OCCASION

This publication has been made available to the public on the occasion of the 50th anniversary of the United Nations Industrial Development Organisation.

DISCLAIMER

FAIR USE POLICY

CONTACT

Please contact publications@unido.org for further information concerning UNIDO publications.

For more information about UNIDO, please visit us at www.unido.org

UNITED NATIONS INDUSTRIAL DEVELOPMENT ORGANIZATION
Vienna International Centre, P.O. Box 300, 1400 Vienna, Austria
Tel: (+43-1) 26026-0 · www.unido.org · unido@unido.org

International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

20953

Collaborative Research Programme

TERMINAL EVALUATION REPORT

UNDO contract # 91/048

ICGEB reef. #: CRP/CHN90-05

Project initiation: 1991

Project termination: 1994

International Centre for Genetic Engineering and Biotechnology
United Nations Industrial Development Organization

Collaborative Research Program

TERMINAL EVALUATION REPORT

Part 1

Title of Project

Studies on Structural Mechani n of Prolonged-Acting and High Potent Human Insulin

Keyword: Insulin mutant, Prolonged action, High Potency, X-ray structural analysis

UNDO contract #	91/048	**ICGEB ref. #:** CRP/CHN	90-05
Project initiation:	1991	**Project termination:**	1994

Principal Investigator's name: Da-Cheng Wang

Affiliate Center mail address:
Department of Protein Engineering
Institute of Biophysics, Chinese Academy of Sciences
Beijing 100101, China

Telephone no.	86-1-2020077 ext. 523	**Telex no.**	2028
Fax no.	86-1-2027837	**Email address**	

Abstract:

In the clinical medicine, insulin is used as an injected therapeutic agent for the treatment of diabetes, therefore is very critical for the relative therapeutics. The present insulin preparations have, however, had some significant disadvantages and the supply of insulin in the market of China is very short. Obviously it is significant to explore some new therapeutic agents with certain attractive properties (e.g. prolonged action, high stability and super potency, low immunogenicity etc.) for improving the clinical use. For these scientific goals it is central to understand the structural mechanism of such new molecular behaviors, namely the relationship between structure and new properties.

The main task of our research project is, by using X-ray crystal structural analysis, to determine the three-dimensional structures of a series of insulin mutant prepared by protein engineering and partly by semisynthesis and possessed some desirable properties related to prolonged-biological action, high potency or highly chemical stability. On basis of these, the structure mechanism of these new features will be elucidate. Furthermore, the rational molecular design and molecular modeling for the more effective prolonged-acting and highly stable or potent insulin derivatives will be investigated. The results will provide new idea and information to the insulin engineering which may benefit the medical practice related to diabetes. The investigation will also give the chance to gain new insight into the structure-function relationship of insulin, especially the new features of insulin as an allosteric protein.

Part 2

OBJECTIVES/METHODOLOGY

(Proposed at the time of the submission of the research proposal)

The set objectives of this project include the following point:

1) Determining X-ray crystal structures of 5~7 insulin mutants and derivatives which are prepared by protein engineering or semi-synthesis and possessed certain attractive properties.

2) On basis of the comparisons between the determined three-dimensional structures of insulin mutants and that of the native insulin, elucidating the structural mechanism of the prolonged action and other engineered properties of mutants and providing new idea and information to further insulin engineering.

3) Gaining new insight into the structure-function relationship of insulin, especially the new allosteric properties of insulin.

The main methods for the investigation is X-ray crystal structure analysis and molecular modeling with the knowledge of the structural similarity, which include the following main methodological steps:

1. Crystallization of insulin mutants: with various techniques (e.g. setting drop, vapor diffusion etc.) to make single crystals large enough (>0.5 mm) for the X-ray diffraction analysis.

2. Diffraction data collection: using four-circle diffractometor, or rotation camera, area detector in our institute to collect the X-ray diffraction data at medium resolution, and also planning to use the X-ray source of synchrotron at the National Laboratory for High Energy Physics of Japan to collect the data at high resolution.

3. Structural analysis and model building: mainly using the molecular replacement and difference Furies methods to solve the phase problem and build the initial model; then to refine the structural model by means of restrained least square procedure programmed in EREF (Jack and Levity) or PROLSQ/PROTIN (W. Hendrckson), or X-PLOERE. All model building will be done on the Graphics PS330 or Silicon Computer Graphics.

4. Structure comparison and molecular modeling: Using least square procedure (MODELFIT) the 3-D structures of various insulin mutants will compaer with that of the native insulin, which will provide information about the relationship between the structures and new properties of insulin mutants so as to gain insight into the understanding of structural mechanism of prolonged action, high potency, stability etc.

RESULTS
(compare against the set objectives)

In comparison with the set objectives (See page 2), the main results we have obtained can be summarized as following:

(1) Crystallographic system of insulin mutants: The X-ray crystal structures of 10 insulin mutants and derivatives with various attractive properties as shown in Tabl 1 have been determined at different resolution, including three of prolonged-acting insulin, two of high potent insulin, three of highly stable insulin, one of low antigenic insulin and one of structural significant mutant. Besides, further 4 structures, including 2 with fast absorption, 1 with super-activity and 1 with structural significance, are still in progress. On basis of these, a crystallographic system of insulin mutants with certain attractive properties has been established in the framework of this project. The details of this system are shown in Table 1.

TABLE 1 Crystallographic System of Insulin Mutants With Various Properties

Property	Mutant	Results
Long Action	+B31-Arg	2.0Å Structure
	+B31-Lys-NH_2	2.0Å Structure
	AsnA21Ser-ThrB27Arg-B30Thr-NH_2	2.1Å Structure
Fast Absorption	SerB9→Asp	1.8Å Data
	SerB9→Glu	Crystallization
Super-Activity	AsnB3→Lys	2.1Å structure
	PheB25→Tyr	2Å structure
	AsnA21→Ala	2Å Data
High Stability	AsnA21→Ser	1.8Å Structure
	AsnA21→Gly	1.8Å Structure (1.3Å Data)
	AsnA21→Asp	1.8Å Structure
Low Antigenicity	ThrB30→Gly	1.6Å Structure
Structure Significant	GlyB8Ser-GluB13Gln	2.1Å Structure 1.6Å Data
	GlyB20→Gln	2.0Å Data

(2) Prolonged-acting Insulin: The structural basis of the prolonged action of insulin have been elucidated through the X-ray structural analysis of three insulin mutants, including B31Arg-human insulin (BAHI), B31Lys-human insulin (BLHI) and AsnA21Ser-ThrB27Arg-B30Thr-NH_2-human insulin (ASBABN-HI) which provide a principle for designing the new type of long-acting insulin.

The refined structures of BAHI, BLHI and ASBABN-HI shown a common striking structural feature that an additional ionic bond or hydrogen bond was formed between the mutant residues ArgB31 (in BAHI) or LysB31 (in BLHI) or ArgB27 (in ASBABN-HI) and

the target residue GluB21 of the neighbor molecules. Thereby, three additional intermonomeric contacts appeared on the surface of the mutant hexamer as shown in Fig. 1, which should slow done the dissociation rate of the hexamers when they were injected into the body. Therefore, the property of protraction for these mutants could be produced by a "depot effect". The results obtained here have been used in protein engineering of insulin as a principle for searching the new insulin preparation.

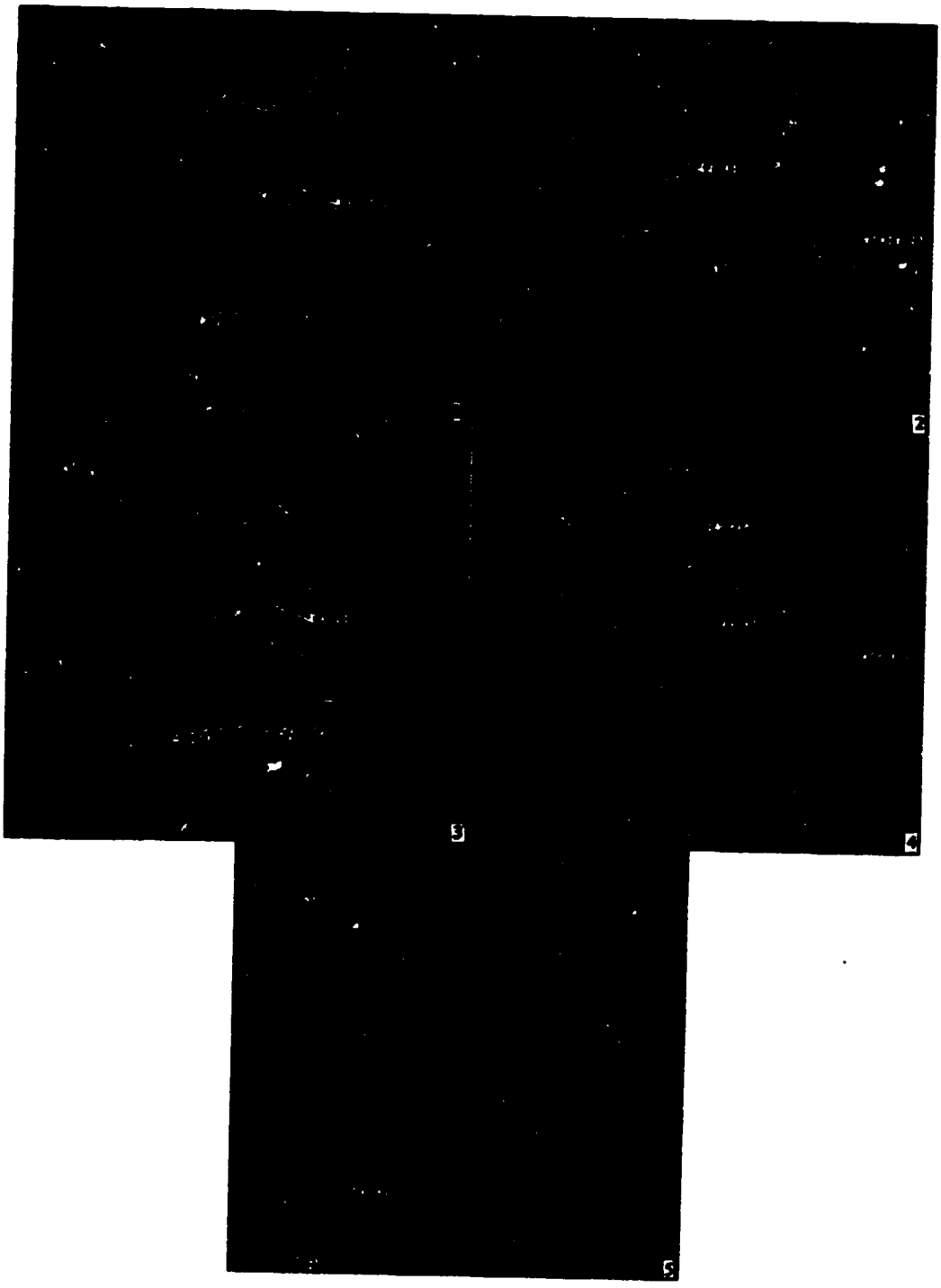
(3) High potent insulin: The crystal structures of two insulin mutants with high biological potency, including AsnB3Lys-insulin (with 180% of insulin potency) and PheB25Tyr-insulin (with 160% of insulin potency), have been determined. Besides, the structure of another one, AsnA21Ala-insulin (with 140% of insulin potency) is in progress. Results observed here revealed that the potency *in Vitro* is related to the binding of insulin with its receptor. The substitution of PheB25 by Tyr may extend the area of hydrophobic interaction and produce a new polar contact provided by the aromatic ring and OH group of Tyr. The Lys at B3 may provide an additional ionic interaction for the binding. The inhencemeant of the biological potency can be attributed to the strengthening of interactions between mutant molecules and receptor. On basis of this some mutant position and residues can be suggested to the rational molecular design searching for the new high potent insulin.

(4) High stable insulin: X-ray crystal structures of three insulin mutant with high stability, including SerA21Gly-, AsnA21Ser- and AsnA21Asp-human insulin have been determined all at 1.8Å resolution. The main structural information from these three mutants can be summarized as: a) The enhancement of the chemical stability in the mutants are just caused by the elimination of the chemically unstable side-chain -CO-NH_2 of A21Asn through substitutions by Gly, Ser and Asp. b) In compression with wild molecule, the potencies of the mutants are reduced in a certain degree (to 0.7~0.8 in FFC assay and to 0.8~0.9 in MBG assay). It indicates that the characteristic of the side-chain at the residue A21 is closely related to the performance of the biological activities. Considering the sensitivity of this position, one may meet a certain substitution which could enhance the potency of the molecule. The high potent mutant AsnA21Ala-human insulin is obtained just from this idea. c) The A21Asn is an invariant residue in all species of insulin known today. The structures of three A21 mutants revealed that the significance of this residue is mainly structural. Especially the hydrogen bond of A21-NH ...OC-B23 may play a very impotent role in retaining the unique conformation of B20-B23 β-turn. These results will contribute to the further engineering of insulin for the practically useful mutants.

(5) New structural type of insulin allosteric intermediate observed in some mutants: It has been a long time to know insulin as a polypeptide hormone and as a pharmaceutical preparation. However, some recent findings from X-ray structural analysis revealed that the insulin hexamer is an allosteric protein which are extending the status of insulin as a classical fundamentally interesting protein. In our X-ray structural analysis of A21Ser- and A21S-B27R-B30NH_2- human insulin, two new types of insulin allosteric structure were observed, which were considered as new forms of insulin allosteric intermediate and designated as 2Zn-T_3R_3 and Zn-free-T_3R_3 structures, as shown in Fig.2 and 3. Our results indictated that the allosteric intermediate structure of insulin hexamer possesses the diversity in both the pattern of oligomeric organization and the types of inducing effect. These observatives will gain insight into the structure-function relationship.

More details about the results summarized above can be gained from the three Annual Report for 1991~1992, 1992~1993 and 1993~1994.

Fig.1 Structure of a Protonged-Acting Insulin ArgB31 Human Insulin (ABHI) (1) Two successive β-turns formed by the segment B27-B31 of Mol. and the surrounding of Arg-B31. (2) Surrounding of Arg-B31 in Mol.II. (3) Space-filling model of ABHI dimer. A chains in both Mol.I and II are colored in green and the B chains of Mol.I and II are colored in purple and yellow, respectively. An ionic pair formed by Arg-B31 (colored in blue of Mol.I and Glu-B21 (colored in red) of Mol.II is highlighted on the dimer surface. (4) Space-filling model of ABHI hexamer. The Mol.I and II are colored in purple and green, respectively and the three ionic links formed by Arg-B31 (in blue) of Mol.I and Glu-B21 (inn red) of Mol.II are highlighted on the hexamer surface which should function like three "locks" so as to strengthen the stability of the oligomer.



Electron density maps around the residue Arg-B31. (1) (Fo-Fc) difference Fourier maps of (a) Mol.I; (b) Mol.II. (2) (2Fo-Fc) maps omitted residues B29-B31 in the calculation for (a) Mol.I (labeled in 229-231); (b) Mol.II (labeled in 429-431). (3) (2Fo-Fc) map in the area of Arg-B31 of Mol.I showing the contact between Arg-B31 (labeled in 231) of Mol.I and Glu-B21 (Labeled in 421) of Mol.II in an ionic link distance.

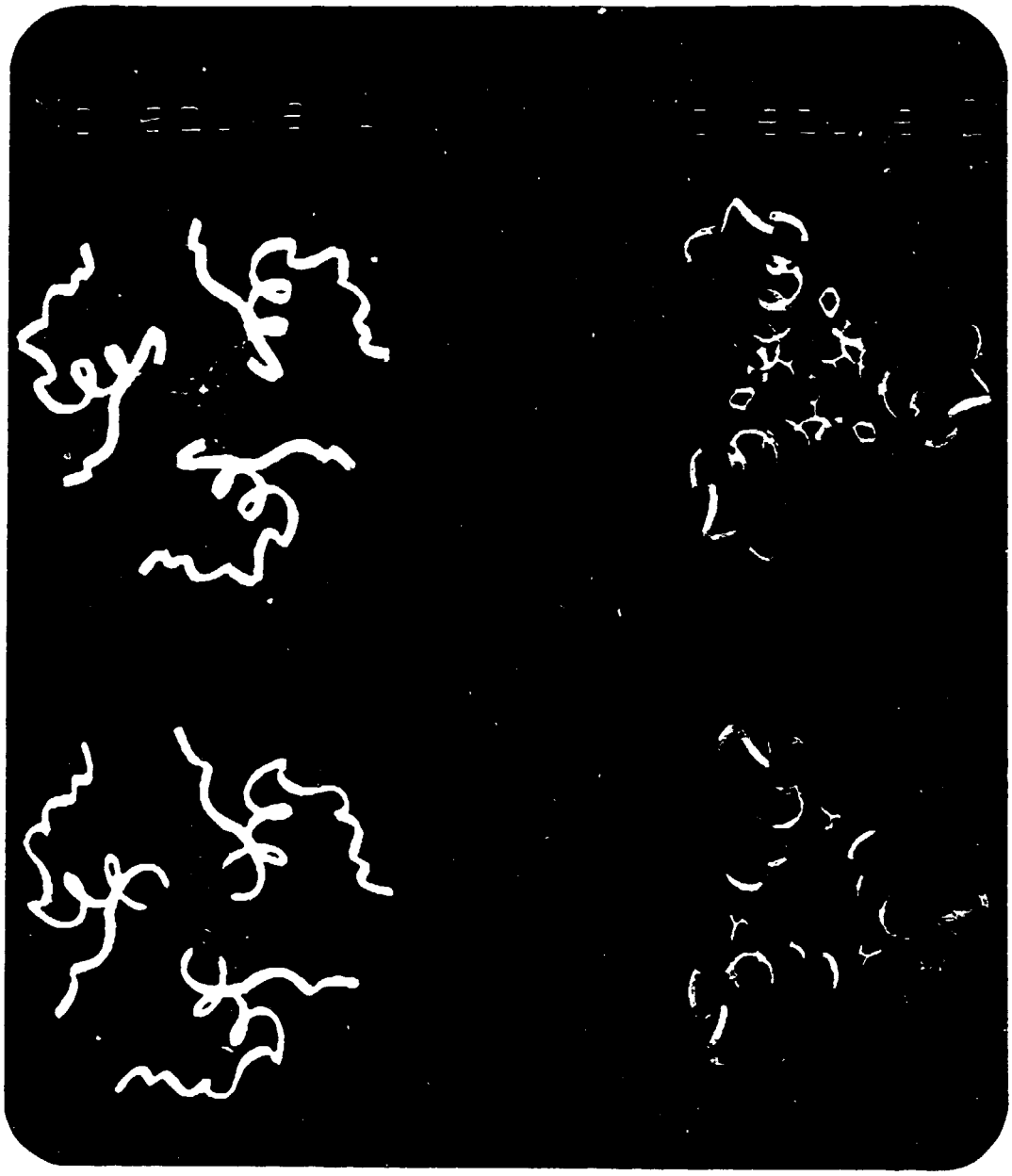


Fig. 2 Dioxane-induced 2Zn-T_3R_3. structure (a) and DMF-induced Zn-free-T_3R_3 structure (b) of insulin allosteric intermediate. Trimers T_3 composed of Molecules 1 (left) and R_3 composed of Molecules 2 (right) are separately showing with B-chains alone. Only those side-chains related to the association are represented. Three dioxane and DMF molecules are shown in yellow as skeleton drawing.

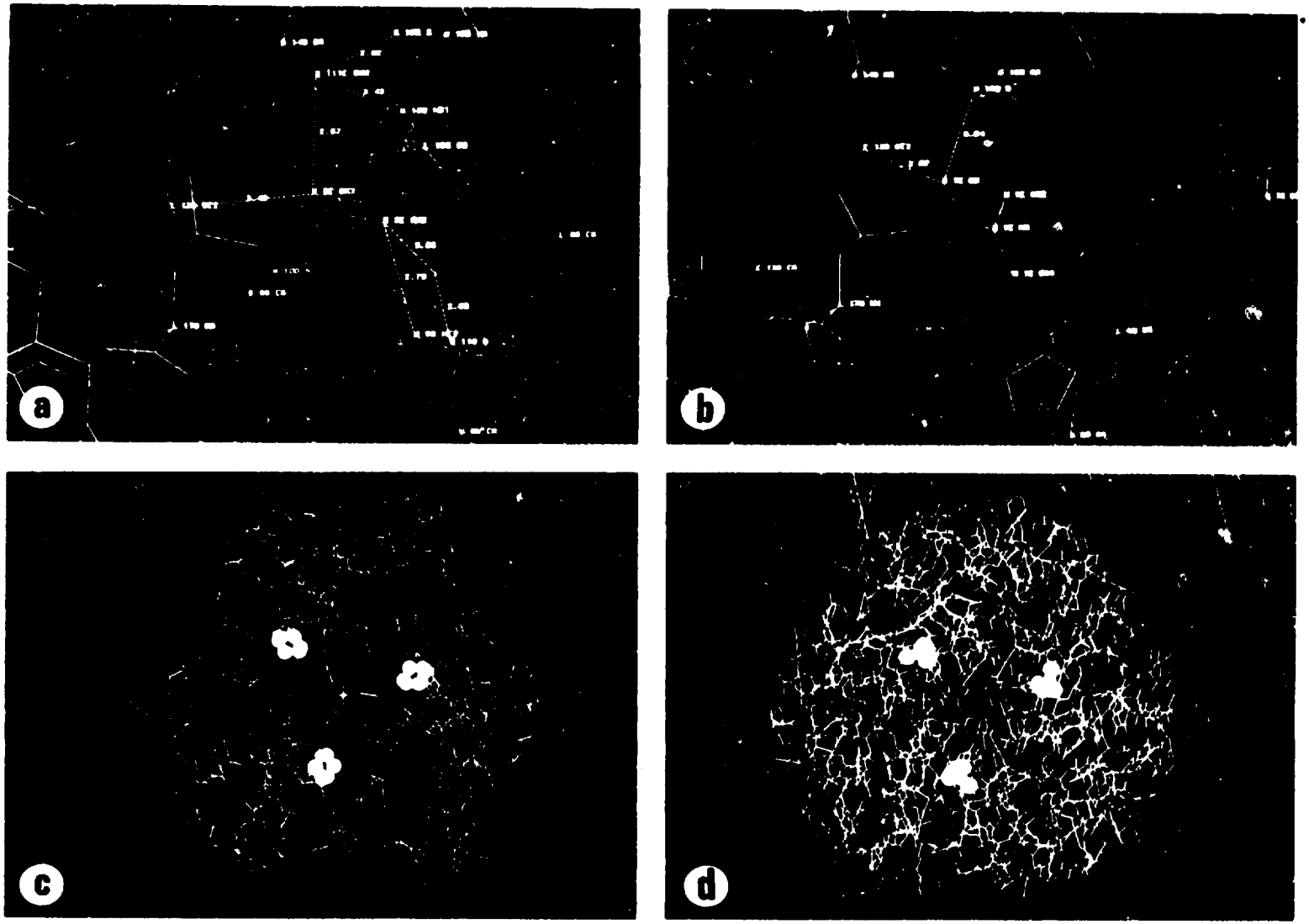


Fig.3 1,4-dioxane and DMF molecules as effectors for inducing 2Zn-T_3R_3 and Zn-free-T_3R_3 structure and its binding pockets on the hexamer surface of A21S and A21S-B27R-B30NH_2. (a) and (b) show sections of ΔF Fourier map (contour levels beginning at 5σ) indicating definitely existences of 1,4-dioxane in A21S (a) and DMF in A21S-B27R-B30NH_2 (b). The whole A21S hexamer with three dioxane molecules (in space-filling drawing) and A21S-B27R-B30NH_2 with three DMF molecules (in space filling drawing) are shown in (c) and (d) respectively, which clearly indicate the locations of effector binding pockets. The details please see the text.

Work plan and time schedule (originally envisaged)

The period of the project will last for three years and the general work plan is listed as follows:

1st Year Crystallization of a series of insulin mutants and preliminary X-ray crystallographic analysis; Partial X-ray data collection.

2nd Year Collection of the X-ray data for 3~5 mutants at medium and partly at high resolution. Solving the phase problem and building the initial structural model for 2~3 mutants.

3rd Year Completing the structural determination of 5 insulin derivatives, as well as the structural comparison between the mutants and the native insulin so as to understand the structural mechanism of prolonged action and other properties; Data collection for some more mutants.

Part 3

Work plan and time schedule

(actual)

Project landmarks, duration of individual tasks (use bar charts); evaluation criteria (publications, patents, services, training)

1991~1992, 1st Year

Crystallization: 3 prolonged-acting HI (B31Arg-, B31-Lys- and A21Ser-B27Arg-B30NH_2-HI);
3 high stable HI (A21Ser-, A21Gly-, A21Asp-HI);
1 low antigenieity HI (B30Gly HI).
Data Collection: 7 samples described above at the resolution 2.5Å, 1.8Å and 1.5Å.
Structure: 1 structure was determined: B31Arg HI at 2.0Å resolution.
Publication: 7 papers (see Annual Report (1991~1992) in details).

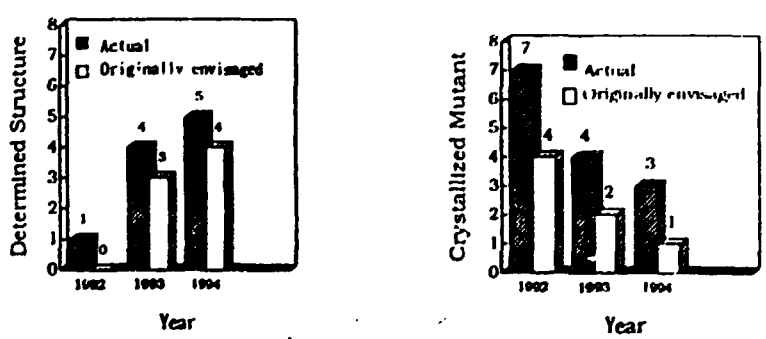
1992~1993, 2nd Year

Crystallization: 2 high potent HI (B3Lys- and A21Ala-HI);
2 structural significant HI (B8Ser-B13Gln HI and GlyB20Gln HI).
Structure: 4 structure were determined:
A21Gly-HI at 1.8Å resolution,
A21Ser-HI at 1.8Å resolution,
A21Asp-HI at 1.8Å resolution,
A21Ser-B27Arg-B30NH_2-HI at 2.1Å resolution.
Publication: 7 papers (see Annual Report (1992~1993) in details).

1993~1994, 3rd Year

Crystallization: 1 high potent HI (B25Tyr-HI);
2 fast absorption HI (B9Asp-HI and B9Glu-HI).
Data Collection: 7 samples crystallized in 1992~1994.
Structure: 5 structure were determined:
B31Lys-HI at 2.0Å resolution
B30Gly-HI at 1.6Å resolution
B3Lys-HI at 2.1Å resolution
B25Tyr-HI at 2.0Å resolution
B8Ser-B13Gln-HI at 2.1Å resolution
Publication: 5 papers (see Annual Report (1993~1994) in details).

* **HI:** Human insulin



The Determined Structures and the Crystallizeed Mutants of Insulin During 1991~1994.

NETWORKING

We have collaborated with the Novo Institute (Dr. Jan Markusser) of Novo-Nordisk AG at Denmark and the shanghai Institute of Biochemistry (Dr. Feng Youmin) for the design and preparation of insulin mutants.

PUBLICATIONS

[1] Crystal Structure Analysis of Human Insulin Mutant at High Resolution: Data Collection. *Photon Factory Acting Report*, National Laboratory for High Energy Physics, pp.87, Japan, 1991.

[2] Preliminary Crystallography Study on GlyB30-Human Insulin, an Analogue with Low Antigenicity, *Chinese Science Bulletin, 37*, 680-683, 1992

[3] Crystallographic Study on Highly Stable Human Insulin (I): A21-Ser Mutant, *Chinese Science Bulletin, 37*, 1390-1393, 1992

[4] Crystallographic Study on Highly Stable Human Insulin (II):Preliminary X-ray Analysis of A21-Gly Mutant, *Chinese Science Bulletin, 37*, 1302-1305, 1992

[5] Crystallographic Study on Highly Stable Human Insulin (III), Preliminary X-ray Analysis of A21-Asp Mutant, *Acta Biophysica Sinica, 8*, 76-79, 1992.

[6] Crystal Structure Analysis of Human Insulin Mutant at high Resolution. *Photon Factory Acting Report*, National Laboratory for High Energy Physics, pp87, Japan, 1992

PUBLICATIONS

[7] Crystal Structure of 4-Zn Bovine Insulin at 1.9 A Resolution. *Science in China*, 22(6), 541-549 (1992)

[8] Studies on Prolonged-Acting Insulin: Rational Molecular Design. *Acta Biophysics Sinica*, 8 (4), 601-609 (1992).

[9] Structural Basis of Prolonged Action of Insulin. *High Technology Letters*, 2, 8-11 (1992).

[10] X-ray Crystal Structure of Arg-B31 Human Insulin at 2.0 A Resolution. *Science in China*, 23 (3), 263-273 (1993).

[11] Crystal Structure Analysis of A21-Asp Human Insulin Mutant at 2.4 A Resolution. *Acta Biophysics Sinica*, 9 (1), 20-25 (1993).

[12] Studies on Crystal Structure of A21-Gly Human Insulin Mutant at 2.6 A Resolution. *Acta Biochimica et Biophysica Sinica*, 25 (3), 223-229 (1993).

[13] A New Structure Type of T_3R_3 Insulin Hexamer Observed in a Mutant A21Ser-Human Insulin. *Peptide: Biology and Chemistry*, Ed. Y.C. Du and J.P.Tam, ESCOM, 1993, pp241-244.

[14] Two Types of Conformational Intermediate of Insulin hexamer. Invited Lecture, *Symposium on Insulin Structure and Function*, Copenhagen, 18-23 May, 1993.

[15] Molecular Design and Target Sample Preparation for a Prolonged-acting Insulin. *Chinese Journal of Biotechnology*, 1994, 10 (2): 142-150.

[16] The Diversity of Insulin Allosteric Intermediate Observed in Some Mutants, *Proceeding of the First Asian Symposium on Biophysics*, May 16-20, 1994 Hyogo, Japan, pp. 129.

[17] Engineered Insulin: From Molecular Design to X-ray Analysis. Invited Lecture. *Workshops on Structural Biophysics*, May 19-20, 1994 Harima, Japan, pp. 20.

[18] Insulin Protein Engineering, Invited Lecture, *China-European Commission Workshop on Protein Engineering*, 22-27 Aug. 1994, Beijing, pp. 179.

[19] Crystallographic Studies on [A21Ser-B27Arg-B30Thr-NH2]-Human Insulin. *Chinese Science Bulletin*, 1994 (Accepted).

Part 4

STATEMENT OF EXPENDITURES

To be filled by ICGEB		To be filled by the Affiliated Center	
Budgets as per original proposal		**Summary of expenditures***	
1) Capital equipment	US$	1) Capital equipment	US$ 60,469.30
2) consumable	US$	2) consumable	US$ 10,450.00
3) training	US$	3) training	US$ 2,500.00
4) literature	US$	4) literature	US$ 527.00
5) miscellaneous	US$	5) miscellaneous	US$ 10,832.80
TOTAL GRANT	**US$**	**TOTAL**	**US$ 84,779.10**

Please itemize the following budget categories (if applicable)

Capital equipment

1. Wild Kombistereo Microscope (Switzerland)	US$	11,162.00
2. TCF2A Micro-Channel System	US$	6,030.00
3. Incubator ABMI (USA)	US$	3,377.00
4. Computer-Graphics System (SGI 4D/30G+)	US$	33,951.00
5. SGI Disk Drive	US$	5,949.30

Training (provide names, duration of training, host laboratory)

Zhang Ying 15 Aug. ~ 15 Sep. 1993
Synergy International center (Hong Kong)
for SGI Computer and Graphics Technique Training.

Literature

for "Current Opinion in Structural Biology" 1992~1994

* Please do not send invoices, receipts etc.: these should be kept by the Affiliated Center for future reference and sent to ICGEB upon request.

** except for invoices that are required in connection with paragraph 5. of the Contract.