The elimination of phosphate binding by m-ClCCP is consistent with this interpretation. Since the binding of inorganic phosphate is inhibited by FMA without uncoupling the generation of the energized state, it is a reasonable inference that sulfhydryl groups may play a determinant role in phosphate binding by the cristal membrane.

In previous communications (1-4) a correlation has been established between the configurational state of the membrane and the energy state of the mitochondrion. It was this correlation which led to postulation of the conformational basis of energy transduction in mitochondria (1). On the basis of this correlation it would be predictable that specific reagents could be found which would interfere with one facet or another of the configurational cycle; indeed, such has been found to be the case for the uncoupler, m-ClCCP, and the mercurial FMA.

ACKNOWLEDGMENTS

This work was supported in part by the U.S. Public Health Service Program Project Grant GM-12,847 and by a training grant GM-88 from the National Institute of General Medical Sciences. Meat by-products were generously furnished by Oscar Mayer and Co., Madison, Wisconsin. Martin J. Lee is a postdoctoral trainee of the University of Wisconsin. The technical assistance provided by Miss Monya Caldwell and Mrs. Terry Spennetta is greatly appreciated.

REFERENCES

1. Green, D.E., Asai, J., Harris, R.A., and Penniston, J.T., Arch. Biochem. Biophys. 125, 684 (1968).
2. Harris, R.A., Williams, C.H., Jolly, W.W., Asai, J., and Green, D.E., in preparation.
3. Harris, R.A., Asbell, M.A., and Green, D.E., Arch. Biochem. Biophys. 131, 316 (1969).
4. Harris, R.A., Asbell, M.A., Jolly, W.W., Asai, J., and Green, D.E., Arch. Biochem. Biophys., July 1969.
5. Hansford, R.G., and Chappell, J.B., Biochem. Biophys. Res. Communs. 30, 643 (1968).

6. Hunter, G.R., and Brierley, G.P., Biochim. Biophys. Acta 180, 68 (1969).
7. Lee, M.J., Harris, R.A., and Green, D.E., in preparation.
8. Fluharty, A.L., and Sanadi, D.R., Biochemistry 2, 519 (1963).
9. Cooper, C., and Lehninger, A.L., J. Biol. Chem. 224, 561 (1957).
10. Fluharty, A.L., and Sanadi, D.R., J. Biol. Chem. 236, 2772 (1961).
11. Sanadi, D.R., Lam, K.W., and Kurup, C.K.R., Proc. Nat. Acad. Sci. U.S. 61, 277 (1968).
12. Tyler, D.D., Biochem. J. 107, 121 (1968).
13. Haugaard, N., Lee, N.H., Kostrzewa, R., Horn, R.S., and Haugaard, E.S., Biochim. Biophys. Acta 172, 198 (1969).
14. Fonyo, A., Biochem. Biophys. Res. Communs. 32, 624 (1968).
15. Knight, V.A., Settlemire, C.T., and Brierley, G.P., Biochem. Biophys. Res. Communs. 33, 287 (1968).
16. Chen, P.S., Jr., Toribara, T.Y., and Warner, H., Analyt. Chem. 28, 1756 (1956).