UNITED STATES DISTRICT COURT
FOR THE SOUTHERN DISTRICT OF NEW YORK

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ASSOCIATION FOR MOLECULAR PATHOLOGY; AMERICAN COLLEGE OF MEDICAL GENETICS; AMERICAN SOCIETY FOR CLINICAL PATHOLOGY; COLLEGE OF AMERICAN PATHOLOGISTS; HAIG KAZAZIAN, MD; ARUPA GANGULY, PhD; WENDY CHUNG, MD, PhD; HARRY OSTRER, MD; DAVID LEDBETTER, PhD; STEPHEN WARREN, PhD; ELLEN MATLOFF, M.S.; ELSA REICH, M.S.; BREAST CANCER ACTION; BOSTON WOMEN'S HEALTH BOOK COLLECTIVE; LISBETH CERIANI; RUNI LIMARY; GENAE GIRARD; PATRICE FORTUNE; VICKY THOMASON; KATHLEEN RAKER,

Plaintiff,

-against-

UNITED STATES PATENT AND TRADEMARK OFFICE; MYRIAD GENETICS; LORRIS BETZ, ROGER BOYER, JACK BRITTAIN, ARNOLD B. COMBE, RAYMOND GESTELAND, JAMES U. JENSEN, JOHN KENDALL MORRIS, THOMAS PARKS, DAVID W. PERSHING, and MICHAEL K. YOUNG, in their official capacity as Directors of the University of Utah Research Foundation,

Defendant.

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No. Civil Action No. 09-4515 (RWS)

ECF Case

DECLARATION OF JOSEPH SCHLESSINGER. PH.D.

I, Joseph Schlessinger hereby declare that:

1. I am the Chairman of the Department of Pharmacology at Yale University School of Medicine and William H. Prusoff Professor since 2001. Prior to my appointment at Yale, I was the Director of the Skirball Institute for Biomolecular Medicine at New York University (NYU) Medical Center from 1998–2001 and the Milton and Helen Kimmelman Professor and Chairman of the Department of Pharmacology at NYU Medical School from 1990–2001. I was also a member of the faculty of the Weizmann Institute from 1978–1991 and the Ruth and Leonard Simon Professor of Cancer Research in the Department of Immunology from 1985–1991. My qualifications, experience and list of my publications are set forth in my curriculum vitae attached hereto as Exhibit 1. (Ex. 1)

2. I received a B.Sc. degree in Chemistry and Physics in 1968 (magna cum laude), and a M.Sc. degree in chemistry (magna cum laude) in 1970 from the Hebrew University in Jerusalem. I obtained my Ph.D. degree in biophysics from the Weizmann Institute of Science in 1974. From 1974–1976, I was a postdoctoral fellow in the Departments of Chemistry and Applied Physics at Cornell University, and from 1977–1978, I was a visiting fellow in the immunology branch of the National Cancer Institute of NIH.

3. In addition to my academic appointments, I was a Research Director at Meloy laboratory in Rockville, Maryland and later on in Rorer Biotechnology in King of Prussia, Pennsylvania from 1985–1990. Thereafter, I co-founded Sugen, Inc. in 1991. One of the pipeline products developed at Sugen (SU11248) was ultimately approved by the FDA (Sutent or Sunitinib) for treating gastrointestinal stromal tumors, renal cell carcinoma and endocrine pancreatic cancer . In 2001, I co-founded Plexxikon, a company that uses a pioneering

structural biology-based platform for drug discovery. I am currently the Chairman of the Board and a member of the Scientific Advisory Board of Plexxikon. In 2008 I founded Kolltan, a company that uses a novel approach for developing a new generation of therapeutic antibodies that block the activity of oncogenic receptor tyrosine kinases

4. I received the following awards: Michael Landau Prize (1973), Sara Leady Prize (1980), Hestrin Prize (1983), Levinson Prize (1984), Ciba-Drew Award (1995), Antoine Lacassagne Prize (1995), The Distinguished Service Award of Miami Biotechnology in (1999), Honorary Membership of the Japanese Biochemical Society (1999), Taylor Prize (2000), Honorary Doctor of Philosophy from the University of Haifa (2002) and the Dan David Prize (2006).

5. I was elected to the National Academy of Sciences in 2000, to the American Academy of Arts and Sciences in 2001, and as Member of the Institute of Medicine of the National Academies in 2005. I am a member of the European Molecular Biology Organization (EMBO) (1982), a fellow of the Neuroscience Research Program (2000) and a member of the European Academy of Sciences (2004).

6. I serve on the editorial boards of numerous journals, including Cell, Molecular Cell., the Journal of Cell Biology, and the Science magazine 'Science Signaling' journal. I have authored over 450 scientific original and review articles in the area of molecular biology, biochemistry, structural biology and drug discovery, mostly in the area of tyrosine kinase signaling.

7. I have delivered many named lectures including The Fourth Kroc Lecture, Harvard Medical School (1988); E.J. Cohn Lecture, Harvard Medical School (1993); E. Fisher

Lecture, University of Geneva (1993); Lamport Lecture, University of Seattle (1993); Harvey Lecture, Rockefeller University (1994); Deans Lecture, Mount Sinai Medical School (1994); Feigen Lecture, Stanford University (1994); Randall Lecture, University of Pennsylvania (1994); Sigma Tau Lecture, Rome, Italy (1995); Lindner Lecture, Weizmann Institute (1996); Burroughs Wellcome Lectures, University of Indiana (1997); Juan March Lecture, Madrid, Spain (1998); Bayer Lecture, Berkeley (1999); Sixth Kroc Lecture, University of Massachusetts (1999); NIH Director Lecture (2000); K.F. Naidorf Lecture, Columbia University (2000); Distinguished Speaker, University of Texas, San Antonio (2000); Severo Ochoa Lecture, Madrid, Spain (2000); First Alton Meister Lecture, Cornell University (2001); Fritz Lipmann Lecture, German Society for Biochemistry and Molecular Biology (2001); Karl Beyer Lectures, University of Wisconsin, Madison (2003); Asher Rothstein Lecture, The Hospital for Sick Children Research Institute, Toronto, Canada (2003); and the Distinguished Lecture on Molecular Targets for Cancer Prevention, AACR, Baltimore, Maryland (2005).

8. My area of research has focused on signaling through tyrosine phosphorylation, which is important in many areas of cellular regulation, especially growth control and cancer. My studies have led to an understanding of the mechanism of transmembrane signaling by receptor tyrosine kinases and how the resulting signals are transmitted within the cell to regulate gene expression, control cell growth and differentiation. Tyrosine kinase signaling plays a critical role in the control of many cellular processes including gene expression, cell proliferation, differentiation, metabolism, as well as cell survival and migration. Tyrosine kinases play a particularly important role in cancer, and several agents that block their activity are now used as anti-cancer drugs, such as Gleevec, Sutent and many others.

9. I reviewed the following documents: Plaintiffs' Memorandum of Law in Support of Motion for Summary Judgment ("Memo"); Plaintiffs' Rule 56.1 Statement of Material Facts ("SMF"); Declaration of Sir John Sulston, Ph.D. of August 17, 2009 ("Sulston"); Declaration of Christopher E. Mason of August 20, 2009 ("Mason"); and Declaration of Myles W. Jackson of August 18, 2009 ("Jackson").

10. As a scientist practicing in both the academic and industrial biotechnology/pharmaceutical arenas, I have experience with patents. I am a named inventor on a number of patents relating to screening methods and therapeutics. I have been asked to share my observations on the impact that patents have on research and development in the biotechnology field, and the role that patents have in bringing diagnostic and therapeutic products to the market making them available to patients. I am not a lawyer and express no legal opinion.

BACKGROUND

11. By way of background, genes are hereditary units contained within chromosomes – threadlike parts of the nucleus of cells in the body. The body does not have a mechanism for isolating genes, contrary to what I read in the Memo at p. 25 citing Jackson, ¶¶ 26-31 and Mason ¶¶ 11-12. A gene is part of a chromosome and is always surrounded by proteins.

12. Chromosomes are composed of DNA (**d**eoxyribo**n**ucleic **a**cid) and proteins. DNA is a chemical composition -- a polymer of building blocks called "nucleotides". Each nucleotide is composed of a deoxyribose sugar, a phosphate and one of four nitrogenous bases: adenine (A), guanine (G), thymine (T), and cytosine (C). DNA is double stranded and the

strands are crosslinked via the bases – A always links with T and G always links with C. A DNA molecule can be characterized by the sequence of the bases in the DNA polymer.

13. A gene on its own cannot do anything – other parts of the cell are required for a gene to have its biological effect. In general, this is to make one or more proteins vital to the structure and function of cells in the body. To do this, the gene's DNA is first copied or "transcribed" into RNA (ribonucleic acid) – a polymer of nucleotides that differ from DNA, each unit composed of a ribose sugar, a phosphate and one of four bases: adenine (A), guanine (G), uracil (U), and cytosine (C). For transcription, the DNA "unzips" and one of the DNA strands serves as the template for RNA assembly – A always links to U; G always links to C. The RNA transcript is then processed to form messenger RNA (mRNA) that is "translated" into a polypeptide (another biopolymer composed of building blocks called amino acids) which is a component of proteins responsible for the functions vital to the cell.

14. The RNA transcribed from a gene is processed into one or more messenger RNAs ("mRNAs") through a process called splicing in which portions of the RNA transcript that are not required for protein production are removed. Alternative splicing is a process in which different portions of the RNA transcript are reconnected in multiple ways. These different mRNAs resulting from transcription of one gene may then be translated into different polypeptides. Thus, a single gene may code for multiple mRNAs and protein products.

15. Using enzymatic reactions, a DNA molecule can be synthesized that is complementary to an mRNA (cDNA). But this DNA molecule would capture only one splice variant expressed from the gene. Thus, contrary to statements made by the plaintiffs, a cDNA is not necessarily informationally identical to the gene in the body. The plaintiffs' basis for this --

the old dogma of "one gene, one enzyme" -- is dead. SMF, ¶¶ 59, 61, 62; Mason, ¶¶ 9, 28-29, 32-33.

DECIPHERING THE STRUCTURE OF A GENE AND FUNCTION OF ITS PRODUCTS

16. Deciphering the structure of a gene and the function of its products is very relevant for diagnostics and drug development. If you do not know the sequence of a gene and the function of its products, you cannot design diagnostics and therapies to treat patients. The genome has been sequenced and published. But the raw sequence data does not tell you where a particular gene is exactly located and most importantly, the structure and function of the protein or proteins expressed from the gene.

17. I am puzzled by Dr. Sulston's view that an isolated DNA molecule cannot be an invention. He seems to rationalize his conclusion by simplifying DNA to an arrangement of letters that carries biological information dictated by nature that is identical to the information of its genomic counterpart. *See* Sulston, ¶¶ 10-19. Categorizing isolated genes as natural products/information that should be relegated to the public domain seems to me a bit superficial.

18. If you look hard enough, all things have natural origins. There are many examples of "natural products" that have been patented. Some examples of patented, small molecule "natural products" are antibiotics (*e.g.*, penicillin), chemotherapeutics (*e.g.*, taxol, mitomycin C) and statins (*e.g.*, lovastatin). Larger biomolecular "natural products," *i.e.*, proteins, have been patented as well, such as antibodies, erythropoietin, interferon and proteins that control blood clotting such as tissue plasminogen activator (TPA), streptokinase, and urokinase. DNA molecules whose biological function has been revealed by research should be treated in the

same manner. There is no difference between discovering the function of a gene and developing a diagnostic, and discovering a new natural chemical product and developing a new drug.

19. I disagree with Dr. Sulston's assessment that DNA is only information. *See* Sulston, ¶ 13-17. DNA is not merely a linear digital code consisting of a sequence of letters. DNA is a chemical composition – a polymer of building blocks called nucleotides. Each nucleotide is composed of a deoxyribose sugar, a phosphate and one of four nitrogenous bases: adenine, guanine, thymine and cytosine. Those bases are represented by the letters A, G, T, and C, respectively. The letters are just designations assigned to the nucleotides so that we can easily depict a DNA molecule. This system is analogous to the chemical formulas we use for describing other chemical compounds. For example, the chemical formula for water is H_2O, where "H" represents a hydrogen atom, "2" represents the number of hydrogen atoms present in the compound, and "O" represents an oxygen atom.

20. Sequencing the genome yields information that is fantastically important, *but* it does not tell you the exact location of the gene and most importantly what function is specified by the gene or by a mutated disease causing gene. I can understand why one should not be allowed to patent DNA without having any knowledge about its function, but an isolated DNA where the function of its products has been determined is a different story - here, an invention has been made. Moreover, if you figure out a direct link or an association between a gene sequence and a cause for or if you figure out the correlations or associations between a gene sequence and an increased risk for a specific disease, this discovery can be used to develop diagnostics and drugs that help people. I do not see any reason why a patent should not be awarded for such inventions.

21. To decipher the function of the proteins expressed from a gene takes a lot of work, ingenuity and luck. We have only 20,000 genes (humans have fewer genes than rice!). To manage all of the traits manifested by a human being, strategies evolved to use the limited supply of genes to provide all the functions needed by the body. For example, alternative splicing of RNA transcribed from a gene is a mechanism the cell uses to generate multiple mRNAs and their encoded protein products from each gene to provide the functions necessary for survival. Alternative splicing greatly increases the diversity of proteins that can be encoded by the genome.

22. In my field, there are approximately 518 genes that encode for protein kinases. On average, five alternatively spliced products result from the expression of each protein kinase gene, and this does not take into account the post-translational modifications of the resulting polypeptides and proteins which lead to regulation. Thus, there are at least 2,500 forms of protein product(s) that result from the expression of over 518 genes that encode the protein kinases.

23. In other situations, fully functional proteins are encoded by two or more genes. For example, antibodies, receptors, ion channels and many other proteins are composed of multiple polypeptides each encoded by different genes, *e.g.*, mammalian cytochrome c oxidase has 13 different polypeptide subunits with 3 being coded by a mitochondrial and nuclear genes and 10 by a nuclear gene. Tomitake Tsukihara, *et al.*, 1995, "Structures of Metal Sites of Oxidized Bovine Heart Cytochrome *c* Oxidase at 2.8 Å" *Science* 269:1069-1074 (Exhibit 2).

24. One approach to deciphering the function of a protein is to isolate or synthesize the DNA containing the gene and use it to produce protein product(s) to study their biochemical

functions. This requires first deciphering the sequence of the gene responsible for expression of the protein.

25. In another approach, you can use genetics to eliminate the gene from the genome of a cell or laboratory animal (sometimes referred to as "knocking out" the gene). If you are lucky and the gene is not redundant, you can figure out what the gene product does by observing the loss or gain of biochemical or physiological function in the cell or animal. For example, for tumor suppressor genes, like the BRCA genes, the elimination of the gene in these experiments should result in a loss of the protective function and increase the animal's risk for developing cancer.

26. In my view, a scientist who, using his ingenuity and aided by a little luck, spends a great deal of time and effort to investigate and discover what a gene product does and how to use it, has made an invention.

27. Once the scientist has deciphered the function of the gene's products, synthetic DNAs can be designed for diagnostics, cells can be engineered for drug screening, and therapeutics can be developed. The isolated DNA acquires functions it did not have as a gene in the body. Unlike genes in the body which are chemically bound in the chromosomes, isolated DNA molecules or their fragments can be used as a probe or primer for diagnostics, to synthesize proteins for therapeutic use, and/or for drug screening.

28. A probe or a primer can be used to detect target DNAs or RNAs based on its ability to bind, *i.e.*, hybridize, to its complementary nucleotide strand. A nucleotide strand is complementary to another when the bases of one strand are properly paired with the bases of the

other, *i.e.*, A always pairs with T (U in RNA) and G always pairs with C. For example, the complementary strand to a DNA molecule represented by ATCG will be depicted as TAGC.

29. An isolated DNA molecule can be used as a probe to identify target DNAs or RNAs in sample that are complementary to the arrangement of the probe's bases. In this application, the DNA probe is tagged with a molecular marker, *e.g.*, a radioactive isotope, and contacted with the sample under appropriate conditions to allow for hybridization. After washing away unbound material, the detection of the radioactive isotope in the sample indicates hybridization of the DNA probe to a complementary target.

30. An isolated DNA can also be used as a primer to amplify a specific region of a DNA strand (the DNA target) by a process called **p**olymerase **c**hain **r**eaction ("PCR"). In this approach, primers complementary to the target region along with an enzyme called DNA polymerase are added to a sample under appropriate reaction conditions. The primer serves as a starting point for synthesis of a new DNA strand complementary to the target DNA which serves as a template. The reaction is repeated to allow for amplification of the synthesized fragments. A gene in the body cannot be used as a probe or primer.

PATENTS ARE CRITICAL FOR RESEARCH AND DEVELOPMENT

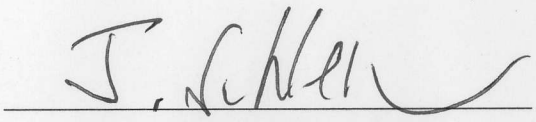
31. In my experience in the biotechnology industry, companies would not be able to raise money to do the research needed to develop diagnostic and therapeutic products without patent protection. Scientists involved in the Human Genome Project have been very fortunate to have the financial backing of organizations such as the Wellcome Trust. Ironically, the income used to establish the Wellcome Trust was derived from Burroughs Wellcome, a pharmaceutical company that amassed a fortune selling patented drugs. Most institutions do not have the luxury

of a trust fund, and need patent protection to obtain investments and funding. If these institutions cannot raise the capital to start companies or invest in research and development, promising new diagnostics and therapeutics will not be developed.

32. Moreover, without patent protection, large pharmaceutical and diagnostic companies would not take a drug or diagnostic test through clinical trials and to market. As a result, discoveries that would otherwise help patients, would remain a mere laboratory curiosity. The exclusivity that patents afford is the incentive for the large pharmaceutical companies to take the risk of investing large quantities of money to take a candidate drug or diagnostic through expensive clinical trials.

33. Pursuant to 28 U.S.C. § 1746, I declare under penalty of perjury that the foregoing is true and correct.

Executed on December 21, 2009



Joseph Schlessinger, Ph.D.

APPENDIX 1

LIST OF EXHIBITS

Exhibit No.	Title
Exhibit 1	*Curriculum Vitae*
Exhibit 2	Tsukihara, T. *et al.*, 1995, "Structures of Metal Sites of Oxidized Bovine Heart Cytochrome *c* Oxidase at 2.8 Å" *Science* 269:1069-1074

CERTIFICATE OF SERVICE

This is to certify that on December 23, 2009, a true and correct copy of the foregoing document has been served on all counsel of record via the court's ECF system.

/s/ Brian M. Poissant
Brian M. Poissant

EXHIBIT 1

CURRICULUM VITAE

NAME: **Joseph Schlessinger, Ph.D.**

BORN: March 26, 1945

LABORATORY ASSIGNMENT:
Yale University School of Medicine
Department of Pharmacology
333 Cedar Street, SHM-B95
P.O. Box 208066
New Haven, CT 06520-8066
(203) 785-7395

EDUCATION:

1965 - 1968	B.Sc. in Chemistry and Physics, Magna Cum Laude The Hebrew University, Jerusalem
1968 - 1969	M.Sc. in Chemistry, Magna Cum Laude The Hebrew University, Jerusalem
1970 -1974	Ph.D. Thesis on "Study of Chemical and Biological Systems by Circular Polarization of Fluorescence," Department of Chemical Physics, The Weizmann Institute of Science, Rehovot, Israel

CAREER:

1974 - 1976	Postdoctoral Associate at the School of Applied and Engineering Physics and Department of Chemistry, Cornell University, Ithaca, NY
1977 - 1978	Visiting Scientist at the Immunology Branch of the National Cancer Institute of the National Institutes of Health, Bethesda, MD
1978 - 1980	Senior Scientist, Department of Chemical Immunology The Weizmann Institute of Science, Rehovot, Israel
1980 - 1985	Associate Professor, Department of Chemical Immunology The Weizmann Institute of Science, Rehovot, Israel
1985 - 1991	Professor, Department of Chemical Immunology The Ruth and Leonard Simon Professor of Cancer Research The Weizmann Institute of Science, Rehovot, Israel
1985 - 1990	Research Director, Rorer Biotech. Inc., Rockville, MD. and King of Prussia, PA
1991	Founder, SUGEN, Inc.
1990 - 2001	Professor and Chairman, Department of Pharmacology The Milton and Helen Kimmelman Chair NYU Medical Center
1998 - 2001	Director of the Skirball Institute for Biomolecular Medicine NYU Medical Center
2001	Founder, Plexxikon Inc.
2001 - Present	Professor and Chairman, Department of Pharmacology The William H. Prusoff Professor and Chair Yale University School of Medicine
2008	Founder, Kolltan, Inc.

PROFESSIONAL HONORS AND NAMED LECTURES:

1973 Michael Landau Prize for Ph.D. Thesis
1980 Sara Leedy Prize
1982 Elected to EMBO
1983 Hestrin Prize
1984 Levinson Prize
1986 Keynote Presidential Lecture of the Endocrine Society
1988 The Fourth Kroc Lecture, Joslin Center, Harvard Medical School
1993 The E.J. Cohn Lecture, Harvard Medical School
1993 The E. Fisher Lecture, University of Geneva
1993 The Lamport Lecture, University of Seattle, Washington
1994 The Harvey Lecture, Rockefeller University, New York
1994 The Deans lecture, Mount Sinai Medical School, New York
1994 Opening Keynote Lecture, American Society for Biochemistry and Molecular Biology, Washington, D.C.
1994 The Feigen Lecture, Stanford University Medical School
1994 The Randall Lecture, University of Pennsylvania
1995 Sigma-Tau Lecture, Rome
1995 Opening Keynote Lecture: HHMI Symposium on Signal Transduction
1995 Opening Keynote Lecture, Whitehead Institute Symposium
1995 The Ciba-Drew Award
1995 Antoine Lacassagne Prize
1996 The Lindner Lecture, Weizmann Institute
1997 The Burroughs Wellcome Lecture, Indiana University
1998 The Juan March Lecture, Madrid, Spain
1999 The Distinguished Service Award, Miami Nature Biotechnology
1999 The Bayer Lecture, University of California, Berkeley
1999 The Sixth Ray A. and Robert L. Kroc Lecture University of Massachusetts Medical School
1999 Honorary Member, The Japanese Biochemical Society
2000 Elected to Neurosciences Research Program, La Jolla, California
2000 The NIH Director's Lecture
2000 The Distinguished Speaker Program, University of Texas Health Science Center
2000 Elected to the National Academy of Science
2000 Sixteenth Annual Kenneth F. Naidorf Memorial Lecture, Columbia University
2000 The Taylor Prize
2000 The Severo Ochoa Lecture, Madrid, Spain
2001 Elected to the American Academy of Arts and Sciences
2001 The First Alton Meister Memorial Lecture, Weil Medical College of Cornell University
2001 The Fritz Lipmann Lecture, German Society for Biochemistry and Molecular Biology
2002 Honorary Doctor of Philosophy, Honoris Causa, University of Haifa
2003 Aventis Perspective Lecture, Frankfurt, Germany
2003 The Karl Beyer Lectures, University of Wisconsin, Madison
2003 The Asher Rothstein Lecture, The Hospital of Sick Children Research Institute, Toronto, Canada
2004 Elected to the European Academy of Sciences
2005 Elected to the Institute of Medicine of the National Academies
2005 Distinguished Lecture on Molecular Targets for Cancer Prevention, AACR, Baltimore, Maryland
2006 The 33rd Annual Calbiochem–UCSD Lectureship Series
2006 Dan David Prize
2006 The 13th John F. Enders Lecture, Harvard Medical School
2006 The 41st Annual Mager Lecture, Hebrew University
2006 Elected as a foreign member of the Russian Academy of Sciences
2006 Inaugural Pfizer Lecture, Ann Arbor, Michigan
2006 The Keith Porter Lecture, ASCB Annual Meeting, San Diego, California

2008 Elected as a foreign member of the Croatian Academy of Science
2008 NIH-WALS Lecture, Bethesda, Maryland.
2008 Helen Coley-Nauts Lecture, Moscow State University
2008 24th Medical Scientist Lecture Series, UC Irvine
2009 Medal of Danica Hrvatska Order, Republic of Croatia
2009 Ef Racker Lecture in Biology and Medicine, Cornell University

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PUBLICATIONS:

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EXHIBIT 2

diffusivities between the SCF and the catalyst surface and of reduced coke deposition, benefits that have no parallel in homogeneous catalysis. The restriction to nonpolar or weakly polar reactants has been overcome through the use of entrainers or surfactants. For example, DeSimone and others have cleverly used soluble fluoropolymers in small amounts as surfactants to achieve greater rates or uniformity of product particle size during polymerization of methylmethacrylate (*47*) or acrylamide (*48*) in $scCO_2$. A similar approach could be used for molecularly catalyzed polar reactions in $scCO_2$.

The industrial outlook for SCFs as reaction media may be favorable because of the beneficial environmental effect of dispensing with organic liquid solvents. High-pressure operations would be facilitated in large-scale production processes and especially continuous-flow systems for which SCFs are well suited. Because of the nontoxicity of $scCO_2$, a future direction for molecular catalysis in that medium could be pharmaceutical synthesis. This is a field in which homogeneous transition metal-based catalysts have had a great impact. It is certain that researchers of homogeneous catalysis will adopt SCF as media for many more reactions and thus broaden the scope of this technique.

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51. We gratefully acknowledge the assistance and advice of Y. Hsiao, J. Xiao, and K. Aoyagi of ERATO.

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RESEARCH ARTICLE

Structures of Metal Sites of Oxidized Bovine Heart Cytochrome c Oxidase at 2.8 Å

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The high resolution three-dimensional x-ray structure of the metal sites of bovine heart cytochrome c oxidase is reported. Cytochrome c oxidase is the largest membrane protein yet crystallized and analyzed at atomic resolution. Electron density distribution of the oxidized bovine cytochrome c oxidase at 2.8 Å resolution indicates a dinuclear copper center with an unexpected structure similar to a [2Fe-2S]–type iron-sulfur center. Previously predicted zinc and magnesium sites have been located, the former bound by a nuclear encoded subunit on the matrix side of the membrane, and the latter situated between heme a_3 and Cu_A, at the interface of subunits I and II. The O_2 binding site contains heme a_3 iron and copper atoms (Cu_B) with an interatomic distance of 4.5 Å; there is no detectable bridging ligand between iron and copper atoms in spite of a strong antiferromagnetic coupling between them. A hydrogen bond is present between a hydroxyl group of the hydroxyfarnesylethyl side chain of heme a_3 and an OH of a tyrosine. The tyrosine phenol plane is immediately adjacent and perpendicular to an imidazole group bonded to Cu_B, suggesting a possible role in intramolecular electron transfer or conformational control, the latter of which could induce the redox-coupled proton pumping. A phenyl group located halfway between a pyrrole plane of the heme a_3 and an imidazole plane liganded to the other heme (heme a) could also influence electron transfer or conformational control.

Bovine heart cytochrome c oxidase is a large multicomponent membrane protein complex with molecular size of 200 kilodaltons comprising 13 different polypeptide subunits. Located in the subunits are two heme A moieties, two redox active copper sites, one zinc, one magnesium, and possibly some phospholipids as the intrinsic constituents (*1*, *2*). This enzyme is one of the most intriguing biological macromolecules in the cell. As the terminal enzyme of biological oxidation, it reduces O_2 to H_2O at an active site with the four redox active transition metals coupling to a proton pumping pro-

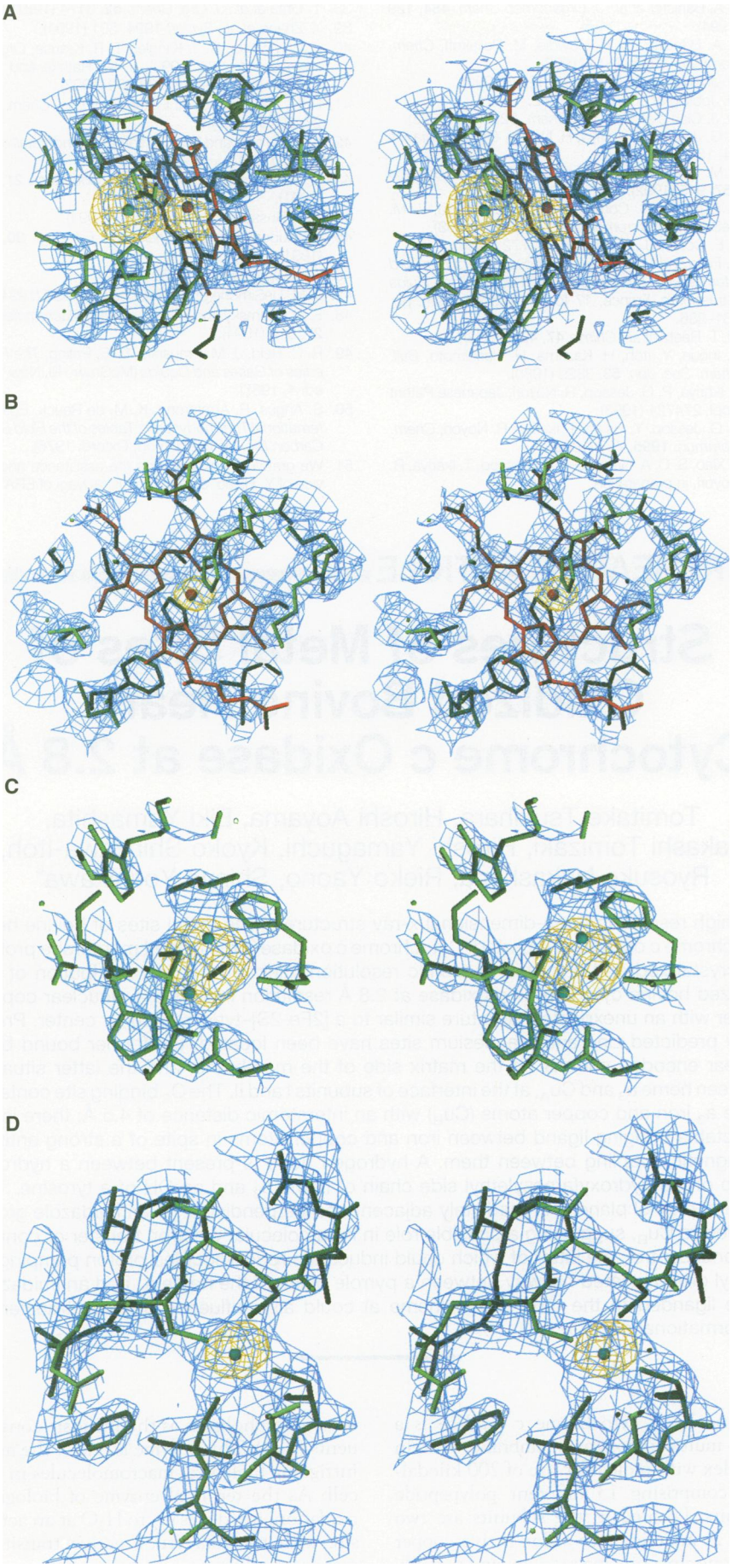

Fig. 1. Stereo pairs of the metal centers fitted in blue cages composed at 2σ of electron density at 2.8 Å resolution together with yellow or brown cages, drawn at 10 σ, of the native anomalous difference Fourier at 4.5 Å resolution. Model fitting was performed by the programs Turbo Frodo (*31*) and X-FIT in the program package QUANTA (CTC Laboratory Systems Co., Ltd.) on SGI workstations. (**A**) The oxygen binding and reduction site. The heme a_3 shown in red bars and a red ball, Cu_B in a green ball, and the amino acid residues in green bars are fitted well in the cages. (**B**) The heme a, two histidine ligands of Fe_a, and some amino acid residues are shown in the same colors as in (A). (**C**) Dinuclear structure of the Cu_A. Two copper atoms are shown with brown balls, amino acid residues with green bars, and tetrahedral coordination of each copper atom in blue dotted lines. (**D**) Tetrahedral coordination structure of the zinc site. Four cysteines and zinc atom position are shown with green bars and a dark yellow ball, respectively.

cess across the mitochondrial inner membrane (*2*, *3*). The bright green color of the enzyme, which is due to the heme iron and which changes sensitively with the redox and ligand-binding states of the active center, has stimulated electronic, resonance Raman, and infrared spectroscopic studies on its kinetic behavior during the enzymatic turnover (*2*–*4*).

Electron spin resonance studies have been made on the paramagnetic iron and copper ions (*2*, *3*), and infrared spectroscopy has been used to study various respiratory inhibitors as probes for the O_2 binding site (*5*). In addition, the varied roles of the large and complex protein moiety, which in the mammalian enzyme contains 13 different polypeptide subunits with 3 being coded by a mitochondrial and nuclear genes and 10 by a nuclear gene, have been widely studied (*1*, *6*). Thus, among the enzymes participating in biological oxidation, this one has been of primary interest ever since it was discovered (*7*). Despite these extensive and successful efforts, the absence of a crystal structure and analysis at atomic resolution has set limits on our understanding of the reaction mechanism of this enzyme. We had previously crystallized the enzyme from bovine heart, thus providing an example of x-ray diffractable crystals of a membrane protein isolated from a higher animal. However, the resolution of the crystals was far lower than suitable for x-ray structural analysis at atomic resolution (*8*). We therefore changed our techniques and have now ob-

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Table 1. Peak heights in the native electron density map at 2.8 Å and those in the anomalous difference Fourier map at 4.5 Å resolution.

Atomic group	Peak heights in σ unit	
	Electron density map	Anomalous difference map
Cu_A	12.1	14.3
Zn	10.9	9.7
Cu_B	11.4	9.3
Fe_{a_3}	10.3	5.1
Fe_a	10.0	5.3
PO^{4*}	9.6	
PO^{4*}	9.3	
PO^{4*}	8.3	
Other highest	7.8	3.5

*PO^4 represents a phosphate group of phospholipid.

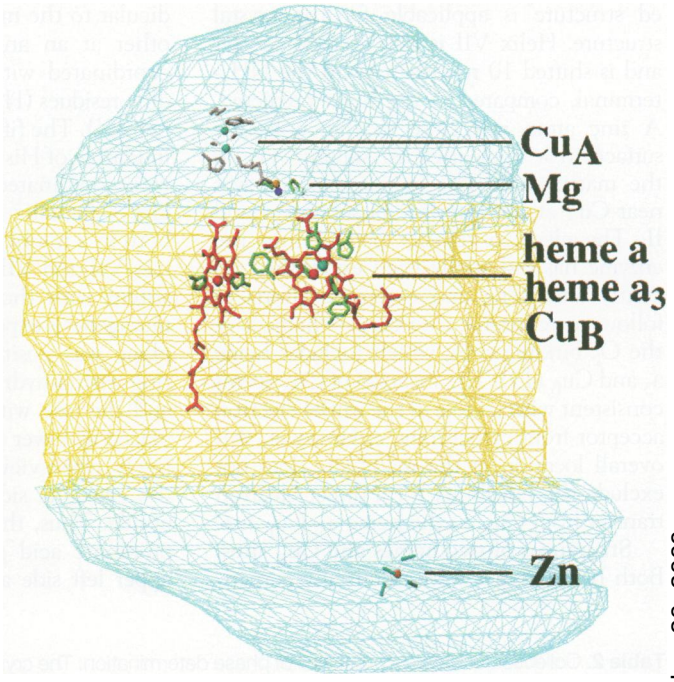

Fig. 2. A schematic representation of metal site location in beef heart cytochrome c oxidase. Molecular surface defined with the electron density map at 5 Å resolution is shown by the cage. The yellow, the upper light blue, and the lower light blue represent a transmembrane part with 48 Å thickness, a hydrophilic part protruding to the cytosolic side with 37 Å, and the other hydrophilic part with 32 Å in the matrix space, respectively. The three metal centers, Fe_a, Fe_{a_3} and Cu_B are located at the same level, 13 Å below the membrane surface on the cytosolic side. Another metal center, Cu_A, close to the molecular surface is 8 Å above the membrane surface level, and magnesium ion is at the membrane surface level. The distances of Cu_A-Fe_a, Cu_A-Fe_{a_3}, Cu_A-Mg, Fe_{a_3}-Fe_a, and Fe_{a_3}-Mg are 19 Å, 22 Å, 9 Å, 14 Å, and 15 Å, respectively.

tained crystals that diffract up to 2.6 Å resolution. During these procedures, it was necessary to adjust the structure of detergent that we used to stabilize the enzyme molecule in aqueous solution (9). We have now obtained from the electron density at 2.8 Å resolution the structures of the metal centers, which provide electron transfer pathways and serve as key structures for proton pumping.

Crystallization and structural determination. The crystals used for our study were prepared from bovine heart cytochrome c oxidase at the fully (resting) oxidized state (9) by the method previously described (*8*, *10*). Briefly, the purified protein was stabilized in aqueous solution with the nonionic detergent, 0.2 percent decyl maltoside in 40 mM sodium phosphate buffer, pH 6.8, and crystallized by the batch method from a solution where the protein concentration was 9.0 percent (w/v) at 4°C, and with polyethylene glycol 4000 (Sigma) being used as the precipitant. The crystals diffracted x-rays up to 2.6 Å resolution. This enzyme preparation reacted with cyanide monophasically, but not so rapidly as the enzyme under turnover conditions, indicating that it is the "fast" form (*11*).

We have obtained electron density of the enzyme at 2.8 Å resolution by the multiple isomorphous replacement (MIR) method; we then used a density modification procedure coupled with noncrystallographic symmetry (NCS) averaging. The electron density distribution obtained was so clear that all the metal center structures were easily built into the map (Fig. 1). The model building was performed with a modeling option REFINE of Turbo Frodo to fit each residue with standard dimensions in appropriate electron density cages. The final refinement comparing the obtained model and x-ray diffraction data has not yet been completed. However, the electron density distribution with extremely high reliability, evidenced by R_{free} factor of 0.258 at 2.8 Å resolution, allows quite satisfactory fitting of the atomic model to the electron density in most parts of the enzyme molecule, including the metal sites. It is remarkable that such a high accuracy of the electron density distribution has been obtained from such a large and multicomponent protein complex. The quality of the atomic model assignments is clearly shown by stereoscopic drawings of the model structures superimposed on electron density cages (Fig. 1).

The following results also indicate that the electron density distribution is plausible and thus reliable.

1) The order in peak height of the electron density map is consistent with the order in number of electrons of a group located at the corresponding peak (Table 1).

2) The anomalous difference Fourier map clearly revealed metal centers in spite of very low anomalous contribution from the metals compared with the structure factor of a whole molecule (Fig. 1 and Table 1). This is strong evidence of extremely high accuracy of phases and electron density.

3) Of 1850 amino acid residues, main chains of 1808 residues were successfully located in the map. More than 90 percent of side chains of nonglycine residues have reasonable electron density cages.

We now present the metal site structures of the enzyme at the fully (resting) oxidized state. Intensity data collection and phase determination are summarized in Table 2.

Overall location of metal sites. The membrane-embedded region evaluated from the start and end of the helices was 48 Å wide in the middle part of the molecule (Fig. 2). The direction of the molecules with respect to the cytosolic and matrix sides was estimated from the location of a copper site, Cu_A (see below), and the positions of the NH_2- and COOH-termini of peptide subunits. All the 13 subunits, each different from the other, were assigned in the crystal structure, which provides conclusive evidence that all these subunits are intrinsic constituents of the enzyme and that each is present in equimolar amount. This enzyme is in a dimeric state in the crystal, and there is no indication for strong interaction between the metal sites of each monomer. One of the redox active copper sites, Cu_A, was located in the third largest subunit, subunit II (6), which protrudes to the cytosolic side 8 Å above the membrane surface. The position of Cu_A, which is a dinuclear copper center (see below), is defined as the middle point between the two copper atoms. The other redox active metal centers, heme a, heme a_3, and Cu_B, the latter two of which constitute the O_2 binding and reduction site (*2*, *3*), were bound to subunit I at an identical level approximately 13 Å below (or inside) the membrane surface at the cytosolic side. Twelve transmembrane helices were found in subunit I, as has been predicted from the amino acid sequence (*1*, *12*). Although most of the helices in the crystal structure are longer than those predicted, the numbering of the helices for the predict-

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ed structure is applicable for the crystal structure. Helix VII is two residues shorter and is shifted 10 residues toward the NH_2-terminal, compared to the helix predicted. A zinc atom was close to the molecular surface in a nuclear-coded subunit Vb on the matrix side. The magnesium site was near Cu_A at the interface of subunits I and II. The electron transfer path within the enzyme has essentially been established recently after long and extensive efforts as follows: cytochrome c → Cu_A → heme a → the O_2 binding sites, which includes heme a_3 and Cu_B (*13*). The location of Cu_A seems consistent with its role as the initial electron acceptor from cytochrome c. However, this overall location of the metal site does not exclude the possibility for a direct electron transfer from Cu_A to the heme a_3–Cu_B site.

Structures of two hemes and Cu_B sites. Both heme planes were essentially perpendicular to the membrane plane facing each other at an angle of 104°. Heme a was coordinated with two imidazoles of histidine residues (His^{61} in helix II and His^{378} in helix X). The fifth ligand of heme a_3 was an imidazole of His^{376} in helix X, whereas Cu_B was coordinated by three imidazoles of His^{240} in helix VI and His^{290} and His^{291}, both in a nonhelical fragment between helices VII and VIII. These ligations are consistent with those proposed as a result of mutagenesis experiments (*12*). The heme a_3 plane was positioned so that the pyrrole ring with a hydroxyfarnesylethyl side chain and the one with a vinyl side group were placed at lower right and left sides, respectively, when viewed from the Cu_B side, with the cytosolic side being considered as the upside. Thus, the pyrrole with methyl and propionic acid groups was located at the upper left side and the one with a formyl and propionic acid groups was located at the upper right. In the case of heme a, the pyrrole with a hydroxyfarnesylethyl side chain was at the lower left, viewed from the Cu_B side (Fig. 2). The hydroxyfarnesylethyl group of heme a was almost in the extended conformation, held by helices I, II, X, XI, and XII, and that of heme a_3 was twisted to

Table 2. Collection of data and statistics of phase determination. The crystals diffracted x-rays up to 2.6 Å resolution. The crystal of the enzyme belongs to the orthorhombic space group of $P2_12_12_1$ with unit cell dimensions a = 189.1 Å, b = 210.5 Å, and c = 178.6 Å, containing two molecules in an asymmetric unit. Intensity data were collected with monochromatized x-ray of 1.0 Å at the Photon Factory, Tsukuba, Japan, by means of a modified Weissenberg camera for macromolecules (*24*). Diffraction intensities were processed with the program DENZO (*25*). Searching more than 50 heavy atom derivatives with MAC Science imaging plate detector (DIP2000) on a Rigaku rotating anode x-ray generator (RU300), we yielded three derivative data sets available for phase determination. Two of three derivatives were prepared by soaking the crystals in Na_2IrCl_6 solutions at various concentrations. The third was a CH_3HgCl derivative. Heavy atom sites of each derivative were located by solving each difference Patterson function at 5 Å resolution. The heavy atom parameters of three derivatives were refined with the program MLPHARE (*26*) of the CCP4 program package. In that MIR (*27*) phase information was poor beyond 5 Å resolution, phases were extended from 5 to 2.8 Å in 200 small steps with the use of solvent flattening (*28*) by means of the program DM of CCP4. The protein mask during the phase extension was determined by the Wang method (*28*) with a sphere of 10 Å and 70 percent solvent content by volume. A free R factor (*29*) for 5 percent of the reflections in each shell was reduced from 0.53 to 0.28. The noncrystallographic symmetry relation between two molecules in an asymmetric unit was determined, and the correlation coefficient $\{\Sigma(\rho1 - \langle\rho1\rangle)(\rho2 - \langle\rho2\rangle)/\Sigma[(\rho1 - \langle\rho1\rangle)^2(\rho2 - \langle\rho2\rangle)^2]^{1/2}\}$ was as high as 0.78. The phase refinement procedure including NCS averaging (*30*) was undertaken, starting at 5 to 2.8 Å resolution. The free R factor and the correlation coefficient at the final stage were 0.26 and 0.92, respectively.

	Native	$IrCl_6$(I)	$IrCl_6$(II)	CH_3HgCl
Resolution ranges (Å)*	100–2.8 (2.9–2.8)	100–3.0 (3.2–3.0)	100–3.0 (3.1–3.0)	100–3.0 (3.1–3.0)
Observed reflections	518,054 (24,542)	319,358 (18,599)	381,828 (28,410)	328,484 (15,186)
Independent reflections	159,742 (13,074)	125,036 (11,545)	133,245 (14,008)	127,548 (9,396)
$I/\sigma(I)$	19.5 (2.2)	10.0 (1.4)	21.3 (2.7)	2.4 (1.5)
Averaged redundancy†	3.2 (1.9)	2.6 (1.6)	2.9 (2.0)	2.6 (1.6)
Completeness (‡)	90.1 (74.4)	86.3 (64.5)	93.0 (79.1)	88.3 (65.7)
R_{merge}§	7.8 (29.0)	9.0 (29.6)	6.2 (23.4)	8.5 (28.4)
R_{iso}‖		7.3 (11.6)	5.7 (8.7)	9.0 (11.4)
R_{Cullis}¶		0.90 (0.97)	0.86 (0.97)	0.79 (0.95)
Phasing power#		0.38 (0.18)	0.54 (0.30)	0.74 (0.34)

*Figures in parentheses are given for the highest resolution shells. †Redundancy is the number of observed reflections for each independent reflection. ‡R_{merge}, $\Sigma_h\Sigma_i|I(h,i) - \langle I(h)\rangle|/\Sigma_h\Sigma_i I(h,i)$, where $I(h,i)$ is the intensity value of the i-th measurement of h and $\langle I(h)\rangle$ is the corresponding mean value of $I(h)$ for all i measurements; the summation is over the reflections with $I/\sigma(I)$ larger than 1.0. ‖R_{iso}, $\Sigma||F_{PH} - F_P|/\Sigma F_{PH}$, where F_{PH} and F_P are the derivative and the native structure factor amplitudes, respectively. ¶R_{Cullis}, $\Sigma||F_{PH} - F_P| - F_H(calc)|/\Sigma|F_{PH} - F_P|$, where $F_H(calc)$ is the calculated heavy atom structure factor. The summation is over the centric reflections only. #Phasing power is root-mean-square (rms) isomorphous difference divided by rms residual lack of closure.

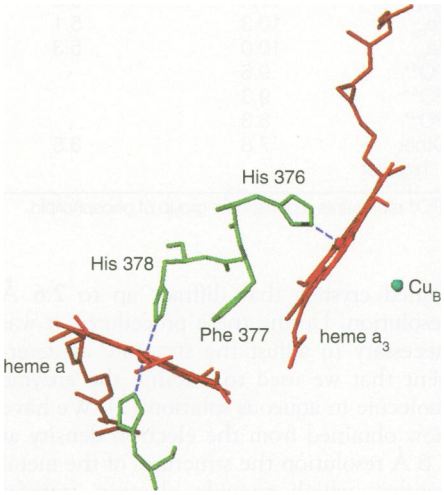

Fig. 3. Structural relation between two hemes in the active site of cytochrome c oxidase. Red structures and green structures represent hemes and amino acid residues in subunit I. A blue ball and red balls define the positions of copper and iron atoms, respectively. The hemes a and a_3 are bridged by three successive residues, His^{376}, Phe^{377}, and His^{378} in the helix X.

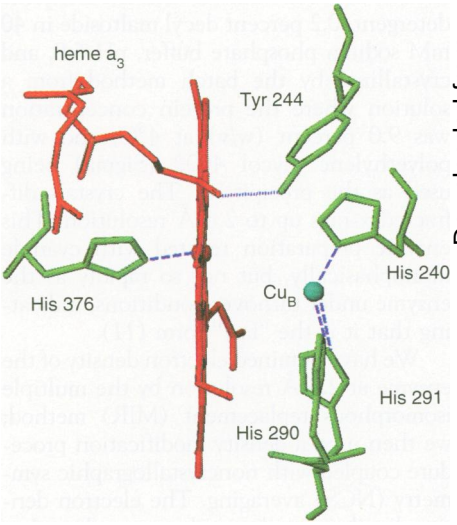

Fig. 4. A side view of the O_2 binding and reduction site. The red structure is heme a_3 and the green ones are amino acids. The dotted line shows a possible hydrogen bond between the hydroxyl of the hydroxyfarnesylethyl group and Tyr^{244}. The broken lines represent coordination bonds. The red and green balls represent the positions of iron and copper atoms, respectively. A close contact between Tyr^{244} and His^{240} with both aromatic rings oriented perpendicular with each other is also shown.

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form a U-shaped arm, located between helices VIII and IX. None of the hydroxyfarnesylethyl side chain interacted with the redox centers or the porphyrin aromatic ring system, contrary to the proposals based on the kinetic behavior of the enzyme (*4*, *14*).

A phenyl plane of a phenylalanine (Phe^{377} in helix X) was observed halfway between the two planes of the heme a_3 and one of the imidazole ligands of heme a (His^{378}) (Fig. 3). The distances between the phenyl plane and the two planes on both sides are as short as 3.5 Å. Thus, any change in the orientation and position of the phenyl group, induced by change in the ligation and oxidation state of Fe_{a_3} from the fifth ligand imidazole of His^{376}, adjacent to Phe^{377}, could control the electron transfer between Fe_a and Fe_{a_3}.

A side view of the heme a_3–Cu_B structure reveals the phenol OH group of tyrosine, Tyr^{244}, in subunit I oriented to the OH of the hydroxyfarnesylethyl group (Fig. 4). The Cu_B site was coordinated only by the three imidazoles of His^{240}, His^{290}, and His^{291}. No other ligand to Cu_B, even water, was detected. The high spin Fe_{a_3} ion was slightly displaced from the heme plane toward the fifth ligand. The Cu_B was 4.5 Å away from Fe_{a_3} and 1.0 Å from the heme normal at Fe_{a_3} position toward the nitrogen atom of the pyrrole with the formyl group. One of the unexpected findings was the absence of a ligand directly bridging the Fe_{a_3} and Cu_B. A bridging ligand has been proposed on the basis of strong antiferromagnetic coupling between the two atoms, a characteristic that was discovered 26 years ago (*15*); other model structures with a bridging ligand have been proposed (*16*). The presence of an amino acid side chain randomly oriented so that the electron density could not be detected by x-ray crystallographic analysis is unlikely because all the amino acid side chains near the dinuclear site have been assigned. However, the presence of water molecules nonspecifically and randomly trapped cannot be excluded.

A typical hydrogen bond is likely to be present between the hydroxyl groups of a tyrosine (Tyr^{244}) and the hydroxyfarnesylethyl side chain of heme a_3 in that the oxygen-oxygen distance is 3.0 Å. Furthermore, the orientation of the phenol plane of Tyr^{244} was perpendicular to the imidazole plane of His^{240}, which liganded to Cu_B. The nitrogen atom of the imidazole of His^{240} was located 3.1 Å above the plane of Tyr^{244}. The system, including the imidazole phenol-hydroxyfarnesylethyl side chain, could facilitate an electron transfer pathway from Fe_{a_3} to Cu_B. A structure that could provide another electron transfer pathway from heme a_3 to Cu_B is the formyl side group at the pyrrole, located 3.0 Å above the imidazole plane of His^{290}, which is a ligand of Cu_B. Although the distance between Fe_{a_3} and Cu_B, as short as 4.5 Å, suggests a facile electron transfer directly between the two metals, multiple electron transfer pathways are still possible for dioxygen reduction. In contrast, these structures may also induce an oxidation state–dependent conformational change to couple O_2 reduction with proton pumping. When Tyr^{244}, which is not bound directly to Cu_B, was replaced by phenylalanine, Cu_B was disrupted (*12*), suggesting some structural role of the amino acid.

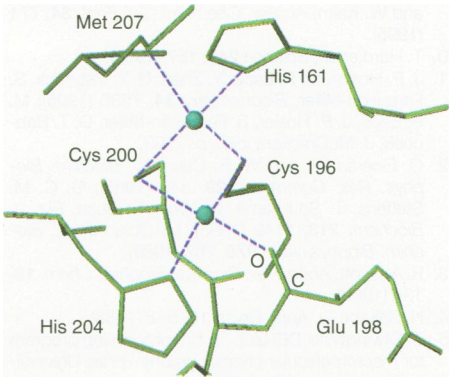

Fig. 5. A representation of Cu_A site showing a [2Cu-2Sγ] structure. Green balls show copper atom positions. Blue broken lines indicate coordination bonds. The peptide carbonyl group of Glu^{198} is illustrated with a bar marked with C and O.

Other interesting structures near the heme a_3 and Cu_B site are a hydrogen bond between the imidazole of His^{376} (the fifth ligand of heme a_3) and the peptide carbonyl of Gly^{351}, and a parallel π-π contact between His^{291} of Cu_B ligand and Trp^{236}. The imidazole of a heme a ligand, His^{61}, is linked to a carbonyl oxygen of Gly^{30} with a hydrogen bond. No close contact with the formyl group of heme a is detectable.

Structure of Cu_A. The Cu_A site in subunit II consists of six ligands, two cysteines (Cys^{196} and Cys^{200}), two histidines (His^{161} and His^{204}), a methionine (Met^{207}), and a peptide carbonyl of a glutamate (Glu^{198}) (Fig. 5). All these ligands, except for the peptide carbonyl, have been proposed as a result of mutagenesis experiments (*17*). The anomalous Fourier map at the central metal site exhibited a single peak (Fig. 1), which suggested a single metal, although both the peak heights in the Fourier and the anomalous Fourier maps, respectively, were too high to be attributable to a single copper atom (Table 1). The arrangement of these six ligands, determined from the electron density distribution near this site, does not provide any reasonable position for a single copper atom, but is fully consistent with a dinuclear copper center, as follows. Two copper atoms are bridged by two sulfur atoms of Cys^{196} and Cys^{200}, placing the four atoms on the same plane with the interatomic distances of 2.7 Å (Cu–Cu), and 3.8 Å (Sγ–Sγ). One of the copper atoms is coordinated by the imidazole nitrogen of His^{161} and the methionine sulfur of Met^{207}, forming a tetrahedral coordination including the two cysteine sulfur atoms. Coordination of the other copper is also tetrahe-

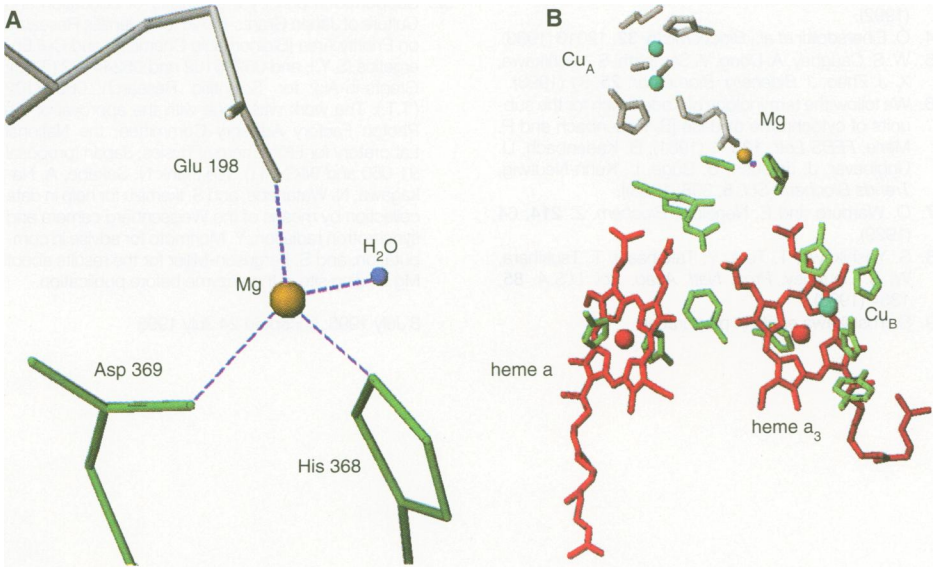

Fig. 6. The magnesium site and its relative orientation to other metal centers. (**A**) Coordination of magnesium. Green and light gray colors denote amino acid residues of subunits I and III, respectively. Dark orange and blue balls are at the positions of magnesium atom and oxygen atom of water, respectively. Blue broken lines are coordination bonds forming a distorted tetrahedron. (**B**) Location of the magnesium site and other active centers. Belonging of amino acids is shown with the same colors as in (A). Positions of iron, copper, magnesium, and oxygen of water are indicated with red, blue, dark orange, and dark blue balls, respectively.

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dral, including the imidazole nitrogen (His^{204}) and the peptide carbonyl (Glu^{198}). The geometry is similar to that of a [2Fe-2S]–type iron-sulfur center (*18*), in which the Fe ions and inorganic sulfur atoms are replaced with Cu ions and cysteine sulfur atoms, respectively. Both dinuclear centers stabilize a one-electron delocalized oxidation state, that is, [$Fe^{2.5+}$---$Fe^{2.5+}$] in the reduced form of [2Fe-2S] center and [$Cu^{1.5+}$---$Cu^{1.5+}$] in the oxidized state of [2Cu-2Sγ] center of Cu_A site. Recently, a dinuclear Cu center for Cu_A has been proposed in analogy to that of the copper enzyme N_2O reductase for which various structures not fully consistent with our structure have been suggested (*19*).

Structures of zinc and magnesium sites. Tetrahedral coordination was detectable for the zinc site where four cysteines (Cys^{60}, Cys^{62}, Cys^{82}, and Cys^{85}) in subunit Vb are coordinated by the central atom (Fig. 1C). The polypeptide fragment from Cys^{60} to Cys^{82} shows a zinc finger motif. However, the fragment does not form any protrusion from a globular core of the protein subunit, contrary to the typical zinc finger structure which interacts with a double-stranded DNA (*20*).

The electron density distribution of the enzyme crystals showed several other coordination structures, each with a central atom, that did not show any anomalous scattering for the x-rays at 1.0 Å. One of them involved an aspartate (Asp^{369}) and His^{368} in subunit I, Glu^{198} in subunit II, and an oxygen atom of water as the ligands in a distorted tetrahedral coordination (Fig. 6A). In agreement, Ferguson-Miller *et al.* discovered that His^{411} and Asp^{412} of *Rhodobacter sphaeroides*, which corresponds to His^{368} and Asp^{369} in beef heart enzyme, respectively, are essential for manganese binding to the enzyme at a site for which magnesium competes (*21*). Furthermore, electron density at this site is at a level reasonable for a magnesium atom. Thus, this site is highly likely to be a magnesium site. The magnesium site was found near (approximately 6 Å away from) two adjacent arginine residues (Arg^{438} and Arg^{439}) that faced diagonally to two propionic acid side groups of pyrroles of both hemes to form a tetrahedron involving two positively charged and two negatively charged groups. One of the ligands of the magnesium site, Glu^{198} of subunit II, was also coordinated to Cu_A at the peptide carbonyl, suggesting an oxidation state–linked conformational change which controls the electron transfer pathways between Cu_A and Fe_a and between Fe_a and Fe_{a_3} via the Arg–propionic acid system as stated above. A structural role of the magnesium for stabilizing the Cu_A site is also possible in that mutagenesis data suggest that Glu^{198} is required for forming the Cu_A site (*17*). The magnesium site is located on the hypotenuse of a right-angled triangle including Fe_a, Fe_{a_3}, and Cu_A with Fe_{a_3}–Cu_A as the hypotenuse (Fig. 6B). The presence of zinc and magnesium in equimolar amount of heme a_3 has been reported from several laboratories (*8*, *22*). However, the roles of these metals have not been clear. Our data clearly indicate the presence of intrinsic zinc and magnesium.

The above metal site structures are not completely consistent with the structures obtained from EXAFS. The inconsistency may be due to differences in the model used for the analysis of EXAFS data (*23*). The active site structures obtained here, although they resolve many controversies, require further refinement for the elucidation of the reaction mechanism of this enzyme.

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32. Supported in part by the Ministry of Education and Culture of Japan Grants-in-Aid for Scientific Research on Priority Area [Bioinorganic Chemistry and Cell Energetics (S.Y.), and 06276102 and 05244102 (T.T.)]; Grants-in-Aid for Scientific Research 06558102 (T.T.). The work was done with the approval of the Photon Factory Advisory Committee, the National Laboratory for High Energy Physics, Japan (proposal 91-050 and 94G-041). We thank N. Sakabe, A. Nakagawa, N. Watanabe, and S. Ikemizu for help in data collection by means of the Weissenberg camera and synchrotron radiation, Y. Morimoto for advise in computation, and S. Ferguson-Miller for the results about Mg binding site in the enzyme before publication.

3 July 1995; accepted 24 July 1995

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