

October 11, 1996

VOLUME 271

NUMBER 41

ISSN 0021-9258

JBCHA3 271(41) 25059-25722 (1996)

STANFORD LIBRARY  
OCT 17 1996

# THE Journal of Biological Chemistry

**Published by the American Society for Biochemistry  
and Molecular Biology**

FOUNDED BY CHRISTIAN A. HERTER

AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

THE JOURNAL OF BIOLOGICAL CHEMISTRY IS AVAILABLE ON THE WORLD WIDE WEB

<http://www-jbc.stanford.edu/jbc/>

# THE JOURNAL OF BIOLOGICAL CHEMISTRY

FOUNDED BY CHRISTIAN A. HERTER AND SUSTAINED IN PART BY THE CHRISTIAN A. HERTER MEMORIAL FUND

PUBLISHED BY THE AMERICAN SOCIETY FOR BIOCHEMISTRY AND MOLECULAR BIOLOGY, INC.

## EDITORIAL BOARD/1996

Herbert Tabor, *Editor*

Ralph A. Bradshaw, *Associate Editor*  
John H. Exton, *Associate Editor*  
Alan G. Goodridge, *Associate Editor*  
Richard W. Hanson, *Associate Editor*  
Vincent C. Hascall, *Associate Editor*  
Robert L. Hill, *Associate Editor*

Claudia Kent, *Associate Editor*  
I. Robert Lehman, *Associate Editor*  
Jerry B Lingrel, *Associate Editor*  
Kathleen S. Matthews, *Associate Editor*  
Kenneth E. Neet, *Associate Editor*  
Stephen M. Prescott, *Associate Editor*

Robert T. Schimke, *Associate Editor*  
Robert D. Simoni, *Associate Editor*  
James T. Stull, *Associate Editor*  
Thomas C. Vanaman, *Associate Editor*  
Kensal E. van Holde, *Associate Editor*  
Martha Vaughan, *Associate Editor*

Sherrill L. Adams  
Robert S. Adelstein  
Bharat Aggarwal  
Joseph P. Albanesi  
Norma M. Allewell  
Paul M. Anderson  
Vernon E. Anderson  
Ifeanyi J. Arinze  
Richard N. Armstrong  
Peter S. Aronson  
Thomas O. Baldwin  
Robert A. Bambara  
Leonard J. Banaszak  
Mariano Barbacid  
Heinz Baumann  
Joseph M. Beechem  
Vann Bennett  
Dale J. Benos  
Jeffrey L. Benovic  
Nathan A. Berger  
Helen M. Berman  
David A. Bernlohr  
Maurice J. Bessman  
Bruce Beutler  
M. Motasim Billah  
Morris J. Birnbaum  
Mariel Birnbaumer  
Diana L. Blithe  
Stephen Bocchino  
Paul E. Bock  
James W. Bodley  
Irving Boime  
David Wayne Bolen  
William F. Bosron  
Lawrence F. Brass  
Richard G. Brennan  
Michael Brenowitz  
Keith Brew  
Robert J. Brooker  
Michael Brownstein  
Steven S. Broyles  
George J. Broze  
Henri Brunengraber  
Joseph Bryan  
Floyd R. Bryant  
Wlodzimierz M. Bujalowski  
Peter M. J. Burgers  
Barbara K. Burgess  
Raymond F. Burk  
John Burke  
Drusilla L. Burns  
Fernando R. Cabral  
Kevin P. Campbell  
John R. Cann  
Roderick A. Capaldi  
Don M. Carlson  
Gerald M. Carlson  
George M. Carman  
Graham Carpenter  
Patrick J. Casey  
Michael Cashel  
Francis J. Castellino  
Kevin J. Catt  
Richard A. Cerione  
Ta-Yuan Chang  
Moses V. Chao  
P. Boon Chock  
David T. Chuang  
Frank C. Church  
Frank Chytil  
Steven G. Clarke  
Dennis O. Clegg  
G. Marius Clore  
Melanie H. Cobb  
Jonathan B. Cohen

Roger J. Colbran  
Joan W. Conaway  
H. Edward Conrad  
J. D. Corbin  
Michael M. Cox  
Richard L. Cross  
Larry W. Daniel  
Douglas Dean  
Donald B. DeFranco  
Pieter L. de Haseth  
George N. DeMartino  
Pierre De Meys  
Melvin L. DePamphilis  
Anna A. DePaoli-Roach  
Channing J. Der  
Robert Deschenes  
Wolfgang H. Dillmann  
Vishva Dixit  
Steven K. Dower  
Kurt Drickamer  
Ronald Dubreuil  
Maria L. Dufau  
Richard L. Eckert  
Dale E. Edmondson  
Peter A. Edwards  
Duane C. Eichler  
Betty A. Eipper  
Edward Eisenstein  
James Douglas Engel  
Harold P. Erickson  
Charles T. Esmon  
Peggy J. Farnham  
Alan P. Fields  
Robert H. Fillingame  
Richard A. Firtel  
David Fitzgerald  
Garret A. FitzGerald  
Michael D. Forgac  
Bernard G. Forget  
Claire M. Fraser  
Colin D. Funk  
John I. Gallin  
David L. Garbers  
Keith D. Garlid  
Lee Gehrke  
Thomas D. Gelehrter  
Peter M. J. Burgers  
Sandra J. Gendler  
Craig Gerard  
Mary Jane Gething  
Jackson Gibbs  
Hiram F. Gilbert  
Henry N. Ginsberg  
Ann Ginsburg  
Tibor T. Glant  
David B. Glass  
Claiborne V. C. Glover III  
David V. Goeddel  
I. David Goldman  
Myron F. Goodman  
Joel M. Gottesfeld  
Daryl K. Granner  
Donald J. Graves  
Michael Green  
Sergio Grinstein  
Richard W. Gross  
Arthur R. Grossman  
Michael Grunstein  
Lorraine J. Gudas  
Richard I. Gumpert  
Arthur L. Haas  
Joel F. Habener  
Margaret E. Haberland  
Stephen L. Hajduk  
Sen-itiroh Hakomori  
Joyce I. Hamlin

Heidi E. Hamm  
Steve Hanks  
Michael R. Hanley  
Robert A. Harris  
Marietta L. Harrison  
Gerald W. Hart  
Robert L. Heinrikson  
Renu A. Heller  
Susan A. Henry  
Brian Herman  
Harvey R. Herschman  
John W. B. Hershey  
Russ Hille  
Ole Hinds Gaul  
Jay Hirsh  
Yaacov Hod  
Robert S. Hodges  
Jeffrey M. Hoeg  
Sandra L. Hofmann  
Paul Horowitz  
M. Marlene Hosey  
Billy G. Hudson  
Richard L. Hugarin  
Tony E. Hugli  
John B. Imboden  
Thomas S. Ingebritsen  
Thomas L. Innerarity  
Keizo Inoue  
Renato V. Iozzo  
Suzanne Jackowski  
Steven J. Jacobs  
Robert J. James  
Michael L. Jennings  
Tae H. Ji  
Eric F. Johnson  
Larry R. Jones  
Donald B. Jump  
Laurie S. Kaguni  
Jack H. Kaplan  
Jerry Kaplan  
Neil Kaplowitz  
Charles B. Kasper  
Howard R. Katz  
Yoshito Kaziro  
Andrius Kazlauskas  
Robert G. Kemp  
Daniel Klionsky  
Walter Kisiel  
Hynda K. Kleinman  
Randall L. Kincaid  
Brian Kobilka  
Richard Kolesnick  
John J. Kopchick  
Ron Rieger Kopito  
Murray Korc  
Ruth M. Kramer  
Monty Krieger  
Thomas A. Kunkel  
John M. Kyriakis  
Gary E. Landreth  
John C. Lawrence  
James C. Lee  
Y. C. Lee  
Stuart F. Le Grice  
Mark A. Lehrman  
Warren J. Leonard  
Christina C. Leslie  
Ellis R. Levin  
Rodney L. Levine  
David E. Levy  
Thomas S. Leyh  
Stephen A. Liebhaber  
Gustav E. Lienhard  
Thomas M. Lincoln

Ulf Lindahl  
Vishwanath R. Lingappa  
Robert J. Linhardt  
John D. Lipscomb  
Laura Liscum  
Irving Listowsky  
Mark O. Lively  
Zvi Livneh  
Lawrence F. Loeb  
Joel Loftus  
Paul W. Ludden  
Richard F. Luduena  
Donal S. Luse  
Samuel E. Lux  
Ian G. Macara  
Robert W. Mahley  
Vince C. Manganiello  
Kenneth G. Mann  
David R. Manning  
James M. Manning  
Kenneth J. Mariani  
Daniel R. Marshak  
Michael A. Marletta  
Nancy C. Martin  
Marino Martinez-Carrion  
Vincent Massey  
Bettie Sue Masters  
Christopher K. Mathews  
Rafael Mattera  
Harry R. Matthews  
Richard E. McCarty  
Rodger P. McEver  
G. Stanley McKnight  
Linda C. McPhail  
Kathryn E. Meier  
Alfred H. Merrill  
Tobias Meyer  
Edith W. Miles  
Edward Mocariski  
Keith Moffat  
William T. Morgan  
Richard I. Morimoto  
John S. Mort  
Glenn E. Mortimore  
Dale W. Mosbaugh  
John Moss  
Shmuel Muallem  
Mike Mueckler  
Marc C. Mumby  
Robert S. Munford  
Philip M. Murphy  
Joanne Murphy-Ullrich  
Hideaki Nagasi  
Angus C. Nairn  
Joseph L. Napoli  
William M. Nauseef  
Benjamin G. Neel  
Francis C. Neuhaus  
Christopher B. Newgard  
Alexandra Newton  
John H. Nichols  
John H. Nilson  
Robert C. Nordlie  
Peter Novick  
Thomas L. Nowak  
Jerrold M. Olefsky  
Joanna B. Olmsted  
Bjorn R. Olsen  
Bradley B. Olwin  
Jack H. Oppenheimer  
Charles P. Ordahl  
Neil Osheroff  
Ida S. Owens  
R. Padmanabhan

Keith L. Parker  
Peter J. Parker  
J. Thomas Parsons  
Sarah J. Parsons  
Nicola C. Partridge  
Mulchand S. Patel  
Phillip H. Pekala  
John T. Penniston  
Ernest G. Peralta  
Anthony Persechini  
Jeffrey E. Pessin  
Kenneth D. Phillips  
Paul F. Pilch  
Thomas L. Poulos  
Susan Powers-Lee  
William B. Pratt  
Jack Preiss  
David H. Price  
Darwin J. Prockop  
Richard L. Proia  
John A. Putkey  
James P. Quigley  
Daniel M. Raben  
Francesco Ramirez  
A. Hari Reddi  
John C. Reed  
Marilyn D. Resh  
Raymond Reeves  
Reinhart A. F. Reithmeier  
Robert E. Rhoads  
John P. Richardson  
Paul D. Rick  
A. Jennifer Rivett  
Peter J. Roach  
Anita B. Roberts  
David D. Roberts  
James M. Roberts  
Diane M. Robins  
Janet D. Robishaw  
Thomas E. Roche  
Charles O. Rock  
William J. Roesler  
Barry P. Rosen  
Jeffrey Ross  
Richard A. Roth  
Fritz M. Rottman  
Enrique Rozenfurt  
Peter Rubenstein  
Frederick B. Rudolph  
Zaverio M. Ruggeri  
David W. Russell  
John C. Saari  
Lawrence E. Samelson  
David Samols  
Charles E. Samuel  
Konrad Sandhoff  
Immo E. Scheffler  
Carl Schildkraut  
Michael Schimerlik  
Bernard P. Schimmer  
Christian Schindler  
Joseph Schlessinger  
Gregory W. Schmidt  
Vern L. Schramm  
Robert D. Schreiber  
Arnold Schwartz  
Martin A. Schwartz  
Robert Schwartz  
John D. Scott  
Jere P. Segrest  
Pravin B. Sehgal  
Michael E. Selsted  
Alan E. Senior  
David Ray Setzer

Jules A. Shafer  
Barry Shane  
Aaron J. Shatkin  
Stephen B. Shears  
Dennis Shields  
Steven E. Shoelson  
Gary E. Shull  
Howard A. Shuman  
David R. Sibley  
S. Stoney Simons, Jr.  
Anna Marie Skalka  
Richard G. Sleight  
Jeffrey W. Smith  
Martin D. Snider  
Roy J. Soberman  
Avril V. Somlyo  
Leonard D. Spicer  
Robert G. Spiro  
Michael Sporn  
Howard Sprecher  
Linda L. Spremulli  
John L. Spudich  
Darrel W. Stafford  
E. Richard Stanley  
Pamela Stanley  
James V. Staros  
Robert E. Steele  
Donald F. Steiner  
Tom H. Stevens  
Jeffrey B. Stock  
Catherine D. Strader  
Thomas W. Sturgill  
Thomas C. Sudhof  
Kathleen J. Sweadner  
Ira Tabas  
Marvin L. Tanzer  
Palmer Taylor  
Simeon Taylor  
Elizabeth C. Theil  
Dennis J. Thiele  
Andrew P. Thomas  
Kenneth A. Thomas  
Larry S. Tobacman  
Allen J. Tobin  
Douglas M. Tollefsen  
Howard C. Towle  
James Travis  
Robert B. Trimble  
Bernard L. Trumpower  
Kathleen M. Trybus  
Robert H. Tukey  
Salvatore J. Turco  
Michael D. Uhler  
Todd A. Verdoorn  
Joseph J. Villafranca  
Dennis R. Voelcker  
Charles J. Waechter  
Donal A. Walsh  
John L. Wang  
Teresa S. F. Wang  
David J. Waxman  
Paul H. Weigel  
Peter Anthony Weil  
Robert J. Wenthold  
Reed B. Wickner  
Bryan R. G. Williams  
Masaki Yanagishita  
Helen L. Yin  
Howard A. Young  
Peter R. Young  
Daniel M. Ziegler  
Sally H. Zimmond  
Guy A. Zimmerman  
R. Suzanne Zukin

John T. Edsall, *Advisor to the Board*  
Charles C. Hancock, *Manager*, 9650 Rockville Pike, Bethesda, Maryland 20814  
Barbara A. Gordon, *Assistant to the Editor*

Copyright © 1996 by the American Society for Biochemistry and Molecular Biology, Inc.  
9650 Rockville Pike, Bethesda, MD 20814 U.S.A.

## CONTENTS Arranged by Subject Categories

- MINIREVIEW
- 25059 **The MutT proteins or "nudix" hydrolases, a family of versatile, widely distributed, "house cleaning" enzymes.** Maurice J. Bessman, David N. Frick, and Suzanne F. O'Handley
- CELL BIOLOGY AND METABOLISM
- 25067 **Communication—Calmodulin binds to and inhibits GTP binding of the Ras-like GTPase Kir/Gem.** Roland Fischer, Yu Wei, John Anagli, and Martin W. Berchtold
- 25107 **Site-directed mutagenesis of nm23-H1. Mutation of proline 96 or serine 120 abrogates its motility inhibitory activity upon transfection into human breast carcinoma cells.** Nicholas J. MacDonald, José M. P. Freije, Mary L. Stracke, Richard E. Manrow, and Patricia S. Steeg
- 25117 **Altered regulation of G<sub>1</sub> cyclins in oxidant-induced growth arrest of lung alveolar epithelial cells. Accumulation of inactive cyclin E-CDK2 complexes.** Sophie Corroyer, Bernard Maitre, Véronique Cazals, and Annick Clement
- 25126 **Identification of histone H2A.X as a growth factor secreted by an androgen-independent subline of mouse mammary carcinoma cells.** Yoshio Watabe, Hiroaki Kuramochi, Yuzo Furuya, Nobuya Inagaki, Susumu Seino, Sadao Kimura, and Jun Shimazaki
- 25131 **Function and expression of flavohemoglobin in *Saccharomyces cerevisiae*. Evidence for a role in the oxidative stress response.** Xiao-Jian Zhao, Desmond Raitt, Patricia V. Burke, Amy S. Clewell, Kurt E. Kuast, and Robert O. Poyton
- 25145 **Specific phospholipid association with apolipoprotein A-I stimulates cholesterol efflux from human fibroblasts. Studies with reconstituted sonicated lipoproteins.** Yuwei Zhao, Daniel L. Sparks, and Yves L. Marcel
- 25157 **Adducin regulation. Definition of the calmodulin-binding domain and sites of phosphorylation by protein kinases A and C.** Yoichiro Matsuoka, Christine A. Hughes, and Vann Bennett
- 25173 ***Clostridium novyi*  $\alpha$ -toxin-catalyzed incorporation of GlcNAc into Rho subfamily proteins.** Jörg Selzer, Fred Hofmann, Gundula Rex, Matthias Wilm, Matthias Mann, Ingo Just, and Klaus Aktories
- 25192 **Phosphopleckstrin inhibits G $\beta\gamma$ -activable platelet phosphatidylinositol-4,5-bisphosphate 3-kinase.** Charles S. Abrams, Jin Zhang, C. Peter Downes, Xiu-wen Tang, Wei Zhao, and Susan E. Rittenhouse
- 25198 **p130<sup>CAS</sup> forms a signaling complex with the adapter protein CRKL in hematopoietic cells transformed by the BCR/ABL oncogene.** Ravi Salgia, Evan Pisick, Martin Sattler, Jian-Liang Li, Naoki Uemura, Wai-Keung Wong, Stephen A. Burky, Hisamaru Hirai, Lan Bo Chen, and James D. Griffin
- 25204 **A tyrosine kinase signaling pathway accounts for the majority of phosphatidylinositol 3,4,5-trisphosphate formation in chemoattractant-stimulated human neutrophils.** Andrzej Ptaszniak, Eric R. Prossnitz, Dan Yoshikawa, Alan Smrcka, Alexis E. Traynor-Kaplan, and Gary M. Bokoch
- 25208 **Covalent attachment of FAD derivatives to a fusion protein consisting of  $\delta$ -hydroxy-D-nicotine oxidase and a mitochondrial presequence. Folding, enzyme activity, and import of the modified protein into yeast mitochondria.** Michaela Stoltz, Joachim Rassow, Andreas F. Bückmann, and Roderich Brandsch
- 25227 **Overexpression of a constitutively active form of phosphatidylinositol 3-kinase is sufficient to promote Glut 4 translocation in adipocytes.** Jean-François Tanti, Thierry Grémeaux, Sophie Grillo, Véronique Calleja, Anke Klippel, Lewis T. Williams, Emmanuel Van Obberghen, and Yannick Le Marchand-Brustel
- 25240 **A predictive scale for evaluating cyclin-dependent kinase substrates. A comparison of p34<sup>cdc2</sup> and p33<sup>cdk2</sup>.** Jennifer K. Holmes and Mark J. Solomon
- 25277 **Secretagogues increase the expression of surfactant protein A receptors on lung type II cells.** Qiping Chen, Sandra R. Bates, and Aron B. Fisher
- 25308 **Interaction of phosphorylated Fc $\epsilon$ RI $\gamma$  immunoglobulin receptor tyrosine activation motif-based peptides with dual and single SH2 domains of p72<sup>src</sup>. Assessment of binding parameters and real time binding kinetics.** Ting Chen, Barbara Repetto, Richard Chizzonite, Christine Pullar, Charles Burghardt, Elizabeth Dharm, Zhicheng Zhao, Robert Carroll, Perla Nunes, Mitali Basu, Waleed Danho, Mike Visnick, Jarema Kochan, David Waugh, and Alasdair M. Gilfillan
- 25327 **Regulation of *obese* (*ob*) mRNA and plasma leptin levels in rhesus monkeys. Effects of insulin, body weight, and non-insulin-dependent diabetes mellitus.** Kikuko Hotta, Thomas A. Gustafson, Heidi K. Ortmeyer, Noni L. Bodkin, Margery A. Nicolson, and Barbara C. Hansen
- 25369 **The antibiotic bicyclomycin affects the secondary RNA binding site of *Escherichia coli* transcription termination factor Rho.** Attila Magyar, Xiangdong Zhang, Harold Kohn, and William R. Widger
- 25400 **Interleukin-8 (IL-8), melanoma growth-stimulatory activity, and neutrophil-activating peptide selectively mediate priming of the neutrophil NADPH oxidase through the type A or type B IL-8 receptor.** Simon P. Green, Anan Chuntharapai, and John T. Curnutte
- 25406 **Modulation of GDP release from transducin by the conserved Glu<sup>134</sup>, Arg<sup>135</sup> sequence in rhodopsin.** Shreeta Acharya and Sadashiva S. Karnik
- 25430 **The regulated secretion and vectorial targeting of neurotrophins in neuroendocrine and epithelial cells.** John V. Heymach, Jr., Alex Krüttgen, Ueli Suter, and Eric M. Shooter
- 25446 **Cytosolic and membrane-associated proteins involved in the recruitment of AP-1 adaptors onto the trans-Golgi network.** Matthew N. J. Seaman, Penelope J. Sowerby, and Margaret S. Robinson
- 25452 **Identification of a novel guanine nucleotide exchange factor for the Rho GTPase.** Matthew J. Hart, Sanju Sharma, Nadia elMasry, Rong-Guo Qiu, Peter McCabe, Paul Polakis, and Gideon Bollag
- 25479 **Double-stranded RNA-dependent protein kinase mediates c-Myc suppression induced by type I interferons.** Tal Raveh, Ara G. Hovanessian, Eliane F. Meurs, Nahum Sonenberg, and Adi Kimchi
- 25492 **Hypoxia-inducible protein binding to vascular endothelial growth factor mRNA and its modulation by the von Hippel-Lindau protein.** Andrew P. Levy, Nina S. Levy, and Mark A. Goldberg
- 25506 **Formation of a ligand-binding site for the acetylcholine receptor *in vitro*.** Svetlana S. Shtrom and Zach W. Hall

The JBC is available on World Wide Web. URL: <http://www-jbc.stanford.edu/jbc/>Full Instructions to Authors will be found in (1996) *J. Biol. Chem.* **271**, 15845–15848, and reprints may be obtained from the editorial office.

- 25533 **Interactions between Src homology (SH) 2/SH3 adapter proteins and the guanylnucleotide exchange factor SOS are differentially regulated by insulin and epidermal growth factor.** *Shuichi Okada and Jeffrey E. Pessin*
- 25569 **Activation of protein-tyrosine phosphatase SH-PTP2 by a tyrosine-based activation motif of a novel brain molecule.** *Hiroshi Ohnishi, Misae Kubota, Atsuko Ohtake, Kazuki Sato, and Shin-ichiro Sano*
- 25598  **$\alpha 7$  integrin mediates cell adhesion and migration on specific laminin isoforms.** *Chung-Chen Yao, Barry L. Ziober, Rachel M. Squillace, and Randall H. Kramer*
- 25630 **Degradation of 3-hydroxy-3-methylglutaryl-CoA reductase in endoplasmic reticulum membranes is accelerated as a result of increased susceptibility to proteolysis.** *Todd P. McGee, Helen H. Cheng, Hidetoshi Kumagai, Satoshi Omura, and Robert D. Simoni*
- 25677 **Phosphorylation of extracellular domains of T-lymphocyte surface proteins. Constitutive serine and threonine phosphorylation of the T cell antigen receptor ectodomains.** *Sergey G. Apasov, Patrick T. Smith, Marie T. Jelonek, David H. Margulies, and Michail V. Sitkovsky*
- 25684 **Cell-surface cytokeratin 8 is the major plasminogen receptor on breast cancer cells and is required for the accelerated activation of cell-associated plasminogen by tissue-type plasminogen activator.** *Todd A. Hembrough, Li Li, and Steven L. Gonias*
- 25692 **The role of phosphatidylcholine biosynthesis in the regulation of the *INO1* gene of yeast.** *Peter Griac, Marci J. Swede, and Susan A. Henry*

#### ENZYMOLGY

- 25071 **Phospholipase C  $\beta 2$  association with phospholipid interfaces assessed by fluorescence resonance energy transfer. G protein  $\beta \gamma$  subunit-mediated translocation is not required for enzyme activation.** *Valerie Romoser, Rebecca Ball, and Alan V. Smrcka*
- 25213 **Inhibition of phospholipase D by a protein factor from bovine brain cytosol. Partial purification and characterization of the inhibition mechanism.** *Jae Ho Kim, Yoon Jung Suh, Tae-hoon G. Lee, Yong Kim, Sun Sik Bae, Myung Jong Kim, J. David Lambeth, Pann-Ghill Suh, and Sung Ho Ryu*
- 25316 **Phosphatidylinositol 4,5-bisphosphate binding to the pleckstrin homology domain of phospholipase C- $\delta 1$  enhances enzyme activity.** *Jon W. Lomasney, Hwei-Fang Cheng, Li-Ping Wang, Y.-S. Kuan, S.-M. Liu, Stephen-W. Fesik, and Klim King*
- 25332 **Ca<sup>2+</sup> binding to the first epidermal growth factor-like domain of human blood coagulation factor IX promotes enzyme activity and factor VIII light chain binding.** *Peter J. Lenting, Olivier D. Christophe, Hans ter Maat, D. Jasper G. Rees, and Koen Mertens*
- 25611 **Allosteric activation of L-lactate dehydrogenase analyzed by hybrid enzymes with effector-sensitive and -insensitive subunits.** *Shinya Fushinobu, Kenji Kamata, So Iwata, Hiroshi Sakai, Takahisa Ohta, and Hiroshi Matsuzawa*
- 25699 **The calmodulin-dependent phosphodiesterase gene *PDE1C* encodes several functionally different splice variants in a tissue-specific manner.** *Chen Yan, Allan Z. Zhao, J. Kelley Bentley, and Joseph A. Beavo*

#### MEMBRANES AND BIOENERGETICS

- 25079 **Selectivity of the renal collecting duct water channel aquaporin-3.** *Miriam Echevarria, Erich E. Windhager, and Gustavo Frindt*
- 25139 **Transport mechanism of the cloned potato H<sup>+</sup>/sucrose cotransporter StSUT1.** *Kathryn J. Boorer, Donald D. F. Loo, Wolf B. Frommer, and Ernest M. Wright*

- 25167 **The ATP binding cassette transporters Pdr5 and Sng2 of *Saccharomyces cerevisiae* can mediate transport of steroids in vivo.** *Yannick Mahé, Yves Lemoine, and Karl Kuchler*
- 25184 **Function of *Xenopus* cystic fibrosis transmembrane conductance regulator (CFTR) Cl<sup>-</sup> channels and use of human-*Xenopus* chimeras to investigate the pore properties of CFTR.** *Margaret P. Price, Hiroshi Ishihara, David N. Sheppard, and Michael J. Welsh*
- 25247 **Interaction of ATP binding sites in the Arsa ATPase, the catalytic subunit of the Ars pump.** *Jiaxin Li, Shusen Liu, and Barry P. Rosen*
- 25338 **Mechanisms for the transport of  $\alpha, \omega$ -dicarboxylates through the mitochondrial inner membrane.** *Guoying Liu, Bryan Hinch, and Andrew D. Beavis*
- 25438 **Probing conserved regions of the cytoplasmic LOOP1 segment linking transmembrane segments 2 and 3 of the *Saccharomyces cerevisiae* plasma membrane H<sup>+</sup>-ATPase.** *Genfu Wang, Markus J. Tamás, Michael J. Hall, Amparo Pascual-Ahuir, and David S. Perlin*
- 25582 **Membrane topology of the sodium ion-dependent citrate carrier of *Klebsiella pneumoniae*. Evidence for a new structural class of secondary transporters.** *Marleen van Geest and Juke S. Lolkema*
- 25590 **Determination of the transmembrane topology of yeast Sec61p, an essential component of the endoplasmic reticulum translocation complex.** *Barrie M. Wilkinson, Angela J. Critchley, and Colin J. Stirling*
- 25604 **Mechanism responsible for oligomycin-induced occlusion of Na<sup>+</sup> within Na/K-ATPase.** *Teruyo Arato-Oshima, Hideo Matsui, Akira Wakizaka, and Haruo Homareda*

#### NUCLEIC ACIDS, PROTEIN SYNTHESIS, AND MOLECULAR GENETICS

- 25089 **Recognition of DNA adducts by human nucleotide excision repair. Evidence for a thermodynamic probing mechanism.** *Daniela Gunz, Martin T. Hess, and Hanspeter Naegeli*
- 25178 **Molecular design of inhibitors of *in vitro* oriC DNA replication based on the potential to block the ATP binding of DnaA protein.** *Tohru Mizushima, Shigeki Sasaki, Hiroko Ohishi, Masakatsu Kobayashi, Tsutomu Katayama, Takeyoshi Miki, Minoru Maeda, and Kazuhisa Sekimizu*
- 25233 **The chick  $\alpha 2(I)$  collagen gene contains two functional promoters, and its expression in chondrocytes is regulated at both transcriptional and post-transcriptional levels.** *Kim M. Pallante, Zeling Niu, Yufeng Zhao, Arthur J. Cohen, Hyun-Duck Nah, and Sherrill L. Adams*
- 25253 **The interferon (IFN)-stimulated gene *Sp100* promoter contains an IFN- $\gamma$  activation site and an imperfect IFN-stimulated response element which mediate type I IFN inducibility.** *Thilo Gröttinger, Kirsten Jensen, and Hans Will*
- 25269 **Characterization of the human cytochrome P4502D6 promoter. A potential role for antagonistic interactions between members of the nuclear receptor family.** *William Cairns, Christopher A. D. Smith, Aileen W. McLaren, and C. Roland Wolf*
- 25292 **Two distinct promoters drive transcription of the human D<sub>1A</sub> dopamine receptor gene.** *Sang-Hyeon Lee, Mari T. Minowa, and M. Maral Mouradian*
- 25300 **Molecular characterization of a novel human endothelin receptor splice variant.** *Nabil A. Elshourbagy, John E. Adamou, Alison W. Gagnon, Hsiao-Ling Wu, Mark Pullen, and Ponnal Nambi*
- 25345 **The related molecular chaperones calnexin and calreticulin differentially associate with nascent T cell antigen receptor proteins within the endoplasmic reticulum.** *Jeroen E. M. Van Leeuwen and Kelly P. Kearse*

- 25350 **The v-Ki-Ras oncogene alters cAMP nuclear signaling by regulating the location and the expression of cAMP-dependent protein kinase II $\beta$ .** A. Feliciello, P. Giuliano, A. Porcellini, C. Garbi, S. Obici, E. Mele, E. Angotti, D. Grieco, G. Amabile, S. Cassano, Y. Li, Anna M. Musti, Charles S. Rubin, Max E. Gottesman, and Enrico V. Avvedimento
- 25360 **A partially functional DNA helicase II mutant defective in forming stable binary complexes with ATP and DNA. A role for helicase motif III.** Robert M. Brosh, Jr. and Steven W. Matson
- 25375 **Inducible cAMP early repressor can modulate tyrosine hydroxylase gene expression after stimulation of cAMP synthesis.** Cristina Tinti, Bruno Conti, Joseph F. Cubells, Kwang-Soo Kim, Harriet Baker, and Tong H. Joh
- 25423 **Identification of the *cpdA* gene encoding cyclic 3',5'-adenosine monophosphate phosphodiesterase in *Escherichia coli*.** Ryu Imamura, Kunitoshi Yamanaka, Teru Ogura, Sota Hiraga, Nobuyuki Fujita, Akira Ishihama, and Hironori Niki
- 25459 **Identification of functional elements of the chicken  $\epsilon$ -globin promoter involved in stage-specific interaction with the  $\beta/\epsilon$  enhancer.** Mark M. Mason, Joseph A. Grasso, Oksana Gavrilova, and Marc Reitman
- 25485 **Characterization of human B creatine kinase gene regulation in the heart *in vitro* and *in vivo*.** Michael E. Ritchie
- 25498 **Structure and characterization of the human tissue inhibitor of metalloproteinases-2 gene.** Khalil Hammani, Andrew Blakis, Delmore Morsette, Anne M. Bowcock, Christoph Schmutte, Patrick Henriot, and Yves A. DeClerck
- 25515 **Molecular cloning and characterization of a novel mouse macrophage gene that encodes a nuclear protein comprising polyglutamine repeats and interspersing histidines.** George W. Cox, Lynn S. Taylor, Jonathan D. Willis, Giovanni Melillo, Robert L. White III, Stephen K. Anderson, and Jih-Jing Lin
- 25524 **Identification of a novel retinoic acid response element in the promoter region of the retinol-binding protein gene.** Luigi Panariello, Loredana Quadro, Sergio Trematerra, and Vittorio Colantuoni
- 25539 **Evidence that a specific interaction between an 18-base *cis*-element in the 5'-untranslated region of human folate receptor- $\alpha$  mRNA and a 46-kDa cytosolic *trans*-factor is critical for translation.** Xin-Lai Sun and Asok C. Antony
- 25548 **Cloning and developmental expression of a membrane-type matrix metalloproteinase from chicken.** Maozhou Yang, Kimiko Hayashi, Masando Hayashi, Joanne T. Fujii, and Markku Kurkinen
- 25555 **Interleukin-4-induced STAT6 recognizes and activates a target site in the promoter of the interleukin-4 receptor gene.** Helen Kotanides and Nancy C. Reich
- 25562 **Characterization of Elongin C functional domains required for interaction with Elongin B and activation of Elongin A.** Yuichiro Takagi, Ronald C. Conaway, and Joan Weliky Conaway
- 25617 **Redox regulation of GA-binding protein- $\alpha$  DNA binding activity.** Mark E. Martin, Yurii Chinenov, Mi Yu, Tonya K. Schmidt, and Xiu-Ying Yang
- 25624 **Different inducibility of expression of the two xylanase genes *xyn1* and *xyn2* in *Trichoderma reesei*.** Susanne Zeilinger, Robert L. Mach, Martin Schindler, Petra Herzog, and Christian P. Kubicek
- 25639 **Enhanced tumorigenic behavior of glioblastoma cells expressing a truncated epidermal growth factor receptor is mediated through the Ras-Shc-Grb2 pathway.** Sally A. Prigent, Motoo Nagane, Hong Lin, Ivana Hugar, Gerry R. Boss, James R. Feramisco, Webster K. Cavenee, and H.-J. Su Huang
- 25657 **Specific mutations near the amino terminus of double-stranded RNA-dependent protein kinase (PKR) differentially affect its double-stranded RNA binding and dimerization properties.** Rekha C. Patel, Paul Stanton, and Ganes C. Sen
- 25671 **Factor VIII C2 domain missense mutations exhibit defective trafficking of biologically functional proteins.** Steven W. Pipe and Randal J. Kaufman
- 25715 **Parathyroid hormone induces *c-fos* promoter activity in osteoblastic cells through phosphorylated cAMP response element (CRE)-binding protein binding to the major CRE.** A. Terrece Pearman, Wan-Yin Chou, Kimberly D. Bergman, Malini R. Pulumati, and Nicola C. Partridge

#### PROTEIN CHEMISTRY AND STRUCTURE

- 25063 **Communication—Nucleotides reveal polynucleotide phosphorylase activity from conventionally purified GroEL.** Jesse Ybarra and Paul M. Horowitz
- 25083 **Heterologous expression of three plant serpins with distinct inhibitory specificities.** Søren W. Dahl, Søren K. Rasmussen, and Jørn Hejgaard
- 25099 **A novel activating anti- $\beta 1$  integrin monoclonal antibody binds to the cysteine-rich repeats in the  $\beta 1$  chain.** Randall J. Faull, Jian Wang, David I. Leavesley, Wilma Puzon, Graeme R. Russ, Dietmar Vestweber, and Yoshikazu Takada
- 25152 **A recombinant sickle hemoglobin triple mutant with independent inhibitory effects on polymerization.** Juha-Pekka Himanen, Urooj A. Mirza, Brian T. Chait, Robert M. Bookchin, and James M. Manning
- 25220 **Mutations at domain II, loop 3, of *Bacillus thuringiensis* CryIAa and CryIAb  $\delta$ -endotoxins suggest loop 3 is involved in initial binding to lepidopteran midguts.** Francis Rajamohan, Syed-Rehan A. Hussain, Jeffrey A. Cottrill, Fred Gould, and Donald H. Dean
- 25261 **Modulation of structure and antibacterial and hemolytic activity by ring size in cyclic gramicidin S analogs.** Leslie H. Kondejewski, Susan W. Farmer, David S. Wishart, Cyril M. Kay, Robert E. W. Hancock, and Robert S. Hodges

#### GENERAL INFORMATION

This publication is available on CD-ROM. Inquire about availability through your subscription agent or contact: THE JOURNAL OF BIOLOGICAL CHEMISTRY, P.O. BOX 830399, BIRMINGHAM, AL 35283-0399, U.S.A. Minireviews are reprinted in January of the succeeding year in a Minireview Compendium. Compendia for 1988 through 1995 are available through the ASBMB office: ASBMB Office, 9650 Rockville Pike, Bethesda, MD 20814, U.S.A.

Submit all manuscripts in triplicate to

Editor, The Journal of Biological Chemistry  
9650 Rockville Pike  
Bethesda, MD 20814, U.S.A.

Accepted manuscripts will be published with the implicit understanding that the author(s) will pay a charge per page. Current page charges may be obtained by contacting the JBC office. Under exceptional circumstances, when no source of grant or other support exists, the author(s) may apply, at the time of submission, for a grant-in-aid to Chairman, Publications Committee, American Society for Biochemistry and Molecular Biology, Inc., 9650 Rockville Pike, Bethesda, MD 20814. All such applications must be countersigned by an appropriate institutional official stating that no funds are available for page charges.

Queries on matters of general editorial policy, requests for reprints of the "Instructions to Authors," or of the "Editorial Policy and Practices," or for

permission to reproduce any part of a previously published article should be directed to the Journal Editorial Office in Bethesda, telephone 301-530-7150; fax 301-571-1824.

The Journal of Biological Chemistry publishes papers on a broad range of topics of interest to biochemists. The views expressed are those of the author(s) and not of The Journal of Biological Chemistry or the American Society for Biochemistry and Molecular Biology.

The Journal of Biological Chemistry is copyrighted by the American Society for Biochemistry and Molecular Biology, Inc. Reprographic copying beyond that permitted by Sections 107 or 108 of the U.S. Copyright Law is allowed, provided that the \$2.50 per-copy fee is paid through the Copyright Clearance Center, 222 Rosewood Drive, Danvers, MA 01923. For those organizations that have been granted photocopy license by CCC, a separate system of payment has been arranged. The fee code for users of the Transactional Reporting Service is: 0021-9258/96/\$2.50. © Printed on acid-free paper effective with Volume 254, Issue No. 1 (1979). Reproduction of any portion of an article for subsequent republication requires permission of the copyright owner. Requests should be made in writing to the American Society for Biochemistry and Molecular Biology, Inc., Attn.: Editorial Office, 9650 Rockville Pike, Bethesda, MD 20814, and should include a statement of intended use as well as explicit specifications of the material to be reproduced.

Address all correspondence and orders relative to subscriptions and back copies to: The Journal of Biological Chemistry, P.O. Box 830399, Birmingham, AL 35283-0399, U.S.A., telephone 800-633-4931. Subscriptions are entered on a calendar year basis only. Allow at least six weeks for address changes. Claims for replacement copies must be received within three months of the issue date.

- 25284 **Identification of a transferable sorting domain for the regulated pathway in the prohormone convertase PC2.** John W. M. Creemers, Elena F. Usac, Nicholas A. Bright, Jan-Willem Van de Loo, Erik Jansen, Wim J. M. Van de Ven, and John C. Hutton
- 25382 **Subunit structure of rod cGMP-phosphodiesterase.** Nikolai O. Artemyev, Rajendran Surendran, James C. Lee, and Heidi E. Hamm
- 25389 **A novel acidic allergen, Hev b 5, in latex. Purification, cloning and characterization.** Akira Akasawa, Li-Shan Hsieh, Brian M. Martin, Teresa Liu, and Yuan Lin
- 25394 **Identification, cloning, and sequence of a major allergen (Hev b 5) from natural rubber latex (*Hevea brasiliensis*).** Jay E. Slater, Thomas Vedvick, Ann Arthur-Smith, Diane E. Trybul, and Roy G. O. Kekwick
- 25412 **Defining the arachidonic acid binding site of human 15-lipoxygenase. Molecular modeling and mutagenesis.** Qing-Fen Gan, Michelle F. Browner, David L. Sloane, and Elliott Sigal
- 25419 **High resolution crystal structures of the deoxy, oxy, and aquomet forms of cobalt myoglobin.** Eric Allen Brucker, John S. Olson, George N. Phillips, Jr., Yi Dou, and Masao Ikeda-Saito
- 25468 **Regulation of mutant p53 temperature-sensitive DNA binding.** Philip Friedlander, Yann Legros, Thierry Soussi, and Carol Prives
- 25575 **Stonustoxin is a novel lethal factor from stonefish (*Synanceja horrida*) venom. cDNA cloning and characterization.** Farid John Ghadessy, Desong Chen, R. Manjunatha Kini, Maxey C. M. Chung, Kandiah Jeyaseelan, Hoon Eng Khoo, and Raymond Yuen
- 25646 **Identification of Itk/Tsk Src homology 3 domain ligands.** Stephen C. Bunnell, Pamela A. Henry, Rikki Kolluri, Tomas Kirchhausen, Richard J. Rickles, and Leslie J. Berg
- 25664 **Purification and characterization of a novel restricted antigen expressed by normal and transformed human colonic epithelium.** B. Catimel, G. Ritter, S. Welt, L. J. Old, L. Cohen, M. A. Nerrie, S. J. White, J. K. Heath, B. Demediuk, T. Domagala, F. T. Lee, A. M. Scott, G. F. Tu, H. Ji, R. L. Moritz, R. J. Simpson, A. W. Burgess, and E. C. Nice
- 25707 **Mutagenesis analysis of functionally important domains within the C-terminal end of smooth muscle caldesmon.** Ze Wang and Samuel Chacko

ADDITIONS AND CORRECTIONS

- 25722 **Direct or C5a-induced activation of heterotrimeric G<sub>12</sub> proteins in human neutrophils is associated with interaction between formyl peptide receptors and the cytoskeleton.** Vol. 271 (1996) 15267-15271. Eva Särndahl, Gary M. Bokoch, François Boulay, Olle Stendahl, and Tommy Andersson

AUTHOR INDEX

- Abrams, Charles S., 25192  
 Acharya, Shreeta, 25406  
 Adamou, John E., 25300  
 Adams, Sherrill L., 25233  
 Akasawa, Akira, 25389  
 Aktories, Klaus, 25173  
 Amabile, G., 25350  
 Anagli, John, 25067  
 Anderson, Stephen K., 25515  
 Andersson, Tommy, 25722  
 Angotti, E., 25350  
 Antony, Aśok C., 25539  
 Apasov, Sergey G., 25677  
 Arato-Oshima, Teruyo, 25604  
 Artemyev, Nikolai O., 25382  
 Arthur-Smith, Ann, 25394  
 Avvedimento, Enrico V., 25350
- Bae, Sun Sik, 25213  
 Baker, Harriet, 25375  
 Ball, Rebecca, 25071  
 Basu, Mitali, 25308  
 Bates, Sandra R., 25277  
 Beavis, Andrew D., 25338  
 Beavo, Joseph A., 25699  
 Bennett, Vann, 25157  
 Bentley, J. Kelley, 25699  
 Berchtold, Martin W., 25067  
 Berg, Leslie J., 25646  
 Bergman, Kimberly D., 25715  
 Bessman, Maurice J., 25059  
 Blakias, Andrew, 25498  
 Bodkin, Noni L., 25327  
 Bokoch, Gary M., 25204, 25722  
 Bollag, Gideon, 25452  
 Bookchin, Robert M., 25152  
 Boorer, Kathryn J., 25139  
 Boss, Gerry R., 25639  
 Boulay, François, 25722  
 Bowcock, Anne M., 25498  
 Brandsch, Roderich, 25208  
 Bright, Nicholas A., 25284  
 Brosh, Robert M., Jr., 25360  
 Browner, Michelle F., 25412  
 Brucker, Eric Allen, 25419  
 Bückmann, Andreas F., 25208  
 Bunnell, Stephen C., 25646  
 Burgess, A. W., 25664  
 Burghardt, Charles, 25308  
 Burke, Patricia V., 25131
- Burky, Stephen A., 25198
- Cairns, William, 25269  
 Calleja, Véronique, 25227  
 Carroll, Robert, 25308  
 Cassano, S., 25350  
 Catimel, B., 25664  
 Cavenee, Webster K., 25639  
 Cazals, Véronique, 25117  
 Chacko, Samuel, 25707  
 Chait, Brian T., 25152  
 Chen, Desong, 25575  
 Chen, Lan Bo, 25198  
 Chen, Qiping, 25277  
 Chen, Ting, 25308  
 Cheng, Helen H., 25630  
 Cheng, Hwei-Fang, 25316  
 Chinenov, Yuri, 25617  
 Chizzonite, Richard, 25308  
 Chou, Wan-Yin, 25715  
 Christophe, Olivier D., 25332  
 Chung, Maxey C. M., 25575  
 Chuntharapai, Anan, 25400  
 Clement, Annick, 25117  
 Clewell, Amy S., 25131  
 Cohen, Arthur J., 25233  
 Cohen, L., 25664  
 Colantuoni, Vittorio, 25524  
 Conaway, Joan Weliky, 25562  
 Conaway, Ronald C., 25562  
 Conti, Bruno, 25375  
 Corroyer, Sophie, 25117  
 Cottrill, Jeffrey A., 25220  
 Cox, George W., 25515  
 Creemers, John W. M., 25284  
 Critchley, Angela J., 25590  
 Cubells, Joseph F., 25375  
 Curnutte, John T., 25400
- Dahl, Søren W., 25083  
 Danho, Waleed, 25308  
 Dean, Donald H., 25220  
 DeClerck, Yves A., 25498  
 Demediuk, B., 25664  
 Dharm, Elizabeth, 25308  
 Domagala, T., 25664  
 Dou, Yi, 25419  
 Downes, C. Peter, 25192
- Echevarria, Miriam, 25079
- elMasry, Nadia, 25452  
 Elshourbagy, Nabil A., 25300
- Farmer, Susan W., 25261  
 Faull, Randall J., 25099  
 Feliciello, A., 25350  
 Feramisco, James R., 25639  
 Fesik, Stephen W., 25316  
 Fischer, Roland, 25067  
 Fisher, Aron B., 25277  
 Freije, José M. P., 25107  
 Frick, David N., 25059  
 Friedlander, Philip, 25468  
 Frindt, Gustavo, 25079  
 Frommer, Wolf B., 25139  
 Fujii, Joanne T., 25548  
 Fujita, Nobuyuki, 25423  
 Furuya, Yuzo, 25126  
 Fushinobu, Shinya, 25611
- Gagnon, Alison W., 25300  
 Gan, Qing-Fen, 25412  
 Garbi, C., 25350  
 Gavrilova, Oksana, 25459  
 Ghadessy, Farid John, 25575  
 Gilfillan, Alasdair M., 25308  
 Giuliano, P., 25350  
 Goldberg, Mark A., 25492  
 Gonias, Steven L., 25684  
 Gottesman, Max E., 25350  
 Gould, Fred, 25220  
 Grasso, Joseph A., 25459  
 Green, Simon P., 25400  
 Grémeaux, Thierry, 25227  
 Griac, Peter, 25692  
 Grieco, D., 25350  
 Griffin, James D., 25198  
 Grillo, Sophie, 25227  
 Grötzinger, Thilo, 25253  
 Gunz, Daniela, 25089  
 Gustafson, Thomas A., 25327
- Hall, Michael J., 25438  
 Hall, Zach W., 25506  
 Hamm, Heidi E., 25382  
 Hammani, Khalil, 25498  
 Hancock, Robert E. W., 25261  
 Hansen, Barbara C., 25327  
 Hart, Matthew J., 25452  
 Hayashi, Kimiko, 25548
- Hayashi, Masando, 25548  
 Heath, J. K., 25664  
 Hejgaard, Jørn, 25083  
 Hembrough, Todd A., 25684  
 Henriët, Patrick, 25498  
 Henry, Pamela A., 25646  
 Henry, Susan A., 25692  
 Herzog, Petra, 25624  
 Hess, Martin T., 25089  
 Heymach, John V., Jr., 25430  
 Himanen, Juha-Pekka, 25152  
 Hinch, Bryan, 25338  
 Hiraga, Sota, 25423  
 Hirai, Hisamaru, 25198  
 Hodges, Robert S., 25261  
 Hofmann, Fred, 25173  
 Holmes, Jennifer K., 25240  
 Homareda, Haruo, 25604  
 Horowitz, Paul M., 25063  
 Hotta, Kikuko, 25327  
 Hovanessian, Ara G., 25479  
 Hsieh, Li-Shan, 25389  
 Huang, H.-J. Su, 25639  
 Hughes, Christine A., 25157  
 Hussain, Syed-Rehan A., 25220  
 Hutton, John C., 25284  
 Huvar, Ivana, 25639
- Ikeda-Saito, Masao, 25419  
 Imamura, Ryu, 25423  
 Inagaki, Nobuya, 25126  
 Ishihama, Akira, 25423  
 Ishihara, Hiroshi, 25184  
 Iwata, So, 25611
- Jansen, Erik, 25284  
 Jelonek, Marie T., 25677  
 Jensen, Kirsten, 25253  
 Jeyaseelan, Kandiah, 25575  
 Ji, H., 25664  
 Joh, Tong H., 25375  
 Just, Ingo, 25173
- Kamata, Kenji, 25611  
 Karnik, Sadashiva S., 25406  
 Katayama, Tsutomu, 25178  
 Kaufman, Randal J., 25671  
 Kay, Cyril M., 25261  
 Kearsse, Kelly P., 25345  
 Kekwick, Roy G. O., 25394

- Khoo, Hoon Eng, 25575  
 Kim, Jae Ho, 25213  
 Kim, Kwang-Soo, 25375  
 Kim, Myung Jong, 25213  
 Kim, Yong, 25213  
 Kimchi, Adi, 25479  
 Kimura, Sadao, 25126  
 King, Klim, 25316  
 Kini, R. Manjunatha, 25575  
 Kirchhausen, Tomas, 25646  
 Klippel, Anke, 25227  
 Kobayashi, Masakatsu, 25178  
 Kochan, Jarema, 25308  
 Kohn, Harold, 25369  
 Kolluri, Rikki, 25646  
 Kondejewski, Leslie H., 25261  
 Kotanides, Helen, 25555  
 Kramer, Randall H., 25598  
 Krüttgen, Alex, 25430  
 Kuan, Y.-S., 25316  
 Kubicek, Christian P., 25624  
 Kubota, Misae, 25569  
 Kuchler, Karl, 25167  
 Kumagai, Hidetoshi, 25630  
 Kuramochi, Hiroaki, 25126  
 Kurkinen, Markku, 25548  
 Kwast, Kurt E., 25131  
  
 Lambeth, J. David, 25213  
 Leavesley, David I., 25099  
 Lee, F. T., 25664  
 Lee, James C., 25382  
 Lee, Sang-Hyeon, 25292  
 Lee, Taehoon G., 25213  
 Legros, Yann, 25468  
 Le Marchand-Brustel, Yannick, 25227  
 Lemoine, Yves, 25167  
 Lenting, Peter J., 25332  
 Levy, Andrew P., 25492  
 Levy, Nina S., 25492  
 Li, Jian-Liang, 25198  
 Li, Jiaxin, 25247  
 Li, Li, 25684  
 Li, Y., 25350  
 Lin, Hong, 25639  
 Lin, Jih-Jing, 25515  
 Lin, Yuan, 25389  
 Liu, Guoying, 25338  
 Liu, Shusen, 25247  
 Liu, S.-M., 25316  
 Liu, Teresa, 25389  
 Lolkema, Juke S., 25582  
 Lomasney, Jon W., 25316  
 Loo, Donald D. F., 25139  
  
 MacDonald, Nicholas J., 25107  
 Mach, Robert L., 25624  
 Maeda, Minoru, 25178  
 Magyar, Attila, 25369  
 Mahé, Yannick, 25167  
 Maitre, Bernard, 25117  
 Mann, Matthias, 25173  
 Manning, James M., 25152  
 Manrow, Richard E., 25107  
 Marcel, Yves L., 25145  
 Margulies, David H., 25677  
 Martin, Brian M., 25389  
 Martin, Mark E., 25617  
 Mason, Mark M., 25459  
  
 Matson, Steven W., 25360  
 Matsui, Hideo, 25604  
 Matsuoaka, Yoichiro, 25157  
 Matsuzawa, Hiroshi, 25611  
 McCabe, Peter, 25452  
 McGee, Todd P., 25630  
 McLaren, Aileen W., 25269  
 Mele, E., 25350  
 Melillo, Giovanni, 25515  
 Mertens, Koen, 25332  
 Meurs, Eliane F., 25479  
 Miki, Takeyoshi, 25178  
 Minowa, Mari T., 25292  
 Mirza, Urooj A., 25152  
 Mizushima, Tooru, 25178  
 Moritz, R. L., 25664  
 Morsette, Delmore, 25498  
 Mouradian, M. Maral, 25292  
 Musti, Anna M., 25350  
  
 Naegeli, Hanspeter, 25089  
 Nagane, Motoo, 25639  
 Nah, Hyun-Duck, 25233  
 Nambi, Ponnal, 25300  
 Nerrie, M. A., 25664  
 Nice, E. C., 25664  
 Nicolson, Margery A., 25327  
 Niki, Hironori, 25423  
 Niu, Zeling, 25233  
 Nunes, Perla, 25308  
  
 Obici, S., 25350  
 Ogura, Teru, 25423  
 O'Handley, Suzanne F., 25059  
 Ohishi, Hiroko, 25178  
 Ohnishi, Hiroshi, 25569  
 Ohta, Takahisa, 25611  
 Ohtake, Atsuko, 25569  
 Okada, Shuichi, 25533  
 Old, L. J., 25664  
 Olson, John S., 25419  
 Omura, Satoshi, 25630  
 Ortmeier, Heidi K., 25327  
  
 Pallante, Kim M., 25233  
 Panariello, Luigi, 25524  
 Partridge, Nicola C., 25715  
 Pascual-Ahuir, Amparo, 25438  
 Patel, Rekha C., 25657  
 Pearman, A. Terrece, 25715  
 Perlin, David S., 25438  
 Pessin, Jeffrey E., 25533  
 Phillips, George N., Jr., 25419  
 Pipe, Steven W., 25671  
 Pisick, Evan, 25198  
 Polakis, Paul, 25452  
 Porcellini, A., 25350  
 Poyton, Robert O., 25131  
 Price, Margaret P., 25184  
 Prigent, Sally A., 25639  
 Prives, Carol, 25468  
 Prossnitz, Eric R., 25204  
 Ptasznik, Andrzej, 25204  
 Pullar, Christine, 25308  
 Pullen, Mark, 25300  
 Pulumati, Malini R., 25715  
 Puzon, Wilma, 25099  
  
 Qiu, Rong-Guo, 25452  
 Quadro, Loredana, 25524  
  
 Raitt, Desmond, 25131  
 Rajamohan, Francis, 25220  
 Rasmussen, Søren K., 25083  
 Rassow, Joachim, 25208  
 Raveh, Tal, 25479  
 Rees, D. Jasper G., 25332  
 Reich, Nancy C., 25555  
 Reitman, Marc, 25459  
 Repetto, Barbara, 25308  
 Rex, Gundula, 25173  
 Rickles, Richard J., 25646  
 Ritchie, Michael E., 25485  
 Rittenhouse, Susan E., 25192  
 Ritter, G., 25664  
 Robinson, Margaret S., 25446  
 Romoser, Valerie, 25071  
 Rosen, Barry P., 25247  
 Rubin, Charles S., 25350  
 Russ, Graeme R., 25099  
 Ryu, Sung Ho, 25213  
  
 Sakai, Hiroshi, 25611  
 Salgia, Ravi, 25198  
 Sano, Shin-ichiro, 25569  
 Särndahl, Eva, 25722  
 Sasaki, Shigeki, 25178  
 Sato, Kazuki, 25569  
 Sattler, Martin, 25198  
 Schindler, Martin, 25624  
 Schmidt, Tonya K., 25617  
 Schmutte, Christoph, 25498  
 Scott, A. M., 25664  
 Seaman, Matthew N. J., 25446  
 Seino, Susumu, 25126  
 Sekimizu, Kazuhisa, 25178  
 Selzer, Jörg, 25173  
 Sen, Ganes C., 25657  
 Sharma, Sanju, 25452  
 Sheppard, David N., 25184  
 Shimazaki, Jun, 25126  
 Shooter, Eric M., 25430  
 Shtrom, Svetlana S., 25506  
 Sigal, Elliott, 25412  
 Simoni, Robert D., 25630  
 Simpson, R. J., 25664  
 Sitkovsky, Michail V., 25677  
 Slater, Jay E., 25394  
 Sloane, David L., 25412  
 Smith, Christopher A. D., 25269  
 Smith, Patrick T., 25677  
 Smrcka, Alan, 25204  
 Smrcka, Alan V., 25071  
 Solomon, Mark J., 25240  
 Sonenberg, Nahum, 25479  
 Soussi, Thierry, 25468  
 Sowerby, Penelope J., 25446  
 Sparks, Daniel L., 25145  
 Squillace, Rachel M., 25598  
 Stanton, Paul, 25657  
 Steeg, Patricia S., 25107  
 Stendahl, Olle, 25722  
 Stirling, Colin J., 25590  
 Stoltz, Michaela, 25208  
 Stracke, Mary L., 25107  
 Suh, Yoon Jung, 25213  
 Sun, Xin-Lai, 25539  
 Surendran, Rajendran, 25382  
 Suter, Ueli, 25430  
  
 Swede, Marci J., 25692  
 Takada, Yoshikazu, 25099  
 Takagi, Yuichiro, 25562  
 Tamás, Markos J., 25438  
 Tang, Xiu-wen, 25192  
 Tanti, Jean-François, 25227  
 Taylor, Lynn S., 25515  
 ter Maat, Hans, 25332  
 Tinti, Cristina, 25375  
 Traynor-Kaplan, Alexis E., 25204  
 Trematerra, Sergio, 25524  
 Trybul, Diane E., 25394  
 Tu, G. F., 25664  
 Uemura, Naoki, 25198  
 Usac, Elena F., 25284  
  
 Van de Loo, Jan-Willem, 25284  
 Van de Ven, Wim J. M., 25284  
 Van Geest, Marleen, 25582  
 Van Leeuwen, Jeroen E. M., 25345  
 Van Obberghen, Emmanuel, 25227  
 Vedvick, Thomas, 25394  
 Vestweber, Dietmar, 25099  
 Visnick, Mike, 25308  
  
 Wakizaka, Akira, 25604  
 Wang, Genfu, 25438  
 Wang, Jian, 25099  
 Wang, Li-Ping, 25316  
 Wang, Ze, 25707  
 Watabe, Yoshio, 25126  
 Waugh, David, 25308  
 Wei, Yu, 25067  
 Welsh, Michael J., 25184  
 Welt, S., 25664  
 White, Robert L., III, 25515  
 White, S. J., 25664  
 Widger, William R., 25369  
 Wilkinson, Barrie M., 25590  
 Will, Hans, 25253  
 Williams, Lewis T., 25227  
 Willis, Jonathan D., 25515  
 Wilm, Matthias, 25173  
 Windhager, Erich E., 25079  
 Wishart, David S., 25261  
 Wolf, C. Roland, 25269  
 Wong, Wai-Keung, 25198  
 Wright, Ernest M., 25139  
 Wu, Hsiao-Ling, 25300  
  
 Yamanaka, Kunitoshi, 25423  
 Yan, Chen, 25699  
 Yang, Maozhou, 25548  
 Yang, Xiu-Ying, 25617  
 Yao, Chung-Chen, 25598  
 Ybarra, Jesse, 25063  
 Yoshikawa, Dan, 25204  
 Yu, Mi, 25617  
 Yuen, Raymond, 25575  
  
 Zeilinger, Susanne, 25624  
 Zhang, Jin, 25192  
 Zhang, Xiangdong, 25369  
 Zhao, Allan Z., 25699  
 Zhao, Wei, 25192  
 Zhao, Xiao-Jian, 25131  
 Zhao, Yufeng, 25233  
 Zhao, Yuwei, 25145  
 Zhao, Zhicheng, 25308  
 Ziober, Barry L., 25598

## A Recombinant Sickle Hemoglobin Triple Mutant with Independent Inhibitory Effects on Polymerization\*

(Received for publication, June 13, 1996, and in revised form, July 19, 1996)

Juha-Pekka Himanen‡, Urooj A. Mirza‡, Brian T. Chait‡, Robert M. Bookchin§, and James M. Manning‡§¶

From the ¶Northeastern University, Boston, Massachusetts 02115, the ‡The Rockefeller University, New York, New York 10021, and the §Albert Einstein College of Medicine, Bronx, New York 10461

As part of a comprehensive effort to map the most important regions of sickle hemoglobin that are involved in polymerization, we have determined whether two sites previously shown to be involved, Leu-88( $\beta$ ) and Lys-95( $\beta$ ), had additive effects when substituted. The former site is part of the hydrophobic pocket that binds Val-6( $\beta$ ), the natural mutation of HbS, and the latter site is a prominent part of the hemoglobin exterior. A sickle hemoglobin triple mutant with three amino acid substitutions on the  $\beta$ -chain, E6V/L88A/K95I, has been expressed in yeast and characterized extensively. Its oxygen binding curve, cooperativity, response to allosteric effectors, and the alkaline Bohr effect showed that it was completely functional. The polymer solubility of the deoxy triple mutant, measured by a new micromethod requiring reduced amounts of hemoglobin, was identical to that of the E6V( $\beta$ )/K95I( $\beta$ ) mutant, *i.e.* when the K95I( $\beta$ ) substitution was present on the same tetramer together with the naturally occurring E6V( $\beta$ ) substitution, the L88A( $\beta$ ) replacement had no additive effect on polymer inhibition. The results suggest that Lys-95( $\beta$ ) on the surface of the tetramer and its complementary binding region on the adjoining tetramer are potential targets for the design of an effective antisickling agent.

Sickle cell anemia results from a single point mutation in the gene encoding  $\beta$ -globin, whereby the Glu-6( $\beta$ ) residue in hemoglobin A (HbA) is substituted by Val in sickle hemoglobin (HbS) (1, 2). This hydrophobic side chain initiates a process by which the densely packed deoxyhemoglobin tetramers inside the red blood cells interact through other sites to form long polymer fibers that distort the cells into a characteristic sickle shape. Although the identity of many of these amino acid sites involved in polymer formation and the extent to which they participate is known (3–8), the quantitative contributions to polymerization of many other sites are unknown. A goal of this study was to provide such information for selected polymerization contact sites for which natural mutants either do not exist or have not been reported. Recombinant sickle double and triple mutants are used for this purpose.

Studies describing the hydrophobicity and stereochemistry of deoxy HbS have shown that Val-6( $\beta$ ) binds tightly between Phe-85 and Leu-88 in the acceptor pocket on an adjacent  $\beta$ -chain. According to computer-generated models, the three-

dimensional fit of the side chain of Val into the acceptor pocket is much better than that of Ala (7), explaining the inability of Hb Makassar with Ala-6( $\beta$ ) to polymerize (8), even though the hydrophobicity of Ala and Val do not differ drastically. Other studies have suggested that substitutions by larger hydrophobic residues at the position 6, readily promote polymerization (9). These findings point out the complexity of the polymerization process, which cannot be explained simply by the hydrophobicity and stereochemistry of the  $\beta$ -6 site and its corresponding acceptor pocket. Indeed, it has been established that other contact sites in the gelation process reinforce the initial contact (3–5, 9, 10). In addition, studies with noncovalent chemical inhibitors have shown that these compounds do not act as predicted by their hydrophobic nature (11), implying a significant contribution of other interactions.

In our efforts to understand the mechanism of sickle hemoglobin gelation and to identify the critical sites in the gelation process, we use a yeast expression system (6, 12–15) to produce HbS double and triple mutants as an adjunct to chemical modification studies (16–18). Unlike the *Escherichia coli* expression system, the yeast system produces a native hemoglobin molecule, as judged by many biochemical criteria (15). In addition, since yeast incorporates its own heme group into globin, there are no time-consuming manipulations, such as reconstituting hemoglobin with exogenous heme. Thus, it is feasible to study the involvement of any site on the hemoglobin molecule in the gelation process and to judge the significance of any differences between the crystal structure (5) and the electron microscope structure (4, 19) of HbS. For example, we recently determined that the contact site Lys-95( $\beta$ ) on the outside of the tetramer distant from the hydrophobic pocket, which was implicated in one structure (4) but not the other (5), was significantly involved in the gelation process (12). Indeed, its substitution by Ile inhibits gelation twice as much as a mutation at a site in the acceptor pocket, L88A( $\beta$ ) (6). The diverse locations of these two sites prompted us to design a recombinant Hb having both K95I( $\beta$ ) and L88A( $\beta$ ) in addition to the Val-6( $\beta$ ) mutation in order to measure whether the influence of the two substitutions on gelation is additive. Such a study may reveal important details of the gelation process and could influence efforts for developing well targeted clinically effective inhibitors. For these studies, we employ a new method based on the drastic decrease in the solubility of hemoglobin S upon addition of dextran (20).

### MATERIALS AND METHODS

**Reagents**—The restriction endonucleases, T4 polynucleotide kinase, alkaline phosphatase, and DNA ligase were from Boehringer Mannheim. The DNA sequencing kit and the T7 DNA polymerase (Sequenase version 2.0) were obtained from U. S. Biochemical Corp. The <sup>35</sup>S-labeled dATP was from DuPont NEN. The oligonucleotides were synthesized by Operon Technologies (Alameda, CA). CM-cellulose 52 was from What-

\* This work was supported in part by National Institutes of Health Grant HL-18819 (to J. M. M.) and HL28018 (to R. M. B.) and the Academy of Finland (to J.-P. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¶ To whom correspondence should be addressed: Northeastern University, Dept. of Biology, 360 Huntington Ave., Boston, MA 02115.



man, and HPLC<sup>1</sup> columns (C-4 and C-18) from Vydac. L-1-tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin, dextran, DPG, and IHP were purchased from Sigma. The construction of pGS189 and pGS389 plasmids is described elsewhere (12, 14). All the other reagents were of analytical purity.

**Site-directed Mutagenesis**—To prepare the E6V( $\beta$ )/L88A( $\beta$ )/K95I( $\beta$ ) triple mutant, we used the M13mp18 recombinant phage as a template. The construction of this phage containing the  $\beta$ -globin cDNA with the E6V( $\beta$ ) and the L88A( $\beta$ ) coding mutations has been described earlier (6). The oligonucleotide 5'-ATC CAC GTG CAG GAT GTC ACA GTG CAG-3' was used to create the Lys-95( $\beta$ )  $\rightarrow$  Ile mutation by the method of Kunkel (21). The underlined bases were those used to create the desired mutation. The presence of the mutations was screened by partial sequencing of the mutation site. The mutation frequency was increased to 65% by supplementing the reaction mixture with the Gene 32 Protein and by prolonging the reaction time, as described previously.<sup>2</sup> The mutated  $\beta$ -globin region was subcloned to pGS189sickle, which contains the native  $\alpha$ -globin and the Glu-6( $\beta$ )  $\rightarrow$  Val mutated  $\beta$ -globin cDNAs, by digesting with *Sph*I enzyme. Finally, the  $\alpha$ - and  $\beta$ -globin gene cassette was isolated as a *Not*I fragment after digesting the newly synthesized pGS189sickle-Ala-88-Ile-95 with *Not*I and *Bgl*II and inserted into pGS389 previously digested with *Not*I. The correct insertional direction was verified by restriction mapping and the entire  $\beta$ -globin gene was sequenced using a fluorescence-based detection system (Perkin-Elmer/Applied Biosystems) to show that the Glu-6( $\beta$ )  $\rightarrow$  Val, Leu-88( $\beta$ )  $\rightarrow$  Ala, and Lys-95( $\beta$ )  $\rightarrow$  Ile were the only mutations in the globin chain.

**Yeast Expression System**—The yeast cells were transformed by the pGS389sickle-Ala-88-Ile-95 plasmid using the lithium acetate method (22). The transformants were selected and the copy number of the plasmid increased by growing the yeast on a complete minimal medium first without uracil, then without uracil and leucine (14). To express the E6V( $\beta$ )/L88A( $\beta$ )/K95I( $\beta$ ) triple mutant hemoglobin, the yeast was grown in YP medium for 4 days with ethanol as the carbon source. The promoter controlling the transcription of the globin genes was induced by adding 3% galactose 20 h prior to the harvesting of the yeast cells. The cells were disrupted in a Bead Beater homogenizer and the Hb triple mutant was purified in the CO form on a CM-Cellulose 52 column as described earlier (12, 15). The average yield was 3 mg of the purified hemoglobin/liter of culture medium. The preparations of the E6V( $\beta$ )/L88A( $\beta$ ) and the E6V( $\beta$ )/K95I( $\beta$ ) recombinant hemoglobins were described earlier (6, 12).

**Mass Spectrometry Analysis**—Electrospray mass spectrometric analysis was performed with a Finnigan-MAT TSQ-700 triple quadrupole mass spectrometer (23, 24). Seventy pmol of the hemoglobin sample was loaded onto a desalting protein cartridge (Michrom BioResources, Inc., Auburn, CA) and washed with 1 ml of deionized water. The sample was eluted from the cartridge using a solution of water/acetonitrile/acetic acid, 30/67.5/2.5 (v/v/v) and electrosprayed directly into the mass spectrometer. The flow of the eluting solution was maintained at 6  $\mu$ l/min through a 100- $\mu$ m inner diameter fused silica capillary. The spectrum given in Fig. 1 is an average of 16 scans, obtained at a rate of 3 s/scan.

**Analytical Methods**—Isoelectric focusing, amino acid analysis, and other procedures were performed as described earlier (12, 15, 25). To isolate the  $\alpha$ - and  $\beta$ -globin chains, a Vydac C-4 column was equilibrated with acetonitrile in 0.1% trifluoroacetic acid and eluted as described under "Results." The isolated  $\beta$ -globin chains were digested with trypsin, and the resulting peptides were separated on a Vydac C-18 reversed phase column using an acetonitrile gradient in 0.05% HCl, a modification from previous studies (12, 15, 25).

**Functional Studies**—The oxygen dissociation curves were determined at 37°C on a modified Hem-O-Scan instrument (Aminco) as described previously in 50 mM bis-Tris, pH 7.4 (26). Before the measurements, the Hb samples were dialyzed, converted from the CO form to the oxy form (27), and concentrated using CentriPrep, Centricon, and MicroCon ultrafiltration devices (Amicon; molecular weight cutoff 10,000). The final protein concentration (0.5–2.2 mM) was established by amino acid analysis on a Beckman 6300 analyzer. The pH dependence of the oxygen affinity (Bohr effect) and the effects of allosteric modulators were determined at a Hb concentration of 0.5 mM in the same buffer.

**Determination of  $C_{\text{sat}}$** —Polymer solubilities of the hemoglobins under study were determined by the "Dextran- $C_{\text{sat}}$ " micromethod of Bookchin

<sup>1</sup> The abbreviations used are: HPLC, high performance liquid chromatography; DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

<sup>2</sup> J.P. Himanen, A. M. Popowicz, and J. M. Manning, submitted for publication.

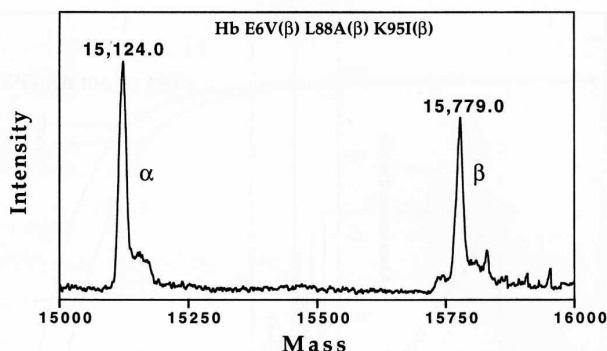


FIG. 1. Reconstituted electrospray ionization mass spectrum of the  $\alpha$ - and  $\beta$ -chains of the recombinant E6V( $\beta$ )/L88A( $\beta$ )/K95I( $\beta$ ) triple mutant. Peaks designated  $\alpha$  and  $\beta$  represent the protonated  $\alpha$ - and  $\beta$ -globin chains of the triple mutant and their molecular masses.

*et al.* (20), with minor modifications. This method is based on the marked decrease in the solubility of deoxy-HbS on admixture with 70-kDa dextran, at physiological ionic strength and pH (a preliminary account of this method and results is described in Ref. 20; full report in preparation). A comparison of the results found with this procedure with those reported previously using another method is described below. Concentrated solutions of the test hemoglobin in 0.05 M potassium phosphate, pH 7.5, were mixed with concentrated dextran solutions in the same buffer to give a final dextran concentration of 120 mg/ml. The solutions were overlaid with paraffin oil, chilled on ice, and deoxygenated by adding (with a Hamilton syringe) a deoxygenated solution of sodium dithionite to give a final concentration of 50 mM. After stirring and incubation for 30 min in a 37°C water bath, the resulting gel under the oil layer was carefully but vigorously disrupted with a narrow plunger or wire loop, and the tubes were centrifuged at room temperature in a microcentrifuge at 14,000 rpm for 20 min. The gel disruption and centrifugation procedure was repeated twice. After confirming the presence of a solid Hb phase by viewing the tube in front of a bright light, the oil was aspirated. The hemoglobin concentration of the supernatant ( $C_{\text{sat}}$ ) was determined by amino acid analysis in duplicate. Each determination of the gelation concentration was performed three to four times with a precision of +10% or less.

## RESULTS

**Mass Spectrometry**—The expected molecular mass (15,779.0 Da) was obtained for the purified E6V( $\beta$ )/L88A( $\beta$ )/K95I( $\beta$ ) Hb mutant  $\beta$ -chain by matrix-assisted laser desorption mass spectrometry (Fig. 1). This value agrees well with the calculated value of 15,781.2 for a  $\beta$ -chain having the three substitutions, *i.e.* the difference of 89.3 mass units between the mass of the  $\beta$ -chain of HbA (15,868.3 Da) is within the experimental error of the combined calculated differences of 87.1 mass units for the substitutions Glu  $\rightarrow$  Val, Leu  $\rightarrow$  Ala, and Lys  $\rightarrow$  Ile (30.0, 15.0, and 42.1 mass units difference, respectively). The measured molecular mass for the  $\alpha$ -chain of E6V( $\beta$ )/L88A( $\beta$ )/K95I( $\beta$ ) (15,124.0) is in accord with the calculated value (15,126.4 Da) for the natural  $\alpha$ -chain of HbA within the experimental error.

**Isoelectric Focusing**—This analysis was performed for the triple mutant by the method described previously (15). The triple mutant showed a similar pI value as the E6V( $\beta$ )/K95I( $\beta$ ) mutant (12) in agreement with the expected mutations, since the L88A( $\beta$ ) mutation has previously been shown not to have an altered pI value (6).

**HPLC Separation of Globin Chains**—The  $\alpha$ - and  $\beta$ -globin chains were separated by reversed phase HPLC on a Vydac C4 column using acetonitrile in 0.1% trifluoroacetic acid as the mobile phase. With an acetonitrile gradient from 40 to 45%, the  $\alpha$ - and  $\beta$ -chains did not separate due to the combined effects of the slightly increased elution of the  $\beta$ -chain having the L88A( $\beta$ ) mutation (6) and the considerably decreased elution of the  $\beta$ -chain with the K95I( $\beta$ ) mutation (12). The chains were suc-

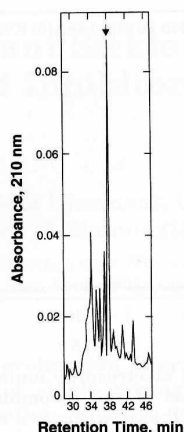


FIG. 2. Tryptic peptide map of the  $\beta$ -globin chain of the triple mutant. The isolated  $\beta$ -globin chain of E6V( $\beta$ )/L88A( $\beta$ )/K95I( $\beta$ ) was carboxymethylated and digested with trypsin as described in the text. The resulting peptides were chromatographed on a Vydac C-18 column using a linear acetonitrile gradient of 12–44% in 0.05% HCl.

cessfully separated by using an isocratic elution with 38.8% acetonitrile. Each had the expected amino acid composition relative to Pro (set at 7.0): Asx (12.1), Thr (6.4), Ser (5.3), Glx (10.0), Gly (12.7), Ala (15.6), Val (18.3), Ile (0.9), Leu (16.7), Tyr (1.9), Phe (6.4), His (8.0), Lys (9.5), Arg (3.1). Cys, Met, and Trp are destroyed partially or completely during acid hydrolysis in non-evacuated hydrolysis tubes.

**Peptide Mapping**—Tryptic peptide mapping of the isolated  $\beta$ -chain was performed as described previously (6, 15, 25, 26) to verify the expected mutations. The peptides were separated on a Vydac C-18 column using a shallow gradient of acetonitrile (from 12 to 44% in 50 min) in 0.05% HCl (Fig. 2). The major peak eluting at about 38 min was collected and its amino acid composition was found to be consistent with that of the expected 22-residue mutant peptide comprising  $\beta$  83–104 within experimental error. The values relative to Val (set at 1.0) were: Asx (3.0), Thr (1.3), Ser (1.8), Glx (1.6), Gly (1.9), Ala (1.5), Cys (0.5), Ile (0.5), Leu (1.9), Phe (2.6), His (0.7); however, Pro and Arg were not detected.

**Tetramer-Dimer Dissociation Constant**—Considerable dimerization may occur in hemoglobin even when the Hill coefficient shows high cooperativity, as discussed by Forsen and Linse (28). Using a method recently developed in our laboratory (29), we determined that the tetramer-dimer dissociation constant for the triple mutant was 0.4  $\mu$ M as compared with the  $K_d$  value of 0.7  $\mu$ M for HbS, indicating that the newly produced mutant hemoglobin did not undergo increased dissociation, *i.e.* at the Hb concentrations used for the functional studies (0.5–2.2 mM) the hemoglobin is predominantly tetrameric.

**Functional Properties**—The oxygen affinity of the triple mutant at a hemoglobin concentration of 0.5 mM showed an average  $P_{50}$  value of 10 mm Hg with a Hill coefficient of 2.7, indicating that the triple mutant retained full cooperativity (Fig. 3). DPG at a 1.2:1 ratio to Hb shows a significant response. The effects of two other anionic effectors, chloride and inositol hexaphosphate, were comparable with those measured earlier for HbS (15), for E6V( $\beta$ )/L88A( $\beta$ ) (6), and for E6V( $\beta$ )/K95I( $\beta$ ) (12): the maximum  $P_{50}$  value was 22 mm Hg in the presence of 1000 mM Cl<sup>-</sup>, and 58 mm Hg using an [IHP]:[Hb] ratio of 1.2 (Table I).

**Bohr Effect**—A plot of the change in  $P_{50}$  versus pH for the triple mutant had a slope of 0.37 (correlation coefficient,  $r = 0.972$ ) (Fig. 4), compared with the value of 0.41 ( $r = 0.995$ ) found for HbA. Thus, within experimental error, the alkaline

Bohr coefficient was unchanged compared with HbA, consistent with the native structure of the recombinant hemoglobin.

**Polymerization**—Comparison of the new micromethod used for direct measurement of gelation in these studies with the previous procedure using oxygen affinity changes (6, 12) indicates similar effectiveness of each mutation on inhibition of polymerization, even though the absolute values differ. Thus, ratios of 1.36 and 1.27 for the gelation concentrations of K95I/L88A recombinant hemoglobins were calculated for the oxygen affinity method and the present method, respectively. The dextran- $C_{sat}$  determinations of the following deoxyhemoglobins are shown in Fig. 5, natural HbS, the double mutant E6V( $\beta$ )/L88A( $\beta$ ), the double mutant E6V( $\beta$ )/K95I( $\beta$ ) and the triple mutant E6V( $\beta$ )/L88A( $\beta$ )/K95I( $\beta$ ). The results shown are an average of three to four determinations with a precision of + 10% or less. At initial concentrations below the  $C_{sat}$ , the final concentrations of each Hb at equilibrium, when plotted as a function of the initial concentrations, fell on a line with a slope of 1.03, indicating that the procedure itself did not result in precipitation (denaturation) of the hemoglobins. When initial Hb concentrations exceeded the  $C_{sat}$ , the final supernatant Hb concentrations ( $C_{sat}$  values) remained constant, independent of further increases in initial Hb concentrations over the ranges tested. The mean dextran- $C_{sat}$  value of the triple mutant was 91 mg/ml, indicating that it requires a considerably higher concentration than deoxy-HbS (mean: 34 mg/ml) for polymerization. The  $C_{sat}$  of the triple mutant was not significantly different from the value found for E6V( $\beta$ )/K95I( $\beta$ ) double mutant (90 mg/ml); the value for the E6V( $\beta$ )/L88A( $\beta$ ) (67 mg/ml) was between those of deoxy-HbS and those containing the Lys-95( $\beta$ ) substitutions. Deoxy HbA remained soluble at concentrations up to 149 mg/ml with no evidence of precipitation during the procedure. Thus, the new micromethod described here measures the true gelation of deoxy HbS.

#### DISCUSSION

In this study the recombinant triple mutant E6V( $\beta$ )/L88A( $\beta$ )/K95I( $\beta$ ) produced in yeast was shown to have the predicted amino acid composition, molecular mass, isoelectric point, and trypsin cleavage sites. Its oxygen affinity, cooperativity, response to negatively charged effectors, alkaline Bohr effect, and the tetramer/dimer dissociation constant were the same as those for HbS. These results, together with extensive characterization of recombinant hemoglobins by a variety of biochemical criteria (15, 25, 26, 29), are consistent with the expression by the yeast system of a native hemoglobin molecule with the correct N-terminal processing. Thus, we have no evidence for any misfolding of the triple mutant as reported for other recombinant hemoglobins made using *E. coli* as a production host (30). Hence, the gelation of the native HbS and the recombinant double and triple mutants by the procedure described here can be taken as reliable measurements of the gelation concentrations.

We reported previously that Lys-95( $\beta$ ), which is distant from the hydrophobic pocket in the region of Phe-85( $\beta$ )-Leu-88( $\beta$ ) comprising the acceptor site for Val-6( $\beta$ ), inhibits gelation much more than the substitution of a residue in the pocket itself (6, 12). Our results agreed with some previous reports implicating Lys-95( $\beta$ ) in the gelation process (33) and as an intermolecular contact site in the polymer (3, 4), although this site was not involved in the Wishner-Love double strand crystal of deoxy-HbS (5). The strong influence of the  $\beta$ -95 site, which is located on the exterior of the tetramer at the lateral contact site of the HbS tetramer, on gelation strongly suggests that the K95I( $\beta$ ) mutant of HbS has different protein self-assembly properties than HbS itself (32). The role of the Val-6( $\beta$ ) and its hydrophobic acceptor pocket may be to provide a

FIG. 3. The oxygen binding curve of E6V( $\beta$ )/L88A( $\beta$ )/K95I( $\beta$ ). The oxygen binding of the triple mutant (0.5 mM in 50 mM bis-Tris buffer, pH 7.4) in oxy form in the presence or absence of DPG was measured at 37°C using a modified Hemo-Scan instrument. The  $n$  value is an average of the two determinations.

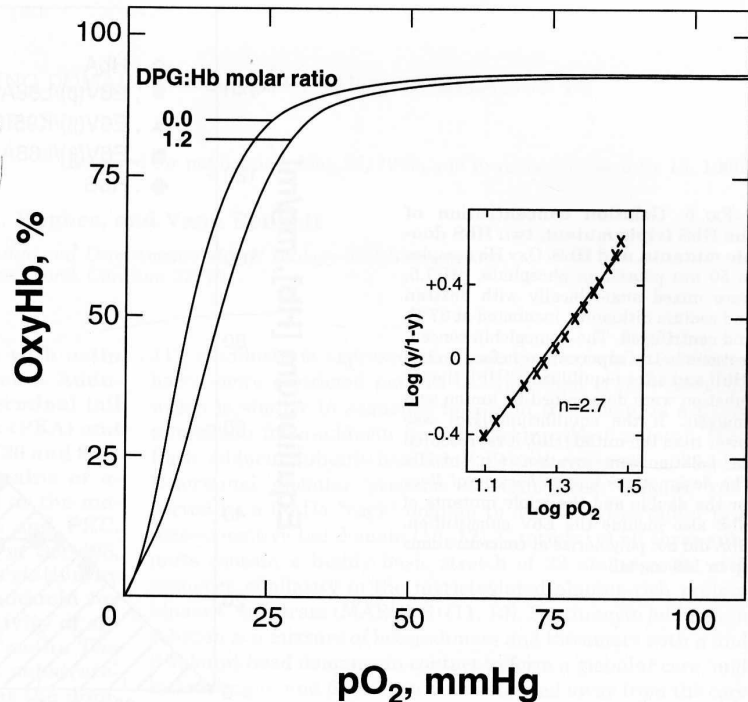


TABLE I  
The influence of allosteric effectors on the oxygen affinity of E6V( $\beta$ )/L88A( $\beta$ )/K95I( $\beta$ ) triple mutant  
The Hb concentration was 0.5 mM in 50 mM bis-Tris, pH 7.4.

	$P_{50}$ , E6V/L88A/K95I	HbS <sup>a</sup>
[Cl <sup>-</sup> ] (mM)		
0	11	10
50	14	
100		15
200	17	16
500	19	21
1000	22	25
[IHP]/[Hb]		
0.4	13	16
0.8	39	37
1.2	58	80
1.6	55	80
2.0	55	

<sup>a</sup> The values for the effect of Cl<sup>-</sup> on HbS are from Ref. 15.

molecular switch to turn the gelation either on or off. If this position is mutated to Ala (Hb Makassar), no gelation occurs because Ala prevents sufficient stabilization of the primary nuclei. Our results on the gelation of E6V( $\beta$ )/L88A( $\beta$ ) mutant (6, 34) also suggest that the Leu to Ala substitution in the acceptor pocket mainly affects the initial nucleation process, but once nucleation has taken place other residues stabilize the polymer. These findings also further emphasize the importance of certain ionizable surface amino acids. Their potential importance as well as that of their complementary sites on adjacent tetramers lies in the possible development of clinical intervention against sickle cell disease. The results presented here demonstrate that two sites on the HbS tetramer exert significantly different and independent effects on the inhibition of polymerization.

Since the polymer solubility of the triple mutant was the same as that of the double mutant without the L88A( $\beta$ ) substitution, *i.e.* E6V( $\beta$ )/K95I( $\beta$ ), the present results demonstrate that the inhibitory effects of the two  $\beta$ -chain substitutions (L88A and K95I) on HbS, are not additive. Although the

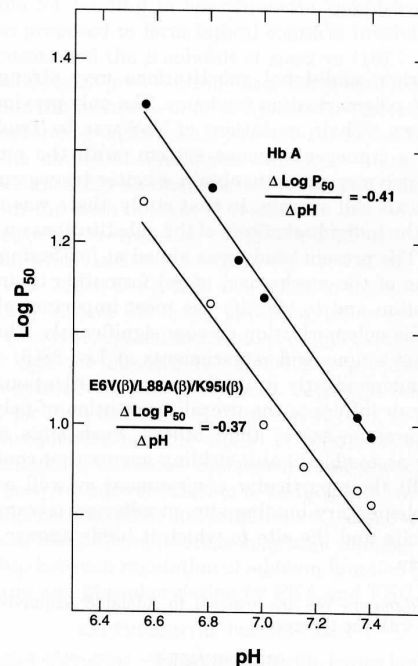
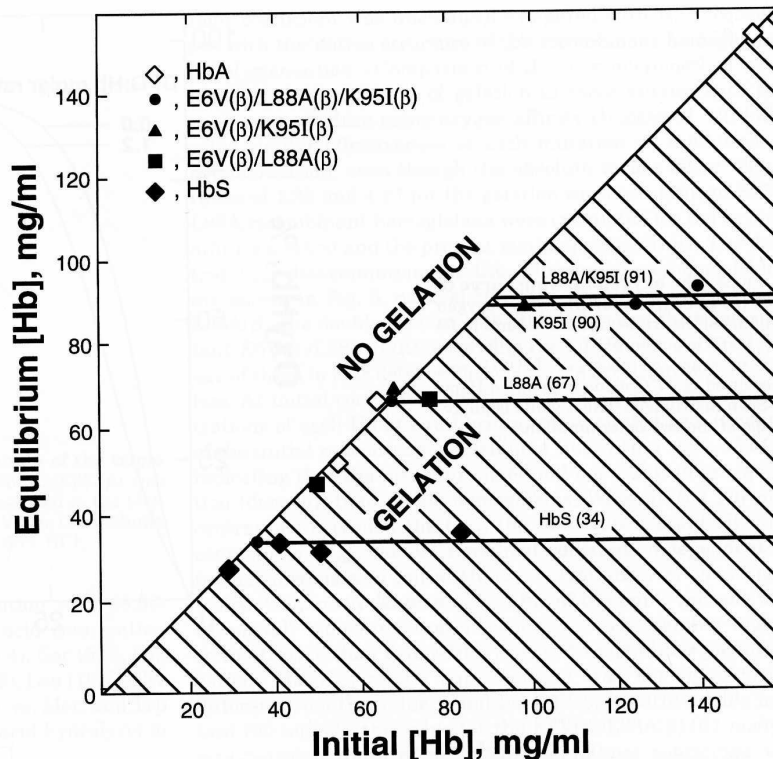


FIG. 4. The alkaline Bohr effect of E6V( $\beta$ )/L88A( $\beta$ )/K95I( $\beta$ ). The purified triple mutant in oxy form was diluted with bis-Tris buffers of different pH values to a final concentration of 0.5 mM Hb in 50 mM bis-Tris, and the  $P_{50}$  values were determined.

L88A( $\beta$ ) mutant, in which the substitution is in the hydrophobic acceptor pocket, has a gelation concentration about midway between the K95I( $\beta$ ) mutant and HbS itself, it does not appear to influence the overall behavior of the triple mutant.

The results of recent studies on recombinant mutants are consistent with the notion that once the initial contact site is established by the Glu-6  $\rightarrow$  Val substitution in the sickle Hb

FIG. 5. Gelation concentration of the HbS triple mutant, two HbS double mutants, and HbA. Oxy Hb samples in 50 mM potassium phosphate, pH 7.5, were mixed anaerobically with dextran and sodium dithionite, incubated at 37°C, and centrifuged. The hemoglobin concentrations in the supernatant before (initial [Hb]) and after (equilibrium [Hb]) the incubation were determined by amino acid analysis. If the equilibrium [Hb] was lower than the initial [Hb], it represented the gelation concentration ( $C_{sat}$ ) of Hb. The designations on the horizontal lines for the double and the triple mutants of HbS also include the E6V substitution. HbA did not polymerize at concentrations up to 149 mg/ml.



tetramer, then additional substitutions may strengthen or weaken the polymerization tendency. The only previous study involving two  $\beta$ -chain mutations of HbS was by Trudel *et al.* (13) using a transgenic mouse system, with the purpose of promoting polymerization to obtain a better transgenic mouse model of sickle cell anemia. In that study, there was no quantitation of the individual effects of the substitutions on polymer solubility. The present study was aimed at furthering our understanding of the mechanism of gel formation by inhibiting polymerization and to identify the most important sites that influence the polymerization process significantly. The results indicate that amino acid replacements at Leu-88( $\beta$ ) and Lys-95( $\beta$ ) act independently in inhibiting polymerization, *i.e.* certain sites can influence the overall prevention of polymerization to a greater extent than others. Such sites might be potentially accessible to anti-sickling agents that could be designed to fit their particular environment as well as that of their complementary binding site on adjacent tetramers. The Lys-95( $\beta$ ) site and the site to which it binds appear to fulfill such criteria.

**Acknowledgment**—We are grateful to Adelaide Acquaviva for her expert help with the typescript.

#### REFERENCES

- Pauling, L., Itano, H., Singer, S. J., and Wells, J. C. (1949) *Science* **110**, 543–548
- Ingram, V. M. (1956) *Nature* **178**, 792–794
- Nagel, R. L., and Bookchin, R. M. (1978) in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities* (Caughey, W. S., ed) pp. 195–201, Academic Press, New York
- Watowich, S. J., Gross, L. J., and Josephs, R. (1989) *J. Mol. Biol.* **209**, 821–828
- Padlan, E. A., and Love, W. E. (1985) *J. Biol. Chem.* **260**, 8280–8291
- Martin de Llano, J. J., and Manning, J. M. (1994) *Protein Sci.* **3**, 1206–1212
- Dickerson, R. E., and Geis, I. (1983) *Hemoglobin: Structure, Function, Evolution and Pathology*, pp. 133–137, Benjamin Cummings, Reading, MA
- Nagel, R. L., and Bookchin, R. M. (1974) in *Sickle Cell Anemia and Other Hemoglobinopathies* (Lever, R. D., ed) pp. 51–66, Academic Press, New York
- Baudin-Chich, V., Pagnier, J., Marden, M., Cohn, B., Loraze, N., Kister, J., Schaad, O., Edelstein, S. J., and Poyart, C. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 1845–1849
- Adachi, K., Konitzer, P., Kim, J., Welch, N., and Surrey, S. (1993) *J. Biol. Chem.* **268**, 21650–21656
- Ross, P. D., and Subramanian, S. (1978) in *Biochemical and Clinical Aspects of Hemoglobin Abnormalities* (Caughey, W. W., ed) pp. 629–645, Academic Press, New York
- Himanen, J.-P., Schneider, K., Chait, B., and Manning, J. M. (1995) *J. Biol. Chem.* **270**, 13885–13891
- Trudel, M., Saadane, N., Garel, M.-C., Bardakdjian-Michau, J., Blouquit, Y., Guerin-Kern, J.-L., Rouyer-Fessard, P., Vidaud, D., Pachnis, A., Romeo, P.-H., Beuzard, Y., and Costantini, F. (1991) *EMBO J.* **10**, 3157–3165
- Wagenbach, M., O'Rourke, K., Vitez, L., Wiecezorek, A., Hoffman, S., Durfee, S., Tedesco, J., and Stetler, G. (1991) *Bio/Technology* **9**, 57–61
- Martin de Llano, J. J., Jones, W., Schneider, K., Chait, B. T., Manning, J. M., Rodgers, G., Benjamin, L. J., and Weksler, B. (1993) *J. Biol. Chem.* **268**, 27004–27011
- Cerami, A., and Manning, J. M. (1971) *Proc. Natl. Acad. Sci. U. S. A.* **68**, 1180–1183
- Njikam, N., Jones, W. M., Nigen, A. M., Gillette, P. N., Williams, R. C., Jr., and Manning, J. M. (1973) *J. Biol. Chem.* **248**, 8052–8056
- Manning, J. M. (1991) *Adv. Enzymol. Mol. Biol.* **64**, 55
- Edelstein, S. J., and Crepeau, R. H. (1979) *J. Mol. Biol.* **134**, 851–855
- Bookchin, R. M., Balazs, T., and Lew, V. L. (1994) *Blood* **86**, 473a
- Kunkel, T. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 488–492
- Ito, H., Fukuda, Y., Murata, K., and Kimura, A. (1983) *J. Bacteriol.* **153**, 163–168
- Beavis, R. C., and Chait, B. T. (1989) *Rapid Commun. Mass Spectrom.* **3**, 233–237
- Beavis, R. C., and Chait, B. T. (1990) *Anal. Chem.* **62**, 1836–1840
- Yanase, H., Cahill, S., Martin de Llano, J. J., Manning, L. R., Schneider, K., Chait, B. T., Vandegriff, K. D., Winslow, R. M., and Manning, J. M. (1994) *Protein Sci.* **3**, 1213–1223
- Martin de Llano, J. J., Schneewind, O., Stetler, G., and Manning, J. M. (1993) *Proc. Natl. Acad. Sci. U. S. A.* **90**, 918–922
- Manning, J. M. (1981) *Methods Enzymol.* **76**, 159–167
- Forsen, S., and Linse, S. (1995) *Trends Biochem. Sci.* **20**, 495–497
- Manning, L. R., Jenkins, W. T., Hess, J. R., Vandegriff, K., Winslow, R., and Manning, J. M. (1996) *Protein Sci.* **5**, 775–781
- Hernan, R. A., and Sligar, S. G. (1995) *J. Biol. Chem.* **270**, 26257–26264
- Eaton, W. A., and Hofrichter, J. (1990) *Adv. Protein Chem.* **40**, 63–279
- Eaton, W. A., and Hofrichter, J. (1994) in *Sickle Cell Disease: Basic Principles and Clinical Practice* (Embury, S. H., Hebbel, R. P., Mohandas, N., and Steinberg, M. H., eds.) pp. 53–87, Raven Press, New York
- Bookchin, R. M., Nagel, R. L., Balazs, T., and Harris, J. W. (1974) *Clin. Res.* **22**, 384A
- Liao, D., Martin de Llano, J. J., Himanen, J. P., Manning, J. M., and Ferrone, F. A. (1996) *Biophys. J.* **70**, 2442–2447