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THREE YEAR PROGRESS REPORT AEC CONTRACT NO. AT(04-3)-34 ERDA

Period Covered by Report

May 1972 to October 1975

"ISOTOPIC STUDIES ON STRUCTURE-FUNCTION RELATIONSHIPS OF NUCLEIC ACIDS AND ENZYMES"

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DISCLAIMER

SUMMARY OF PROGRESS

AEC CONTRACT AT(04-3)-34

Period Covered: May 1972 to October 1975

This summary lists the principal accomplishments reported in published scientific papers during the period together with other accomplishments that are being prepared for publication or will make major contributions to later publications. The contributions are grouped as those that appear to be the most important and as other major contributions.

Most important accomplishments

Developed experimental and theoretical support for an entirely new concept for oxidative phosphorylation by mitochondria and for photophosphorylation by chloroplasts, based on energy-linked conformational changes modifying reactant affinities (see bibliographic citations 1,2,4-7,21-23).

Developed the first definitive chemical technique for the characterization of acyl phosphate groups in the membrane bound transport ATPases and demonstrated that the phosphoryl group was attached to an aspartate residue in the sarcoplasmic reticulum Ca^{++}-activated ATPase and in the Na^{+},K^{+}-ATPases of microsomal and nerve membranes (13,14).

Discovered and characterized a rapid $P_i \rightleftharpoons HOH$ exchange reaction catalyzed by the transport ATPases and demonstrated phosphorylation of the membrane carboxyl group by displacement of water from inorganic phosphate (9,10,12).

Developed techniques for assessment of the molecular integrity of parental DNA during replication of E. coli, including evidence based on ^{18}O incorporation for single strand hydrolytic cleavage to solve the DNA duplex unwinding problem of replication (publication in preparation).

Demonstrated that ADP is the initial phosphoryl acceptor in photophosphorylation, obtained additional evidence for phosphorylated intermediates, and showed the kinetic competency of a proton gradient for energy transmission in ATP synthesis.

Other major contributions

Demonstrated specific alkylation of a methionine residue of fumarase correlated with enzyme inactivation, together with development of a bifunctional reagent that crosslinks residues at the fumarase catalytic site (see abstract in report).

Demonstrated that glutamine synthetase catalyzes a transfer of oxygen from P_i to glutamine that is more rapid than any other exchanges, and together with other data allows important mechanistic deductions (publication in preparation).

Demonstrated the formation of enzyme-bound pyrophosphate from inorganic phosphate with yeast pyrophosphatase, and obtained evidence that such formation may offer a molecular explanation for the oxygen exchange reaction (publication in preparation).

Developed procedures for and made an assessment of the exchangeability with solvent water of the water molecules demonstrated by x-ray crystallography to be in the interior of the chymotrypsinogen molecule (18).

Developed theoretical approaches for the use of equilibrium exchanges to probe mechanisms of enzyme action and control.

Demonstrated an interesting steric acceleration of thiol reactivity in model systems (19, 20).

Characterized a purported acyl-S derivative of myosin as a mixed-disulfide (16).

Developed models for the coupling of energy transductions to proton translocation (24).

RESEARCH ACCOMPLISHMENTS

Relationships of Accomplishments to Originally Stated Objectives

The originally stated objectives as taken directly from our application of May 1972 were as follows:

"CARBOXYL GROUP ACTIVATION IN MEMBRANE AND IN MUSCLE FUNCTION

These studies are part of a long range program to search for covalent bond formation in various energy-transducing processes of membranes and related systems. Approaches are as follows:

The possibility of conformationally-linked activation of a specific myosin carboxyl group. Indications that a carboxyl group may be activated during ATP hydrolysis will be studied using ^{18}O techniques.

Development of methods for measurement of labile acyl derivatives in membranes. The first objective will be to develop quantitative procedures that will allow detection of small amounts of acyl-S groups in various biological systems.

Development of means of derivatization of labile acyl derivatives. Methods of chemical characterization of acyl-P, acyl-S and acyl-imidazole linkages are deemed essential.

Development of methods for examination of ^{18}O in carboxyl groups. Lack of satisfactory methods limits a variety of experimental approaches.

ISOTOPIC AND CHEMICAL PROBES OF ENZYME MECHANISM

Two phases of work are proposed for major attention as follows:

Multiple isotopic probes of control and mechanism. Applications of equilibrium rate measurements ad developed for glutamine synthetase will be extended to phosphorylase and pyruvate kinase.

Possible specific site labeling of fumarase. Preliminary investigations indicate that benzylbromide may give an unusually specific labeling for a catalytically-essential methionine.

BIOPOLYMER TURNOVER

A continuation of the present program and initiation of some new studies are proposed as follows:

The molecular integrity of parental DNA. With the additional use of ^{13}C to aid labeling of parental DNA, further studies of the molecular integrity of DNA during replication of E. coli are outlined.

Turnover studies with neural proteins. Exploratory studies on turnover of brain and nerve proteins based on ^{18}O incorporation from $H^{18}OH$ are proposed."

The above objectives were covered as indicated in the following paragraphs.

The possibility of conformationally linked activation of a specific myosin carboxyl group was based on the report of Japanese workers that p-nitrothiophenol could be induced to form an acyl-S derivative during ATP hydrolysis. We had taken this work as likely sound in making our application. But as our studies progressed, it became apparent that the original characterization was incorrect. Because our evidence indicated the binding was through a disulfide group, probably formed by air oxidation, this approach was abandoned (16). The summary of the paper reproduced later in this application gives further information.

The development of methods for measurement of labile acyl derivatives in membranes as a stated objective was carried out in the laboratory, but because of limitations of financial support through the AEC contract, was supported by other sources. We did develop methods for detection of small amounts of acyl-S groups, and have published separately on their occurrence and lack of relationship to oxidative phosphorylation.

The development of means for derivatization of labile acyl derivatives was, as mentioned earlier, an important accomplishment under the present project. The application of the borohydride reduction technique gave us a successful characterization (13, 14). The technique is being used in a number of other laboratories for related studies. The accompanying copies of paper summaries give details of this accomplishment.

With respect to the suggested development of methods for examination of ^{18}O in carboxyl groups, our demonstrations that an acyl phosphate was likely not involved in oxidative phosphorylation, that myosin did not contain an activated carboxyl group, and that the carboxyl group in membrane ATPases likely did not become labeled with water oxygen during catalysis, took away the prime motivations for development of such methods. Thus possible methods were not explored.

For the proposed isotopic and chemical probes of enzyme mechanisms, we developed a theory of the equilibrium exchange measurements (15) and probed in depth exchanges accompanying net cleavage of glutamine and ATP synthesis. However, the developments of our new concept of oxidative phosphorylation, not anticipated at the time of the original project request, took precedence over further extensions of isotopic-exchange techniques to phosphorylase and pyruvate kinase as originally proposed. The suggested studies on possible specific site labeling of fumarase progressed nicely, but somewhat more slowly because

as indicated above, our principal emphasis was elsewhere. But these studies have yielded some very fine results, as summarized in the copy of an abstract given later in this report.

Our progress on biopolymer turnover has moved to a very interesting conclusion, although it took more time than originally anticipated, in part because of the experimental difficulties involved, and in part because of the limited number of capable personnel available for this project. As summarized later in this report, these studies have given evidence for an apparent hydrolytic cleavage of parental DNA during replication.

The suggested exploratory studies on turnover in brain and nerve proteins remain as useful possible approaches, but such approaches were not undertaken because of the other more promising developments that focused our attention elsewhere.

A retrospective assessment of accomplishments in relation to objectives, as given above, I believe emphasizes the value of some flexibility in support. Some of our most important accomplishments resulted from problems listed in the original three-year proposal. But other very important accomplishments were ones that were not visualized at that time. They were made possible because support was available to encourage a viable research group to pursue the most meaningful leads obtained.

Summaries of Published Contributions

Probably the most visible and durable record of accomplishments is given by the published papers. Both as a convenience in reporting results and because they provide the user of this report with a resume that has been editorially and scientifically reviewed, summaries of papers published in research journals are given in the Appendix to this application. Also included in the Appendix is an abstract of some of the recent work on fumarase being assembled for publication.

Summaries of More Important Unpublished Contributions

The early events in ATP formation by chloroplasts. The appearance of ^{32}P added as $^{32}P_i$ into ADP and ATP has been measured in millisecond mixing and quenching experiments, using both light-activated chloroplasts and chlorplasts activated for ATP synthesis acid-base transition first described by Jagendorf and Uribe. For this we developed methods more sensitive than previously used that allowed detection of initial ^{32}P incorporation in amounts considerably less then the amount of the CF_1 ATPase present. With light-activated chlorplasts a very small amount of ^{32}P-ADP appeared in a few milliseconds but without further labeling (see symposium report, reference 7). In contrast, rapid ^{32}P incorporation into ATP continued. Such a result is consistent with AMP as an initial phosphoryl acceptor and a bound ^{32}P-ADP as an intermediate. However, more precise and extensive studies were made with the initial phosphorylation accompanying the acid-base transition. These showed no detectable ^{32}P present in ADP until after considerable ATP had been labeled. A remarkably small lag (less than 5 milliseconds) was observed in the onsent of the maximal rate of ATP synthesis, quite consistent with the absence of

any phosphorylated or non-phosphorylated presucrosors to P_i uptake. The results give strong evidence that the initial acceptor of P_i for the ATP synthesis is actually ADP. Whether the initial ADP comes from the medium, or is membrane bound to be replaced in the next reaction cycle by medium ADP is not known.

Another interesting facet of these studies is the demonstration that the initial rate of ATP synthesis in the acid-base transition is about the same as the initial maximal rate of steady-state ATP synthesis when light is the energy source. This means that the pH gradient is kinetically competent to serve as the transmitter of energy from the electron transport reactions driven by light to the ATP synthesis complex. These and other related studies are in first-draft manuscript form and have been reported in part in symposium paper in press (23).

Energy linkage in reaction steps of oxidative phosphorylation. As noted earlier, an important part of the conformational coupling concept for oxidative phosphorylation developed as part of this work (references 1-7) is that energy input is necessary for release of ATP formed at the catalytic site. Further probing of the effect of various conditions on the exchange reactions gave evidence of energy input at an earlier step in the cycle (see summary of reference in the appendix). One possibility was that energy was directly involved in the covalent bond forming step, i.e. when ADP joins the phosphoryl moiety of P_i with displacement of water, as noted in a symposium paper (7) and research publication (5). Additional observations not given here suggested that the determination of the effect of energy on the two components of the $P_i \rightleftharpoons HOH$ exchange that occurs with submitochondrial particles in presence of added P_i and ATP might be useful. These are the exchanges that occur at the catalytic site with the P_i formed from ATP prior to its release to the medium (intermediate exchange), and the exchange that occurs with P_i of the medium (medium exchange). Measurement of the appearance of ^{18}O from $H^{18}OH$ into P_i under similar conditions gives a measure of the medium exchange.

Our results show the rather dramatic effect that, as energy is depleted by addition of uncoupler, the very rapid medium exchange (initially the most rapid of the exchanges) is sharply inhibited, but the intermediate exchange continues. This means that with depletion of energy, P_i can still be rapidly released from the catalytic site, as demonstrated by the rapid ATPase action, but that the previously rapid binding of P_i to the catalytic site is lost. Energy input is thus necessary for P_i binding. The results also give evidence that reversal of the covalent bond forming and breaking steps continues even in presence of high uncoupler concentrations. This means that energy is not directly coupled to the covalent bond formation and cleavage. Such lack of coupling is incompatible with a number of suggestions for oxidative phosphorylation, including those of Peter Mitchell on how a proton gradient might be used for ATP synthesis (see reference 6 for a criticism of this suggestion based on the chemistry involved).

These results taken with other data point to the function of energy input in oxidative phosphorylation as increasing the binding of P_i (and possibly of ADP) and decreasing the affinity of ATP once formed. Such events can readily

be visualized as occuring through energy-linked protein conformational. changes. These and other related studies are being assembled for publication. They have been presented at symposia (21,23).

The molecular integrity of parental DNA. The original motivation for these studies was to attempt to find if the problem of the unwinding of the DNA double helix that must occur during replication might be solved by single-strand hydrolytic cleavages of the parental strand, allowing free rotation, followed by resealing. If the cleavage were hydrolytic, water oxygen would be introduced into the phosphoryl end group formed, with 2 chances out of 3 that the oxygen from water would be retained in the DNA when the phosphodiester bond was resealed. These studies have required a considerable methodological development to allow separation of sufficient parental DNA strand and for the ^{18}O and analyses. They have been conducted as part of the Ph.D. and postdoctoral work of Dr. David Strayer. They were further delayed because Dr. Strayer in the same period has also completed his M.D. degree.

The rationale for the studies is simple but the actual experimental requirements are demanding. E. coli cells are grown with ^{15}N-ammonia and ^{13}C-glucose to give a density labeled DNA, then allowed to further grow and divide in medium containg $^{32}P_i$, ^{3}H-thymine and ^{18}O-water. Subsequently, the DNA is isolated, the parental strand is separated by density centrifugation, and examined for the presence of ^{3}H, ^{32}P, and ^{18}O. Experiments up to a year ago had suffice to establish a very low level of ^{32}P and of ^{3}H incorporation, indicative of very little replacement of entire nucleotide residues (e.g. 1 per 10,000 residues). Such low replacement made it feasible to examine for any ^{18}O incorporation from water by hydrolytic cleavage. In experiments completed over a year ago, there appeared to be ^{18}O incorporation just above the experimental error. Thus arrangements were made for Dr. Strayer to continue the growth and DNA separations at NIH, where he is now doing postdoctoral work, with DNA degradations and ^{18}O analyses at UCLA. A series has just been completed, using over $2,000 worth of ^{18}O-water and nearly a gram of highly labeled ^{13}C-glucose, kindly made available through the Atomic Energy Commission. After many days of experimental work, final triplicate analyses for ^{18}O in CO_2 from phosphate of control and experimental DNA were made. The results show that an incorporation of ^{18}O into parental DNA, without accompanying ^{32}P from P_i of ^{3}H from thymine incorporation has occured. The findings give clear evidence of hydrolytic cleavage followed by resealing, and offer a solution to the unwinding problem.

Data are being assembled for preparation of a paper reporting these findings, together with a paper on some of the NaI density gradient centrifugation techniques developed to allow the experiments to be performed. There is, however, one additional control that must be repeated before a final conclusion can be made. The possibility exists that the ^{13}C-glucose used might have been inadvertenly enriched in ^{18}O. This could arise if the $^{13}CO_2$ used for growth were ^{18}O enriched and did not fully exchange with water before incorporation into glucose. In addition, if the ^{18}O in the glucose appeared in the 2'- and 3'-hydroxyl groups of the deoxyribose of the parental DNA, then such ^{18}O would appear in the inorganic phosphate derived from degradation of DNA and used for ^{18}O analyses. Such a series of events seems unlikely, but controls must be run. One control on this has been completed and a more

sensitive control is underway. If these controls are satisfactory, the occurrence of hydrolytic cleavage discussed above will be very likely, and the entire effort will have been worthwhile.

Bound pyrophosphate and ^{18}O-exchanges by inorganic pyrophosphatase. Some years ago, Mildred Cohn reported the capacity of inorganic pyrophosphatase to catalyze and rapid exchange of P_i oxygens with water (J. Biol. Chem., 230, 369, 1958). This exchange was shown not to arise from reversal of pyrophosphate formation, and the mechanism has remained unexplained. Our new concept for oxidative phosphorylation suggested that the exchange might be caused by the reversible formation, from medium P_i, of bound pyrophosphate at the catalytic site. Assessment of such a possibility was important because we need to know the mechanism of as many phosphate oxygen exchanges as possible, and, in particular, if they result from reversible hydrolytic cleavage of phosphate compounds. Such a reversible hydrolsis of bound ATP has been suggested by us as an explanation of the oxygen exchanges catalyzed by chloroplasts and mitochondria. An alternate mechanism, based on reversible formation of a pentacovalent species has been proposed (see Young, Korman and McLick, Bioorganic Chem., 3, 1, 1974), and although regarded as unlikely by us, has not been disproved. With pyrophosphatase, it appeared that rapid mixing and quenching experiments might be able to reveal the exchange mechanism. It was thus gratifying when we were able to demonstrate that merely by incubation in presence of $^{32}P_i$, the pyrophosphatase formed a small amount of enzyme-bound pyrophosphate. Over the past few months, we have been measuring the rate of this formation and cleavage. The amount of bound pyrophosphate present is small, and such measurements do not come easily. But they have now sufficed to show that a major part, and possibly all, of the exchange in presence of Mg^{++} results from reversal formation of bound pyrophosphate. After a few more experiments, the material should be ready for publication.

Glutamine synthetase exchanges and mechanism. As a continuation of our experiments on exchange reactions and the catalytic mechanism of glutamine synthetase from E. coli, measurements have been made of the exchanges accompanying the net synthesis of ATP driven by glutamine cleavage. These researches have demonstrated an unexpected rapid transfer of oxygen from inorganic phosphate to the amide group of the glutamine. A paper has been completed for publication entitled "The Rapid Transfer of Oxygens from Inorganic Phosphate to Glutamine Catalyzed by E. coli Glutamine Synthetase". The summary reads as follows:

> Measurements are reported on certain isotopic fluxes during the net conversion of glutamine, ADP and P_i to glutamate, NH_3 and ATP by E. coli glutamine synthetase in presence of a hexokinase-glucose trap to remove the ATP formed during the reaction. The results show that the transfer of oxygens from P_i to glutamine is the most rapid of the measured to isotopic interchanges, over 5 oxygens from P_i being transferred to glutamine for each glutamate formed by net reaction. Under similar conditions, the oxygen transfer from P_i to glutamate is close to 1. The transfer of ^{18}O from P_i to glutamine is increased considerably by the

initial presence of NH_3 and nearly doubled by presence of NH_3 and glutamate. Transfer of ^{14}C from glutamate to glutamine was the slowest of the measured exchanges, and, like the transfer of ^{18}O from P_i to glutamate, was stimulated somewhat by an increase in the glutamate concentration but inhibited by an increase in the ammonia concentration. The enzyme from brain or peas did not show the rapid transfer of ^{18}O from P_i to glutamine shown by the E. coli enzyme.

The results demonstrate a lack of enzymic spatial selectivity between the oxygens of the γ-carboxyl group of bound glutamate by the E. coli enzyme but are consistent with such selectivity by the pea enzyme. Together with previous data they make likely a relative order of certain catalytic steps as follow:

ATP release < NH_3 release < glutamate release < ADP release < substrate interconversion < glutamine release

Plans for Continuation of Present Objectives and Possible New Objectives

As noted in an accompanying renewal application, we are suggesting that work supported through the ERDA contract be focused on the mechanisms of energy capture and use by plants and bacteria, with a major emphasis on the mechanism of ATP synthesis in photophosphorylation. It is suggested that the contract be retitled, with major initial emphasis as follows:

The role of bound nucleotides and conformational changes in photophosphorylation. An important objective will be to find if the ADP and ATP that are tightly bound in the isolated CF_1-ATPase from chloroplasts participate as intermediates in the photophosphorylation process. Rapid mixing and quenching techniques together with sensitive isotope labeling and separations are proposed, with use of plant chloroplast fragments and chromatophores from bacteria.

The effects of energy depletion on different steps of the phosphorylation process. The steps of P_i and ADP binding, ATP release and covalent interconversion at the catalytic phosphorylation site will be probed by measurements of various isotopic exchanges in the energized and deenergized states of chloroplast and chromatophore preparations.

Chemical and physical probes of structure-function relationships of the phosphorylation complex. Experiments are proposed on the use of photoaffinity analogs of ATP combined with laser activation in new approaches that may give information about the extent and affinity of binding to active phosphorylating membrane as well as the isolated ATPase. Photoactivatable membrane crosslinking agents will be synthesized and applied for identifying key protein components in chloroplast membranes and their interactions. Attempts will be made to obtain crystalline or ordered chloroplast

ATPase preparations suitable for electron microscopic and X-ray studies of ultrastructure.

Several important parts of our present work are being prepared for publication, including that of fumarase derivatization, pyrophosphatase formation of bound pyrophosphate and ^{18}O exchanges, the ^{18}O incorporation during DNA replication and characterization of the initial acceptor of phosphate in photophosphorylation. Completion of these studies and their publication will draw some on the requested renewal support.

GRADUATE STUDENTS AND POST-DOCTORAL TRAINING

The project has given an important source of funds both for the direct support of graduate students and postdoctoral fellows, but also for vital research support for students and fellows who have drawn stipend from other sources, such as teaching assistantships, training grants, and competitive special fellowships.

Graduate students who have contributed to the published researches are as follows:

BARRY STOKES	Ph.D. received in 1974. At present staff member with Jet Propulsion Laboratories, California Institute of Technology.
DAVID STRAYER	Now preparing Ph.D. thesis.
CELIK KAYALAR	Training in progress.

Postdoctoral Fellows are as follows:

Name	Ph.D. Training	Support	Present Position
Stephen Dahms	Mich. State Univ.	AEC Fellowship and Research Grant	Asst. Prof., Cal State Univ., San Diego
Torhu Kanzawa	Univ. of Osaka	Research Grants	Staff Member, Osaka Univ.
Richard Cross	Yale Univ.	Jane Coffin Childs Fellowship	Asst. Prof., State Univ. of New York, Syracuse.
Dan Smith	Univ. of Cal. Berkeley	Research Grant	Currently at UCLA
Gary Rogers	Univ. of Cal. Santa Barbara	USPHS Postdoctoral	Currently at UCLA
Jan Rosing	Univ. of Amsterdam	NATO Fellowship	Currently at UCLA

PRESENT STATE OF KNOWLEDGE AND SIGNIFICANCE IN FIELD OF BIOLOGY AND MEDICINE

This topic is covered briefly here only for what appears to be the most significant development of the accomplishments reported.

It is widely recognized that the mechanism of biological energy transductions poses some of the most important unsolved problems of biochemistry. This is particularly recognized for the processes of oxidative phosphorylation by mitochondria and photophosphorylation by chloroplasts. At this stage, it appears that an understanding of these problems may have depended upon the recognition of two important new biological principles. One of these is the concept developed by Peter Mitchell that energy from electron transport reactions of mitochondria or chloroplasts may be captured by establishment of membrane potential or proton gradients, and transmitted as such to phosphorylation sites. This represents the transduction of oxidative energy into membrane gradient energy. The second new principle may be that biological energy transductions involving covalent bond formation and cleavage occur through energy-linked protein conformational changes. Application of this principle, as pioneered by and developed to a large extent in my laboratory, may give the solution as to how the membrane potential or proton gradient is used to make ATP. Much remains to be done before the field as a whole may recognize and accept these principles, and, indeed, they must at this stage only be regarded as probable but not yet proved. Clearly present developments set the stage for important experimentation, with the prospect at gaining far-reaching generalizations.

Closely related to the mechanism of ATP synthesis is the mechanism of other energy transductions coupled to ATP cleavage, particularly the mechanism of active transport. Studies from a number of laboratories, including mine, appear to be converging on the probability that here again energy-linked protein conformational changes have the vital role. A similar role appears to be emerging for energy-linked conformational changes accompanying muscle contraction. Our and other future studies may establish that conformational coupling has a very fundamental role in most biological energy transductions.

PRESENT DIVISION OF FEDERAL SUPPORT FOR THE RESEARCH PROGRAM

Over the past three years, support has come from the present ERDA (AEC) contract ($38,122 per year in direct costs), USPHS grant GM 11094 ($71,144 per year in direct costs) and NSF grant GB 36344X ($25,366 per year in direct costs).

There is some overlap on support of researches, but the principal present use of support is as follows:

USPHS grant	Mitochondrial oxidative phosphorylation and active transport
ERDA (AEC) contract	Photophosphorylation in plants and bacteria
NSF	Myosin ATPase and contractile mechanisms.

During the period covered by this report, 32 publications in total have appeared from my laboratory. Of these 7 carried acknowledgement only to the AEC support, and 19 carried acknowledgement of support from AEC and another federal agency. A considerable number of the latter were reports of invited presentations at symposia.

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