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OAK RIDGE NATIONAL LABORATORY

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ORNL/FTR--3311

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ORNL

FOREIGN TRIP REPORT

ORNL/FTR-3311

DATE: July 11, 1989

SUBJECT: Report of Foreign Travel of Fred C. Hartman, Director, Biology Division

TO: Alvin W. Trivelpiece

FROM: Fred C. Hartman

PURPOSE: To participate in a conference sponsored by the NATO Advanced Study Institute on enzymatic and model carboxylation and reduction reactions for carbon dioxide utilization at Ginosa, Italy, June 17-28, 1989.

SITES VISITED: 6/17-28/89 NATO-ASI, Golf Hotel Riva dei Tessali, Ginosa Marina, Italy

ABSTRACT: The traveler attended a conference organized by the NATO Advanced Study Institute on plant molecular biology and presented two invited addresses entitled "Rubisco: Active-site characterization and mechanistic implications." Presentations concerning biological CO_2 fixation, chemical modifications of proteins, site-directed mutagenesis, CO_2 chemistry, carbonic anhydrase, biotin-requiring enzymes, and the greenhouse effect were relevant to ongoing investigations of the Protein Chemistry Group and the Protein Engineering Program at ORNL's Biology Division.

DISCLAIMER

UCN-2383A
(3 6-88)

MASTER

This report describes a NATO-sponsored conference on "enzymatic and model carboxylation and reduction reactions for carbon dioxide utilization"; the conference was convened from June 17-28 in Ginosa, Italy. Formally, this conference was conducted as a NATO Advanced Study Institute which differs from a typical scientific meeting because of its course-like structure intended to benefit aspiring young researchers. About half of the registrants were students and postdocs, so the organizers (Dr. Michele Aresta, University of Bari, Italy, and Dr. John Schloss, DuPont) encouraged speakers to give broad overviews of their respective fields in addition to specialized coverage of recent advances in research. The 10-day duration is a minimal requirement imposed by NATO to stimulate close interactions among participants. Although more than 50% of the financial support for the conference was provided by NATO, numerous other organizations also contributed (Appendix I).

In addition to providing formal lectures on most aspects of CO_2 chemistry and biochemistry (see attached program, Appendix II), the meeting included numerous poster sessions and brief oral presentations by students and postdoctoral investigators.

I presented two 45-min invited lectures: one a general overview of affinity labeling and protein engineering and the other an update on the active-site of ribulose bisphosphate carboxylase (Rubisco), a major area of research in my laboratory during recent years. Harry Smith, a graduate student in my laboratory, gave an oral presentation of his recent studies on concerted mutagenesis and modification of Rubisco; and Eva Lee, a postdoc in my laboratory, presented a poster describing partial reactions catalyzed by mutants of Rubisco. About 20% of my travel support was generously provided by DOE through ORNL and the other 80% by the NATO Advanced Study Institute.

The major justification for my participation in this conference was the inclusion of Rubisco as a major topic; no other single enzyme received comparable attention. Why is such interest and importance attached to this one enzyme? Rubisco is essential to all higher life forms. Ubiquitous to photosynthetic organisms, the enzyme catalyzes the carboxylation of ribulosebisphosphate to two molar equivalents of phosphoglycerate. Since this reaction provides the only significant route by which photosynthetic organisms utilize energy from sunlight to achieve net synthesis of carbohydrates, the enzyme is directly essential to all plants and thus indirectly required by all animals because of their dependence on plants for food and oxygen.

The natural abundance of Rubisco provides another reason for the attention that it has received. Comprising about 50% of the total soluble protein in green leaves, Rubisco is the most abundant protein on earth. Since the enzyme is nutritionally complete with respect to content of essential amino acids, Rubisco represents a major untapped source of protein for human nutrition.

Research support both in the U.S. and abroad for basic studies on the structure and mechanism of Rubisco have increased dramatically during the past 15 years because of the realization that regulation of this enzyme's activity *in vivo* is a prime determinant of crop yields. The key research finding that thrust this enzyme onto the stage of "relevancy" was that the enzyme catalyzes an oxygenation reaction as well as the CO_2-fixation reaction first recognized by Melvin Calvin. The oxygenase activity was discovered and characterized independently about ten years ago by W. L. Ogren at the University of Illinois and N. E. Tolbert at Michigan State. Ribulosebisphosphate is the acceptor substrate for both CO_2 and oxygen. The condensation between CO_2 and

ribulosebisphosphate results in net synthesis of carbohydrate; however, the condensation of O_2 with ribulosebisphosphate is a nonsynthetic pathway (photorespiration) which is energy wasteful in that it results in the release (into the atmosphere) of previously fixed CO_2. This discovery of the oxygenase activity inherent in Rubisco explained the long-standing mystery as to why plants, as observed by Otto Warburg, grow more rapidly and efficiently when deprived of oxygen. Obviously, it is impractical to grow crops of agricultural significance under low O_2 tension. However, if a means could be found to abolish the oxygenase activity of Rubisco *in vivo* either through genetic manipulation or by a chemical treatment of the whole plant, there is general agreement that crop yields would be increased by about 50%. Thus, the importance of structural and mechanistic studies on the CO_2-fixation enzyme.

Since Rubisco is crucial to yields of biomass in general, not just plants of agronomic significance, an understanding of the enzyme relates to production of biomass as an energy source. Furthermore, the energy intensiveness of agriculture provides a correlation between agricultural efficiency and energy conservation.

Mechanistically, the enzyme is quite intriguing. Although multiple substrate specificities among enzymes are not unusual, the bifunctionality of Rubisco is perhaps unprecedented in that the two reactions catalyzed are the initial steps in competing metabolic pathways -- photosynthetic assimilation of CO_2 and photorespiration. The oxygenase activity of Rubisco has been very difficult to explain, because the enzyme is devoid of all cofactors (iron, cobalt, flavin) that enzymologists normally view as essential to oxygenase.

A final feature of Rubisco that has attracted wide interest is its mode of regulation. In the absence of CO_2 and divalent metal ion, the enzyme adapts a conformation totally lacking in catalytic activity. Incubation of the protein with CO_2 and Mg^{2+} results in a conformational change that results in the appearance of both carboxylase and oxygenase activity. The activation process entails the formation of a carbamate formed by the specific reaction of CO_2 with a protein lysyl ϵ-amino group. The carbamate is then stabilized by the divalent metal ion. Although hemoglobin transports CO_2 as a carbamate, Rubisco is the only enzyme known to be regulated by specific modification by CO_2.

For the numerous reasons just discussed, Rubisco research is interdisciplinary and includes agronomists, plant physiologists, geneticists, molecular biologists, enzymologists, protein chemists, and X-ray crystallographers. Financial support is provided by all major governmental agencies worldwide and by a few select private sources (*e.g.* Monsanto and DuPont in the U.S.). DOE sponsors Rubisco research through two separate offices: the Office of Health and Environmental Research and the Office of Biological Energy Research. My research is supported by the former office, with supplementation from USDA, under a broad justification concerning protein structure and function.

The work that I presented at the meeting reflected a continuation of efforts to pinpoint the function of active-site residues identified earlier in my laboratory. Previous affinity labeling studies and comparative sequence analyses have identified two different lysines and a glutamic acid at the active site of Rubisco and have suggested their essentiality to function. The essential lysines occupy positions 166 and 329 in *Rhodospirillum rubrum* enzyme and positions 175 and 334 in the spinach enzyme, while the glutamic acid is found at positions 48 and 60 in the two species, respectively.

Site-directed mutagenesis has been applied to *R. rubrum* Rubisco specifically to clarify the functions of Lys-166, Lys-329, and Glu-48. More generic issues also addressed include validation (or invalidation) of chemical modification data, active-site location (intra- or intersubunit), subunit-subunit interactions, and prospects for changing substrate specificity by unusually subtle alterations of the active-site microenvironment through concerted mutagenesis and chemical modification.

Position-166 and position-329 mutant proteins, devoid of carboxylase activity, are dimeric (like wild-type enzyme), undergo normal activation chemistry, and bind ribulose-P_2. Catalytic functionalities of Lys-166 and Lys-329 are thus proven. The Gly-166 mutant protein retains the ability to catalyze the conversion of the well-characterized six-carbon, carboxylated reaction intermediate to 3-phosphoglycerate but is unable to catalyze the enolization of ribulose-P_2, the initial step in the overall reaction. These observations are entirely consistent with the lysyl-166 ϵ-amino group as the essential base that initiates catalysis. In contrast, the Gly-329 mutant protein is competent in the enolization reaction; hence, Lys-329 must function at some subsequent step in the overall pathway.

Partial restoration of carboxylase activity to the Cys-166 and Cys-329 mutant proteins is observed upon aminoethylation; the resultant novel carboxylases differ structurally from wild-type enzyme only in the replacement of a γ-methylene group of the respective lysyl side-chain by a sulfur atom.

Glu-48 is also involved, directly or indirectly, in catalysis. Replacement with other amino acids abolishes >99% of the carboxylase activity but does not disrupt subunit-subunit interactions, activation chemistry, nor substrate binding.

Cross-linking studies had raised the possibility that the active site of *R. rubrum* Rubisco is created by distinct, interacting domains of adjacent subunits. This postulate is proven by hybridization of site-directed mutants. The Gly-166 and Gln-48 mutant proteins are devoid of carboxylase activity. However, when the respective genes for these two proteins are coexpressed in *E. coli* from separate plasmids, activity is restored to ~25% of the wild-type level. Analysis of the carboxylase purified from these cells confirms that the activity reflects the presence of a heterodimer (one subunit with the Gly-166 substitution and one subunit with the Gln-48 substitution) with one active site per molecule (as compared to two in wild-type enzyme). This interallelic complementation conclusively demonstrates that interacting domains from separate subunits constitute the active site.

The remainder of this report will briefly describe some of the presentations that were relevant to ongoing studies in Oak Ridge.

3D Structure of Rubisco (Gunter Schneider, Swedish Univ. of Agricultural Sciences, Uppsala, Sweden)

The 3D structure of Rubisco from both *Rhodospirillum rubrum* and spinach has been determined at high resolution (1.7 Å and 2.9 Å, respectively) by the crystallographic group in Uppsala headed by Carl Branden. The crystal structures support most of the conclusions based on modification and mutagenesis studies carried out in Oak Ridge. These include assignment of Lys-166, Lys-329, and Glu-48 to the active site; the intersubunit location of the active site; the contributions of amino acid side chains from adjacent subunits to create the active site (*e.g.* a salt linkage between Glu-48 and Lys-168); and a location of Lys-329 that is consistent with its putative role in enhancing the reactivity of the enediol reaction intermediate toward CO_2. Controversy arises with respect to the postulate that Lys-166 is the base that enolizes ribulose bisphosphate, because the ϵ-amino group

of this lysine appears too far removed from C3 of the substrate to serve as the direct proton acceptor.

Single Turnover Kinetic Analysis of Rubisco (John Schloss, DuPont, Wilmington, Delaware)

The overall reaction in which ribulose bisphosphate is carboxylated to generate phosphoglycerate is a multistep process in which the enediol of ribulose bisphosphate and carboxyketoarabinitol bisphosphate are well-characterized intermediates. By use of rapid quench techniques, the rates of formation and disappearance of these intermediates during single turnovers of ribulose bisphosphate were determined at several bicarbonate concentrations. Unexpectedly, the carboxylation of the enediol to form the carboxyketone intermediate exhibited rate saturation with respect to bicarbonate concentration. This leads to the conclusion that a slow step would be required after the formation of the enediol but before its reaction with CO_2. "Activation" of the enediol is a function that could be fulfilled by Lys-329, as mutant proteins that lack Lys-329 can still catalyze enediol formation but not its reaction with CO_2.

Coupling Between the Light and Dark Reactions of CO_2 Fixation: Early Experiments in Photosynthesis Revisited (Francis K. Fong, Carbon Reduction Laboratory, West Lafayette, Indiana)

Fong has spent considerable time reevaluating the early isotope tracer experiments that led to the universally accepted Calvin cycle, in which as the first step, ribulose bisphosphate is carboxylated to yield two molecules of phosphoglycerate. *In vivo* experiments performed in the dark are consistent with this 1:2 stoichiometry; and if $^{14}CO_2$ is added as the substrate, phosphoglycerate in the first labeled metabolite. However, similar experiments carried out in the light result in the appearance of labeled sucrose on the same time scale at which phosphoglycerate becomes labeled. Thus, one might speculate that the two molecules of phosphoglycerate generated by the action of Rubisco are not equivalent metabolically and are processed through different pathways. Although this hypothesis appears implausible, it cannot be excluded on mechanistic grounds; and, without question, the differences in metabolite labeling patterns as observed in the light *vs.* the dark raise intriguing, unanswered questions.

APPENDIX I

The International Scientific Committee and Organizing Committee gratefully acknowledges the Grant provided by NATO for the organization of this Advanced Study Institute. This generous contribution permitted to gather the Partcipants in the School and to promove integrated discussion and new exchanges.
In addition to the above support, the financial contribution of:

- The Università degli Studi di Bari
- The Consiglio Nazionale delle Ricerche, Roma
- ENEA, Roma
- The Regione Puglia
- The Cassa di Risparmio di Puglia
- Pergine Industrie, Firenze
- Perkin Elmer Italiana

and the assistance provided through services and facilities by:

- The Dipartimento di Chimica , Università degli Studi di Bari
- Enichem Agricoltura
- Metapontum Agrobios, Metaponto
- The Azienda Autonoma Soggiorno e Turismo, Lecce
- The Ente Provinciale per il Turismo, Matera
- The Ente Provinciale per il Turismo, Taranto

are gratefully acknowledged.

Grants from NSF Foundation-USA and from Portugal and Turkey Authorities to Participants from the relative Countries are also acknowledged.
Gratitude is expressed to Dr. Salvatore Leone de Castris for his kind invitation to visit the Azienda Vinicola Leone de Castris-Salice Salentino (LE).

APPENDIX II

Itinerary

June 14-16, 1989	En route from Oak Ridge to Ginosa, Italy
June 17-28, 1989	Participated in Conference
June 29-30, 1989	En route from Ginosa to Oak Ridge

The traveler did not take vacation as planned but returned to Oak Ridge directly from the meeting.

Persons Contacted During Congress

Please see attached List of Participants

Literature Acquired

List of Participants.

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M. ULMANN — Départment de Chimie Minérale Analytique et Appliquée, Quai Ernest, Anserment 30, 1211 Genève 4.

TIMETABLE

Saturday **17 June 1989**

Arrival of the Participants.

Sunday **18 June 1989**

10 - 12 Meeting of the Teaching staff.

10 - 12
17 - 19 Through the bibliographic references.

2030 Welcome drink and dinner

MONDAY **19 JUNE 1989** **Chairman**

9.00 - 10.00 M. Aresta , L1 **F. Hartman**
10.00 - 11.00 S. Funtowicz, L2

11.00 - 11.30 COFFEE BREAK

11.30 - 12.30 R.S. Brown, L3 **V. RUBIO**

17.00 - 19.00 Tutorial Work : The molecular structure of carbon dioxide
Enzymes : how they work.

TUESDAY 20 JUNE 1989

8.30 - 9.30 G. SCHNEIDER, L5 **J.V. SCHLOSS**
9.30 - 1030 F.C. HARTMAN, L6

10.30 - 11.00 COFFEE BREAK

11.00 - 12.00 J.V. SCHLOSS, L8 **R. KLUGER**
12.00 - 13.00 F.K. FONG, L7

17.00 - 19.00 Short Communications C1,C2,C3,C4,C5,C23
P P P P

WEDNESDAY 21 JUNE 1989

8.30 - 9.30	Y. POCKER, L4	R.S. BROWN
9.30 - 10.30	H. HOBERG, L9	
10.30 - 11.00	COFFEE BREAK	
11.00 - 12.00	D. DARENSBOURG, L10	H. HOBERG
1200 - 13.00	P. DIXNEUF, L11	
15.30	TOUR	

THURSDAY 22 JUNE 1989

8.30 - 9.30	V. RUBIO, L12	A.S. MILDVAN
9.30 - 10.30	W.B. KNIGHT, L13	
10.30 - 11.00	COFFEE BREAK	
11.00 - 12.00	A.S. MILDVAN, L14	W. B. KNIGHT
12.00 - 13.00	R. KLUGER, L15	
16.00 - 17.00	Ziessel L23	
17.00 - 18.00	M.N. SIVAK, L25	
18.00 - 19.00	Short Communications C6,C7,C8,C9,C10,C21,C22	
	P P P P P P	
	Tutorial Work	

FRIDAY 23 JUNE 1989

8.30 - 9.30	G. FUCHS, L16	B. KRAEUTLER
9.30 - 10.30	J. DUARTE, L17	
10.30 - 11.00	COFFEE BREAK	
11.00 - 12.00	G. SILVESTRI, L18	P. DIXNEUF
12.00 - 13.00	~~R. ZIESSEL, L23~~	
17.00 - 18.00	A.S. MILDVAN, L14	
18.00 - 19.00	Short Communications C11,C12,C13,C14,C15,C16.	
	P P P P	

SATURDAY 24 JUNE 1989

8.30 - 9.30	B. KRAUTLER, L19	J. DUARTE
9.30 - 10.30	G. FUCHS, L16	
10.30 - 11.00	COFFEE BREAK	
11.00 - 12.00	F.C. HARTMAN, L6	F.K. FONG
12.00 - 13.00	G. SCHNEIDER, L5 — schematic of active site	
15.30	TOUR	

SUNDAY 25 JUNE 1989

8.30 - 9.30	F. FONG, L7	Y. POCKER
9.30 - 10.30	L. BANCI, L21	
10.30 - 11.00	COFFEE BREAK	
11.00 - 12.00	P. GRAZIANO, L20	D. DARENSBOURG
1200 - 13.00	L. VASKA, L22	
	FREE AFTERNOON	

MONDAY 26 JUNE 1989

8.00	TOUR	

TUESDAY 27 JUNE 1989

8.30 - 9.30	B. KRAUTLER, L19	G. FUCHS
9.30 - 10.30	G. SILVESTRI, L18	
1030 - 11.00	COFFEE BREAK	
11.00 - 12.00	D. DARENSBOURG, L10	L. VASKA
12.00 - 13.00	A. DEDIEU, L24	
17.00 - 19.00	Short Communications C17,C18,C20	

P O O

WEDNESDAY 28 JUNE 1989

8.30 - 9.30	H. HOBERG, L9	**M. ARESTA**
9.30 - 10.30	F. CALDERAZZO, L26	
10.30 - 11.30	Conclusions (J.V. SCHLOSS,R.S.BROWN, G.FUCHS,F.K.FONG)	

Distribution

1. Assistant Secretary for International Affairs, DOE, Wash.
2. Dr. Robert W. Wood, Acting Associate Director, OHER, DOE, Wash.
3. R. L. Egli, Acting Assistant Manager, Energy Research and Development, DOE/ORO
4. W. G. Phelps, Director, Safeguards and Security Division, DOE/ORO
5-6. Office of Scientific and Technical Information, P.O. Box 62, Oak Ridge, TN 37831
7. C. R. Richmond
8. A. W. Trivelpiece
9-11. F. C. Hartman
12. J. S. Cook
13. R. J. M. Fry
14. R. Julian Preston
15. L. B. Russell
16-17. Laboratory Records Department
18. Laboratory Records Department - RC
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21. ORNL Public Relations Office