 DOE/ER/13845--T3

FINAL TECHNICAL REPORT

Department of Energy Research Grant

DE-FG03-88ER13845 A005

ENERGY CAPTURE AND USE IN PLANTS AND BACTERIA

Paul D. Boyer, Principal Investigator

University of California, Los Angeles

Grant Period, Jan 1, 1991 to Dec. 31, 1993

CONTENTS:

40

T3

Overall progress

The project has centered on elucidation of the mechanism of ATP synthase. The metabolic importance of ATP and the complexity of the ATP synthase have made the problem particularly important and challenging. Over the past two decades, with DOE and USPHS support, my laboratory has had the principal role in development of what is now designated as the binding change mechanism of ATP synthesis. This mechanism appears to have gained gratifyingly wide acceptance and appears in recent biochemistry texts. The support has thus helped provide useful and probably lasting information in one part of the broad scope of biochemistry.

The development of the binding change mechanism depended upon our recognition of features that were novel in bioenergetics and indeed to the field of enzymology. One important feature of the mechanism is that the principal way that energy input from transmembrane proton movement is coupled to ATP formation is to drive conformational changes that cause the release of ATP readily formed and tightly bound at a catalytic site. Another is that three equivalent catalytic sites on the enzyme show strong catalytic cooperativity as they proceed sequentially through different conformations. A more speculative feature is that this cooperativity and energy coupling involve a rotational movement of minor subunits relative to the catalytic subunits. This speculative hypothesis is likely to stimulate research in the field for some years.

Progress during the past three-year grant period

Publications appearing during this period have extended and clarified aspects of the synthase mechanism. During assessments of interactions of Mg^{2+} and ADP with the synthase we recognized unexpectedly that whether ADP and P_i, or their complexes with Mg^{2+}, served as substrates for ATP formation by photophosphorylation was not known. Our studies showed that MgADP and free P_i act as substrates.

Mg^{2+} has also been known to be a potent inhibitor of ATP hydrolysis by chloroplasts or the separated CF_1 ATPase. Such inhibition may have an important control function. Our studies show that the inhibition depends on the presence of ADP tightly bound at a catalytic site without bound P_i. Two inactive complexes with Mg^{2+} have been found, and their behavior characterized. The inhibitor azide acts by combining with and stabilizing the inhibitory Mg^{2+}-enzyme-ADP complex.

In other related studies the cooperative interactions induced by Mn^{++} were probed by EPR measurements of Mn^{++} binding and exchange. Binding and release rates and equilibria were assessed. The data are consistent with participation of three catalytic sites that show positive cooperativity and proceed sequentially through different conformations.

What are perhaps the most important results came from development and refinement of methods to measure the nature of bound nucleotides during photophosphorylation. This gave evidence that the energization of chloroplasts favors the formation of tightly bound ATP from tightly bound ADP and P_i, and that only two catalytic sites need to be filled for rapid photophosphorylation. The binding of ADP at a second catalytic site appears to allow the utilization of protonmotive force. These and other findings led to a modified scheme for the conformational changes accompanying catalysis that provide a better rational for the coupling of proton translocation to ATP synthesis. These are important refinements in the binding change mechanism.

Other experiments made use of the measurement of ^{18}O isotopomers of phosphate. One series revealed interesting aspects of the inhibitions of MF_1 ATPase by fluorosulfonylbenzoyl inosine and by efrapeptin. The different effects give insights into the nature of the catalytic cooperativity between the three catalytic sites.. Another series of experiments with ^{18}O provided needed information about intermediate steps in catalysis by a vacuolar pyrophosphatase that is coupled to transmembrane H^+ translocation.

List of Publications of Past Three Years

1. Hiller, R., and Carmeli, C. Kinetic analysis of cooperative interactions induced by Mn^{2+} binding to the chloroplast H^+-ATPase (1990) *Biochemistry* **29**, 6186-6192

2. Murataliev, M. B., Milgrom, Y. M., and Boyer, P. D. Characteristics of the combination of inhibitory Mg^{2+} and azide with ATPase from chloroplasts (1991) *Biochemistry* **30**, 8305-8310

3. Zhou, J.-M., and Boyer, P. D. MgADP and Free P_i as the substrates and the Mg^{2+} requirement for photophosphorylation (1992) *Biochemistry* **31**, 3166-3171

4. Kasho, V. N., Allison, W. S., and Boyer, P. D. Study of the mechanism of MF_1-ATPase inhibition by flurosulfonylbenzoyl inosine, quinacrine mustard, and efrapeptin using intermediate ^{18}O exchange as a probe (1993) *Arch. Biochem. Biophys.* **300**, 293-301

5. Zhou, J.-M., and Boyer, P. D. (1993) Evidence that energization of the chloroplast ATP synthase favors ATP formation at the tight binding site and increases the affinity for ADP at another site. *J. Biol. Chem.* **268**, 1531-1538

6. Baykov, A. A., Kasho, V. N., Bakuleva, N. P., and Rea, P. A. Characterization of oxygen exchange reactions catalyzed by mung bean vacuolar H^+-pyrophatase. (1993) *Submitted for publication*

Grant Interrelationships

Researches in my laboratory have been supported by this Department of Energy grant and by grant GM11094 of the U. S. Public Health Service entitled "Studies of ATP Synthase and Related Enzymes". The USPHS grant provided about $116,000 annually in direct costs. Of the publications listed above two carried acknowledgement to only DOE support. In the same time period five publications from my laboratory not listed above carried acknowledgement only to USPHS support.

Summaries of Publications Supported by the DOE Grant

The following direct copies of summaries from publication reprints are provided as a means of giving more information about progress during the last grant period.

Biochemistry (1990) 29, 6186-6192

Kinetic Analysis of Cooperative Interactions Induced by Mn^{2+} Binding to the Chloroplast H^+-ATPase†

R. Hiller and C. Carmeli*

Department of Biochemistry, Tel Aviv University, Tel Aviv 69978, Israel

Received January 30, 1989; Revised Manuscript Received January 8, 1990

ABSTRACT: The kinetics of Mn^{2+} binding to three cooperatively interacting sites in chloroplast H^+-ATPase (CF_1) were measured by EPR following rapid mixing of the enzyme with $MnCl_2$ with a time resolution of 8 ms. Mixing of the enzyme-bound Mn^{2+} with $MgCl_2$ gave a measure of the rate of exchange. The data could be best fitted to a kinetic model assuming three sequential, positively cooperative binding sites. (1) In the latent CF_1, the binding to all three sites had a similar on-rate constants of $(1.1 \pm 0.04) \times 10^4$ M^{-1} s^{-1}. (2) Site segregation was found in the release of ions with off-rate constants of 0.69 ± 0.04 s^{-1} for the first two and 0.055 ± 0.003 s^{-1} for the third. (3) Addition of one ADP per CF_1 caused a decrease in the off-rate constants to 0.31 ± 0.02 and 0.033 ± 0.008 s^{-1} for the first two and the third sites, respectively. (4) Heat activation of CF_1 increased the on-rate constant to $(4.2 \pm 0.92) \times 10^4$ M^{-1} s^{-1} and the off-rate constants of the first two and the third site to 1.34 ± 0.08 and 0.16 ± 0.07 s^{-1}, respectively. (5) The calculated thermodynamic dissociation constants were similar to those previously obtained from equilibrium binding studies. These findings were correlated to the rate constants obtained from studies of the catalysis and regulation of the H^+-ATPase. The data support the suggestion that regulation induces sequential progress of catalysis through the three active sites of the enzyme.

Biochemistry (1991) 30, 8305-8310

Characteristics of the Combination of Inhibitory Mg^{2+} and Azide with the F_1 ATPase from Chloroplasts†

Marat B. Muratalíev, Yakov M. Milgrom, and Paul D. Boyer*

The Molecular Biology Institute and the Department of Chemistry and Biochemistry, University of California, Los Angeles, Los Angeles, California 90024-1570

Received April 3, 1991; Revised Manuscript Received June 10, 1991

ABSTRACT: The interactions between ADP, Mg^{2+}, and azide that result in the inhibition of the chloroplast F_1 ATPase (CF_1) have been explored further. The binding of the inhibitory Mg^{2+} with low K_d is shown to occur only when tightly bound ADP is present at a catalytic site. Either the tightly bound ADP forms part of the Mg^{2+}-binding site or it induces conformational changes creating the high-affinity site for inhibitory Mg^{2+}. Kinetic studies show that CF_1 forms two catalytically inactive complexes with Mg^{2+}. The first complex results from Mg^{2+} binding with a K_d for Mg^{2+} dissociation of about 10–15 μM, followed by a slow conversion to a complex with a K_d of about 4 μM. The rate-limiting step of the CF_1 inactivation by Mg^{2+} is the initial Mg^{2+} binding. When medium Mg^{2+} is chelated with EDTA, the two complexes dissociate with half-times of about 1 and 7 min, respectively. Azide enhances the extent of Mg^{2+}-dependent inactivation by increasing the affinity of the enzyme for Mg^{2+} 3–4 times and prevents the reactivation of both complexes of CF_1 with ADP and Mg^{2+}. This results from decreasing the rate of Mg^{2+} release; neither the rate of Mg^{2+} binding to CF_1 nor the rate of isomerization of the first inactive complex to the more stable form is affected by azide. This suggests that the tight-binding site for the inhibitory azide requires prior binding of both ADP and Mg^{2+}.

Biochemistry (1992) 31, 3166-3171

MgADP and Free P_i as the Substrates and the Mg^{2+} Requirement for Photophosphorylation†

Jun-Mei Zhou and Paul D. Boyer*

Molecular Biology Institute and Department of Chemistry and Biochemistry, University of California, Los Angeles, Los Angeles, California 90024-1570

Received October 15, 1991; Revised Manuscript Received December 23, 1991

ABSTRACT: Previous studies have not provided definitive information about whether ADP or P_i or their complexes with Mg^{2+} serve as substrates for photophosphorylation and whether free Mg^{2+} or ADP is required. Results presented show MgADP, MgGDP, or MgUDP are substrates. At variable Mg^{2+} concentrations, observed velocities are determined by MgADP and not the free ADP concentration. The approximate K_m for MgADP with spinach chloroplasts is about 30 μM, for MgGDP 260 μM, and for MgUDP above 5 mM. The apparent K_m values for added ADP or Mg^{2+} are decreased to constant low values near 30 μM as the added Mg^{2+} or ADP concentrations, respectively, are increased to the millimolar range. With 100 μM added Mg^{2+}, near-maximal velocities can be obtained with excess ADP, but not with excess GDP or UDP. This is explainable by the apparent K_m values for MgGDP and MgUDP being well above 100 μM. High phosphorylation rates with excess of either Mg^{2+} or ADP present show tnat little or no (<2–3 μM) free Mg^{2+} or ADP is required. In addition, the results show that during rapid photophosphorylation, when one or more catalytic sites are always filled with nucleotide, free ADP does not combine and block the combination of MgADP to catalytic sites that become vacant. This is in contrast to the ability of free ADP to combine tightly with one catalytic site when all catalytic sites are empty. The apparent K_m for added ADP above a few micromolar concentration, and with excess Mg^{2+} present, results from binding of MgADP at a second catalytic site. In contrast to the behavior of ADP, the apparent K_m for P_i is independent of the Mg^{2+} concentration, showing that free P_i is the substrate. In addition, some deviations of photophosphorylation rates from simple Michaelis–Menten relationships are noted and discussed.

Arch. Biochem. Biophys. (1993) 300, 293-301

Study of the Mechanism of MF_1 ATPase Inhibition by Fluorosulfonylbenzoyl Inosine, Quinacrine Mustard, and Efrapeptin Using Intermediate ^{18}O Exchange as a Probe[1]

V. N. Kasho,*[,2] W. S. Allison,† and P. D. Boyer*

Department of Chemistry and Biochemistry, and Molecular Biology Institute, University of California, Los Angeles, California 90024; and †Department of Chemistry, University of California, San Diego, California 92093

The mitochondrial F_1-ATPase (MF_1) is known to be largely or totally inhibited by combination or reaction with one fluorosulfonylbenzoyl inosine (FSBI), quinacrine mustard, or efrapeptin per enzyme. Measurements were made with ^{18}O in attempt to ascertain if the weak catalytic activity remaining after exposure to excess of these reagents was due to retention of some activity by the enzyme modified by these inhibitors. Any such activity could have different characteristics that might be revealed by the distribution of [^{18}O]P_i isotopomers formed from [γ-^{18}O]ATP. The MF_1 inhibited by FSBI showed progressive appearance of two new catalytic pathways as inhibition proceeded. Both pathways appeared to be operative in the enzyme after one β subunit per enzyme had been modified by FSBI. A high exchange pathway showed no detectable change as ATP concentration was lowered. The lower exchange pathway showed an increase in the amount of exchange with lowering of the ATP concentration, similar to the cooperative behavior observed with the unmodified enzyme. With excess ATP more product was formed by the low exchange pathway, showing that compulsory alternation between two catalytic sites was not retained. The behavior can be explained by the ability of the modified β subunit to undergo binding changes similar to those occurring in catalysis, with the other two β subunits catalyzing sluggish hydrolysis by different pathways because of the asymmetry introduced by the modification. Inhibition by quinacrine mustard also resulted in the appearance of two new pathways, somewhat similar to those from FSBI inhibition. In contrast, activity remaining with excess efrapeptin present showed only one pathway like that of the native enzyme. This can be attributed to a low equilibrium concentration of free enzyme and total inhibition of MF_1 combined with efrapeptin.

J. Biol. Chem. (1993) 268, 1531-1538

Evidence That Energization of the Chloroplast ATP Synthase Favors ATP Formation at the Tight Binding Catalytic Site and Increases the Affinity for ADP at Another Catalytic Site*

(Received for publication, June 30, 1992)

Jun-Mei Zhou and Paul D. Boyer‡

From the Molecular Biology Institute and the Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024-1570

Previous results have not established whether the attainment of a rapid photophosphorylation rate as ADP concentration is increased in the micromolar range (apparent K_m = ~30 μM) results from the filling of a second or a third catalytic site. Measurements reported here show that the ATP synthase of chloroplast thylakoids, with 2–4 μM medium ADP present during steady-state photophosphorylation, has one catalytic site filled with tightly bound nucleotides, but other catalytic sites are largely empty. Thus, the rapid increase in the photophosphorylation rate with higher ADP concentrations results from the filling of a second catalytic site. Even with 30 μM added ADP in the dark, the binding of more than one ADP per synthase was not detectable. The sensitivity of the assay was such that the K_d for binding of ADP at a second catalytic site of the de-energized synthase is >150 μM, considerably above the apparent K_m for rapid photophosphorylation. This result can be explained by an increase in the affinity of a second catalytic site for ADP upon energization. Other experiments assessed the effect of ADP binding at a second catalytic site on the equilibrium between bound ATP and ADP and P_i at the tight catalytic site. When the rate of photophosphorylation is limited by a low ADP concentration, about equal amounts of ATP and ADP are bound at one catalytic site on the synthase. In contrast, when the rate is limited by a low P_i concentration with 100 μM ADP present, the equilibrium of bound reactants is shifted so that close to one ATP per synthase is present. This is as expected if the binding of ADP at a second catalytic site allows the protonmotive force to promote ATP formation from ADP and P_i at a tight binding catalytic site. A scheme for the binding change mechanism incorporating these results is presented.

Submitted for publication (1993)

1

Characterization of Oxygen Exchange Reactions Catalyzed by Mung Bean Vacuolar H^+-pyrophosphatase

Alexander A. Baykov[a], Vladimir N. Kasho[b], Natalia P. Bakuleva[c] and Philip A. Rea[d]

[a]*A. N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, Russian Federation,*
[b]*Department of Chemistry and Biochemistry, University of California, Los Angeles, CA 90024, USA,*
[c]*A. N. Bakulev Institute of Cardiovascular Surgery, Academy of Medical Sciences, Moscow 117049, Russia and*
[d]*Plant Science Institute, Department of Biology, University of Pennsylvania, Philadelphia 19104-6018, USA*

Vacuolar membrane vesicles prepared from the plant *Vigna radiata* were found to catalyze oxygen exchange between medium phosphate and water at a maximum rate of 16.5 nmol per min per mg protein. Basing on inhibitor sensitivity and cation requirements, this exchange is attributed to vacuolar H^+-pyrophosphatase. The exchange occurs *via* a single pathway, utilizing magnesium phosphate and free phosphate as substrates, and appears to result from reversal of enzyme-bound pyrophosphate synthesis. The extent of the reversal, as deduced from the distribution of ^{18}O-labeled phosphate species, is not greater than for soluble pyrophosphatases.