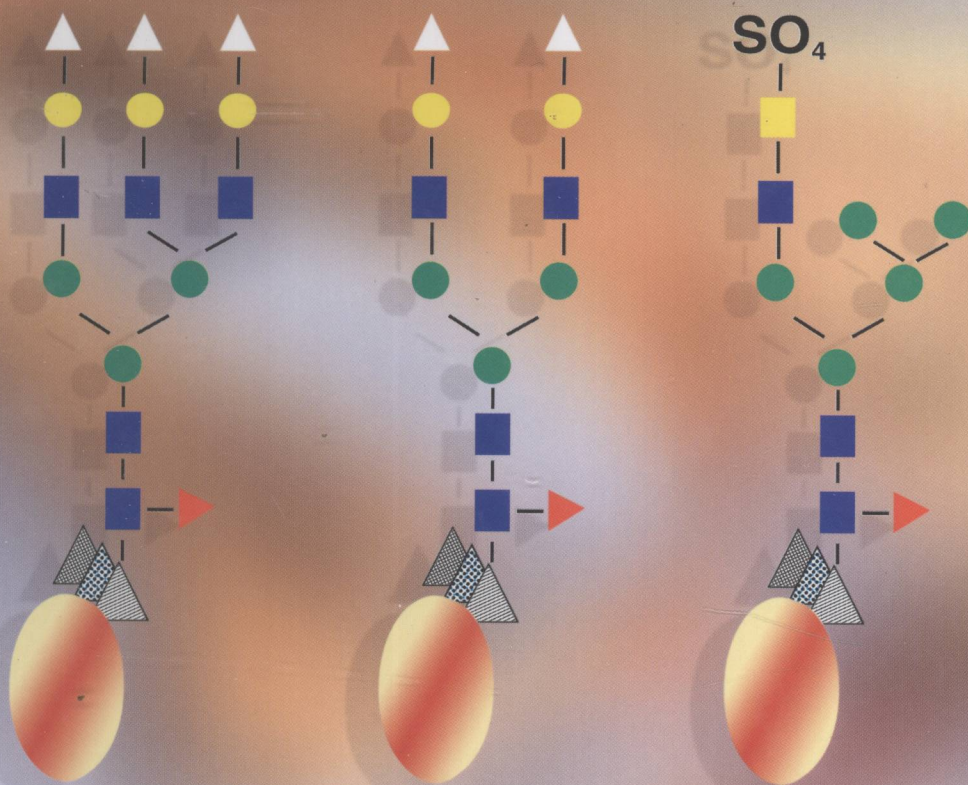




**WILEY-
BLACKWELL**

The figure shows three stages of polymer degradation by a cellulase enzyme. The enzyme is represented by a large orange oval at the bottom, with a blue square and a red triangle indicating its active site. The polymer chain is a linear sequence of green circles, blue squares, yellow circles, and white triangles. In the first diagram, the chain is intact. In the second diagram, the chain is being cleaved at the junction between a blue square and a yellow circle. In the third diagram, the chain is fully degraded into individual monomers.



R914
P857

Post-translational Modification of Protein Biopharmaceuticals

Edited by
Gary Walsh



WILEY-
BLACKWELL



E2009002443

WILEY-VCH Verlag GmbH & Co. KGaA

The Editor

Prof. Dr. Gary Walsh

Industrial Biochemistry Program
CES Department
University of Limerick
Castletroy, Limerick City
Ireland

■ All books published by Wiley-VCH are carefully produced. Nevertheless, authors, editors, and publisher do not warrant the information contained in these books, including this book, to be free of errors. Readers are advised to keep in mind that statements, data, illustrations, procedural details or other items may inadvertently be inaccurate.

Library of Congress Card No.: applied for

British Library Cataloguing-in-Publication Data

A catalogue record for this book is available from the British Library.

Bibliographic information published by the Deutsche Nationalbibliothek

The Deutsche Nationalbibliothek lists this publication in the Deutsche Nationalbibliografie; detailed bibliographic data are available on the Internet at <http://dnb.d-nb.de>.

© 2009 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

All rights reserved (including those of translation into other languages). No part of this book may be reproduced in any form – by photoprinting, microfilm, or any other means – nor transmitted or translated into a machine language without written permission from the publishers. Registered names, trademarks, etc. used in this book, even when not specifically marked as such, are not to be considered unprotected by law.

Composition Thomson Digital, Noida, India

Printing Strauss GmbH, Mörlenbach

Bookbinding Litges & Dopf GmbH, Heppenheim

Cover Design Adam Design, Weinheim

Printed in the Federal Republic of Germany
Printed on acid-free paper

ISBN: 978-3-527-32074-5

**Post-translational Modification
of Protein Biopharmaceuticals**

Edited by
Gary Walsh

Related Titles

Lutz, S., Bornscheuer, U. T. (eds.)

Protein Engineering Handbook

2008

ISBN: 978-3-527-31850-6

Behme, S. (ed.)

Manufacturing of Pharmaceutical Proteins

From Technology to Economy

2009

ISBN: 978-3-527-32444-6

Dübel, S. (ed.)

Handbook of Therapeutic Antibodies

2007

ISBN: 978-3-527-31453-9

Desiderio, D. M., Nibbering, N. M.

Redox Proteomics

From Protein Modifications to Cellular Dysfunction and Diseases

2006

ISBN: 978-0-471-72345-5

Knäblein, J. (ed.)

Modern Biopharmaceuticals

Recent Success Stories

2009

ISBN: 978-3-527-32283-1

Gruber, A. C.

Biotech Funding Trends

Insights from Entrepreneurs and Investor

2008

ISBN: 978-3-527-32435-4

Borbye, L. (ed.)

Industry Immersion Learning

Real-Life Industry Case-Studies in Biotechnology and Business

2009

ISBN: 978-3-527-32408-8

Preface

The majority of approved therapeutic proteins, as well as those currently in development, naturally undergo some form of post-translational modification (PTM). An increasing appreciation of the central importance of such PTMs to the application-relevant properties of these proteins continues to emerge.

This book aims to provide a comprehensive overview of protein post-translational modifications specifically in the context of biopharmaceuticals. Chapter 1 introduces the topic, to provide an appropriate context for the remainder of the book. The subsequent 13 chapters focus upon various specific post-translational modifications. Several chapters are devoted to various aspects of glycosylation, as this PTM is by far the most complex and significant associated with therapeutic proteins. Individual chapter authors are drawn from both academia and industry, and from various global regions.

The book will serve as a reference source for those working or wishing to work in the biopharmaceutical sector. Its scope should also render it a useful reference text for third level students undertaking healthcare-related programs of study (e.g., undergraduate or taught postgraduate programs in pharmacy, pharmaceutical science and biotechnology, as well as in biochemistry). Likewise, it should serve as a useful reference for academic and industry researchers whose research interests relate to biopharmaceuticals.

February 2009

G. Walsh
University of Limerick

List of Contributors

Gian Maria Bonora

University of Trieste
Department of Chemical Science
Via Giorgieri
34126 Trieste
Italy

George R. Bousfield

Wichita State University
Department of Biological Sciences
1845 Fairmount
Wichita, KS 67260
USA

Susan A. Brooks

Oxford Brookes University
School of Life Sciences
Gipsy Lane
Headington
Oxford OX3 0BP
UK

Mark A. Brown

Marine Biological Laboratory
Woods Hole, MA 02543
USA
and

Center for Hemostasis and Thrombosis
Research

Beth Israel Deaconess Medical Center
and Harvard Medical School
Cambridge, MA 02139
USA

Michael Butler

University of Manitoba
Faculty of Science
Department of Microbiology
Winnipeg
Manitoba R3T 2N2
Canada

Sarah E. Carpenter

Unigene Laboratories, Inc.
110 Little Falls Road
Fairfield, NJ 07004
USA

Jean-François Collet

Université catholique de Louvain
de Duve Institute
75-39 Avenue Hippocrate
1200 Brussels
Belgium

Angelo P. Consalvo

Unigene Laboratories, Inc.
110 Little Falls Road
Fairfield, NJ 07004
USA

James A. Dias

State University of New York at Albany
New York State Department of Health
and Department of Biomedical Sciences
Wadsworth Center
David Axelrod Institute for
Public Health
120 New Scotland Ave
Albany, NY 12208
USA

Tim Edmunds

Therapeutic Protein Research
Genzyme Corporation
One Mountain Road
Framingham, MA 01701
USA

Steve Elliott

Amgen Inc.
One Amgen Center Drive
Thousand Oaks, CA 91320
USA

Hayat El Hajjaji

Université catholique de Louvain
de Duve Institute
75-39 Avenue Hippocrate
1200 Brussels
Belgium

Mario Feldman

University of Alberta
Department of Biological Sciences
Alberta Ingenuity Centre for
Carbohydrate Sciences
Edmonton T6G 2E9
Canada

Christoph Geisler

University of Wyoming
Department of Molecular Biology
1000. E. University Avenue
Laramie, WY 82071
USA

Don Jarvis

University of Wyoming
Department of Molecular Biology
1000. E. University Avenue
Laramie, WY 82071
USA

Roy Jefferis

University of Birmingham
The Division of Immunity & Infection
Birmingham B15 2TT
UK

Michael Kowarik

GlycoVaxyn AG
Grabenstrasse 3
8952 Schlieren
Switzerland

Nozer M. Mehta

Unigene Laboratories, Inc.
110 Little Falls Road
Fairfield, NJ 07004
USA

Scott M. Van Patten

Therapeutic Protein Research
Genzyme Corporation
One Mountain Road
Framingham, MA 01701
USA

Natarajan Sethuraman

GlycoFi, a wholly-owned subsidiary of
Merck and Co.
21 Lafayette Street
Suite 200
Lebanon, NH 03766
USA

Terrance A. Stadheim

GlycoFi, a wholly-owned subsidiary of
Merck and Co.
21 Lafayette Street
Suite 200
Lebanon, NH 03766
USA

Leisa M. Stenberg

Marine Biological Laboratory
Woods Hole, MA 02543
USA
and
Center for Hemostasis and Thrombosis
Research
Beth Israel Deaconess Medical Center,
and Harvard Medical School
Cambridge, MA 02139
USA

Francesco Maria Veronese

University of Padova
Department of Pharmaceutical Sciences
Via F. Marzolo
35131 Padova
Italy

Alfredo Ulloa-Aguirre

Hospital de Gineco-Obstetricia "Luis
Castelazo Ayala"
Research Unit in Reproductive
Medicine
Instituto Mexicano del Seguro Social
Mexico D.F.
Mexico

Gary Walsh

University of Limerick
Industrial Biochemistry Program
Limerick
Ireland

Contents

Preface XV

List of Contributors XVII

1	Post-Translational Modifications in the Context of Therapeutic Proteins: An Introductory Overview	1
	<i>Gary Walsh</i>	
1.1	Introduction	1
1.2	Biopharmaceuticals and the Biopharmaceutical Sector	1
1.3	Protein Post-Translational Modification	2
1.4	PTMs in the Context of Biopharmaceuticals	7
1.5	Some Specific PTMs	8
1.5.1	Glycosylation	8
1.5.2	Disulfide Bond Formation and Proteolytic Cleavage	10
1.5.3	γ -Carboxylation and β -Hydroxylation	11
1.5.4	Amidation and Sulfation	12
1.6	Extending and Engineering PTM Profiles	12
1.7	Conclusion	13
	References	14

Part One Glycosylation 15

2	Protein Glycosylation: The Basic Science	17
	<i>Susan A. Brooks</i>	
2.1	Introduction – Glycosylated Proteins	17
2.2	Basic Building Blocks of Glycosylation in Human Cells	18
2.3	Formation of Complex Glycan Structures	19
2.3.1	α and β Glycosidic Bonds	19
2.3.2	Structural Complexity of Glycoprotein Glycans	19
2.4	Glycan Synthesis is Catalyzed by Enzymes of Glycosylation – the “Glycozymes”	22

2.5	Protein Glycosylation – Relationship Between N-linked and O-linked Glycoproteins	23
2.6	N-linked Glycoproteins	24
2.6.1	Where Does N-linked Glycosylation of Proteins Take Place?	24
2.6.2	Why is it Called N-linked?	24
2.6.3	N-Glycosylation Step-by-Step	24
2.6.3.1	Polypeptide Enters the RER	24
2.6.3.2	Building and Positioning the Dolichol Oligosaccharide Precursor	24
2.6.3.3	Attachment of the Dolichol Oligosaccharide Precursor to the Polypeptide Chain	25
2.6.3.4	Trimming the Dolichol Oligosaccharide Precursor in the RER	25
2.6.3.5	Processing of N-linked Oligosaccharides in the Golgi Apparatus	25
2.6.4	Three Classes of N-linked Glycoproteins	25
2.6.5	What Determines Whether a Potential Site of N-Glycosylation on the Polypeptide Chain is Occupied or Not?	27
2.7	O-linked Glycoproteins	27
2.7.1	Why is it Called O-linked?	28
2.7.2	Different Types of O-linked Glycosylation	28
2.7.3	Overview of O-linked Mucin-Type Protein Glycosylation	28
2.7.4	No Consensus Sequence for Mucin-Type O-linked Glycoprotein Glycosylation	29
2.7.5	Synthesis of O-linked Mucin-Type Glycan Core Structures Step-by-Step	29
2.7.5.1	Initiation – Synthesis of GalNAc α 1 \rightarrow Ser/Thr (Tn Epitope)	29
2.7.5.2	Synthesis of NeuNAc(α 2 \rightarrow 6)GalNAc α 1 \rightarrow Ser/Thr (Sialyl Tn)	29
2.7.5.3	Synthesis of Core 1, Gal(β 1 \rightarrow 3)GalNAc α -Ser/Thr, or the Thomsen–Friedenreich (T or TF) Antigen	29
2.7.5.4	Synthesis of Core 2, GlcNAc(β 1 \rightarrow 6)[Gal(β 1 \rightarrow 3)]GalNAc α 1-Ser/Thr	31
2.7.5.5	Synthesis of Core 3, GlcNAc(β 1 \rightarrow 3)GalNAc α 1 \rightarrow Ser/Thr	31
2.7.5.6	Synthesis of Core 4, GlcNAc(β 1 \rightarrow 6)[GlcNAc(β 1 \rightarrow 3)]GalNAc α 1-Ser/Thr	31
2.7.5.7	Less Common Core Structures	31
2.7.6	Regulation of O-Glycan Synthesis	32
2.8	O- and N-linked Glycan Chain Extension and Commonly Occurring Glycan Motifs	32
2.8.1	Type I Chains or Neolactosamine Units, Gal(β 1 \rightarrow 3)GlcNAc/GalNAc	32
2.8.2	Type 2 Chains or Lactosamine Units, Gal(β 1 \rightarrow 4)GlcNAc	33
2.8.3	Termination of Glycan Chains	33
2.8.4	Blood Group Antigens	33
2.9	Analytical Methodologies Developed to Detect and Characterize Glycosylation	33
2.9.1	Glycan Analysis is Complex and Requires a Number of Techniques	33

2.9.2	Detection of Glycans	34
2.9.2.1	Periodic Acid-Schiff (PAS) Reaction	34
2.9.2.2	Recognition Through Lectin Binding	34
2.9.2.3	Pulsed Amperometric Detection (PAD) of Unlabelled Free Glycans	35
2.9.2.4	Labeling Glycans with Radiolabels or Fluorescent Labels	35
2.9.3	Profiling Glycans Using Microarrays	35
2.9.4	One- and Two-Dimensional Gel Electrophoresis – Exploring the Glycome	36
2.9.5	Chemical Release and Analysis of Monosaccharides	36
2.9.6	Chemical Release of Intact Oligosaccharides	37
2.9.7	Enzymatic Release of Intact Oligosaccharides	37
2.9.8	Sequential Exoglycosidase Digestions to Provide Monosaccharide Sequence and Linkage Data	37
2.9.9	Oligosaccharide Separation and Mapping by High-Performance Liquid Chromatography (HPLC)	38
2.9.9.1	Normal Phase HPLC (NP-HPLC)	38
2.9.9.2	Weak Anion Exchange High-Performance Liquid Chromatography (WAX-HPLC)	38
2.9.9.3	High-Performance (or High pH) Anion Exchange Chromatography (HPAEC)	39
2.9.10	Separation and Mapping of Oligosaccharides by Fluorophore-Assisted Carbohydrate Electrophoresis (FACE)	39
2.9.11	Separation and Mapping of Oligosaccharides by Capillary Electrophoresis (CE)	39
2.9.12	Oligosaccharide Analysis by Nuclear Magnetic Resonance (NMR)	40
2.9.13	Determining the Mass of an Oligosaccharide Using Mass Spectrometry (MS)	40
2.9.14	Gas-Liquid Chromatography (GLC)/MS for Determining the Linkage Position of Monosaccharides in Oligosaccharides	41
2.10	Conclusion	42
	References	42
3	Mammalian Cell Lines and Glycosylation: A Case Study	51
	<i>Michael Butler</i>	
3.1	Introduction	51
3.2	The Choice of Cell Line for Glycoprotein Production	51
3.3	Effect of Growth and Protein Production Rate on Glycosylation	54
3.4	Enzymes Associated with Glycan Heterogeneity	55
3.4.1	N-Acetyl Glucosaminyltransferases	55
3.4.2	Fucosylation	57
3.4.3	Sialylation	58
3.5	Immunogenicity of Non-Human Glycans	61
3.6	Culture Parameters that may Affect Glycosylation	61
3.6.1	Nutrient Depletion	61

3.6.2	Fed-Batch Cultures and Supplements	63
3.6.2.1	Glucosamine as a Supplement	64
3.6.2.2	Galactose as a Supplement	65
3.6.3	Ammonia	65
3.6.4	pH	66
3.6.5	Oxygen	66
3.7	Functional Glycomics	67
3.8	Conclusion	68
	References	69
4	Antibody Glycosylation	79
	<i>Roy Jefferis</i>	
4.1	Introduction	79
4.2	Antibodies	80
4.2.1	Basic Structure/Function	80
4.2.2	Antibody (Immunoglobulin) Isotypes	84
4.3	Glycosylation	84
4.3.1	Glycosylation of Normal Human IgG	84
4.3.2	Impact of Glycosylation on Structure	86
4.3.3	Impact of Glycosylation on Stability	87
4.4	IgG-Fc Effector Functions	88
4.4.1	Inflammatory Cascades	88
4.4.2	Catabolism, Pharmacokinetics and Placental Transport	90
4.5	Individual IgG-Fc Glycoforms	91
4.5.1	Individual IgG-Fc Glycoforms and Effector Activities	91
4.5.2	Sialylation of IgG-Fc Oligosaccharides	92
4.5.3	Influence of Galactosylation on IgG-Fc Activities	92
4.5.4	Influence of Fucose and Bisecting <i>N</i> -Acetylglucosamine on IgG-Fc Activities	94
4.6	IgG-Fab Glycosylation	96
4.7	Recombinant Monoclonal Antibodies for Therapy	98
4.8	Conclusions and Future Perspectives	100
	References	100
5	Gonadotropins and the Importance of Glycosylation	109
	<i>Alfredo Ulloa-Aguirre, James A. Dias, and George R. Bousfield</i>	
5.1	Introduction	109
5.2	Structure of Gonadotropins	111
5.3	Glycosylation of Gonadotropins and Structural Microheterogeneity	114
5.4	Role of Glycosylation in the Function of Gonadotropins	121
5.4.1	Role in Folding, Subunit Assembly and Secretion	121
5.4.2	Metabolic Clearance Rate	122
5.4.3	Binding and Signal Transduction	123
5.4.4	LH and FSH Glycoforms and Gonadotropin Function	124

5.4.5	Chorionic Gonadotropin Glycoforms and Function	126
5.5	Regulation of Gonadotropin Glycosylation	127
5.5.1	Effects of Estrogens	127
5.5.2	Effects of Androgens	128
5.5.3	Effects of Gonadotropin-Releasing Hormone	129
5.6	Therapeutic Applications of Gonadotropins	130
5.7	Conclusions	132
	References	133
6	Yeast Glycosylation and Engineering in the Context of Therapeutic Proteins	149
	<i>Terrance A. Stadheim and Natarajan Sethuraman</i>	
6.1	Introduction	149
6.2	N-Glycosylation in Fungi	150
6.3	O-Glycosylation in Fungi and Mammals	152
6.4	Remodeling Yeast Glycosylation for Therapeutic Protein Production	154
	References	160
7	Insect Cell Glycosylation Patterns in the Context of Biopharmaceuticals	165
	<i>Christoph Geisler and Don Jarvis</i>	
7.1	Introduction	165
7.2	Recombinant N-Glycoproteins in the BEVS Product Pipeline	166
7.2.1	Chimigen [™] Vaccines	167
7.2.2	FluBlok [™]	167
7.2.3	Influenza Virus-Like Particles	167
7.2.4	Provenge [®]	167
7.2.5	Specifid [™]	168
7.3	Insect Glycoprotein N-Glycan Structure	168
7.3.1	Typical N-Glycan Structures	168
7.3.2	Hybrid/Complex N-Glycans	170
7.3.3	Sialylated N-Glycans	170
7.3.4	Summary	170
7.4	N-Glycan Processing Enzymes in the BEVS	171
7.4.1	Processing β -N-Acetylglucosaminidase	171
7.4.2	Core α 1,3 Fucosyltransferase	172
7.4.3	Lack of Mannose-6-Phosphate	172
7.5	Lack of Glycosyltransferases	173
7.5.1	N-Acetylglucosaminyltransferases II–IV	173
7.5.2	N-acetylgalactosaminyl-Galactosyl- and Sialyltransferase	173
7.5.3	Glycosyltransferase Donor Substrates	174
7.6	Use of Baculoviruses to Extend BEVS N-Glycosylation	174
7.6.1	Promoter Choice	174
7.6.2	Baculovirus Encoded Glycosyltransferases	175

7.6.2.1	<i>N</i> -Acetylglucosaminyltransferase I	175
7.6.2.2	Galactosyltransferase	175
7.6.2.3	Sialyltransferase	175
7.6.2.4	<i>N</i> -acetylglucosaminyltransferase II	176
7.6.2.5	Trans-Sialidase	176
7.6.3	Baculoviruses Encoded Sugar Processing Genes	176
7.6.3.1	UDP-GlcNAc 2-Epimerase/ <i>N</i> -Acetylmannosamine Kinase	176
7.6.3.2	Sialic Acid Synthetase	177
7.6.3.3	CMP-Sialic Acid Synthetase	177
7.6.4	Summary	177
7.7	Transgenic Insect Cell Lines	178
7.7.1	Proof of Concept	178
7.7.2	Transgenic Glycosyltransferases	179
7.7.2.1	Galactosyltransferase	179
7.7.2.2	Sialyltransferase	179
7.7.2.3	<i>N</i> -Acetylglucosaminyltransferase II	180
7.7.3	Transgenic Sugar Processing Genes	180
7.7.4	Use of Transposon-Based Systems	181
7.7.5	Summary	181
7.8	Sugar Supplementation	182
7.9	Future Directions	182
7.9.1	Completing the <i>N</i> -Glycosylation Pathway	183
7.9.2	Reducing Deleterious Activities	183
	References	184

8 **Getting Bacteria to Glycosylate** 193

Michael Kowarik and Mario F. Feldman

8.1	Introduction	193
8.1.1	Overview and Background	193
8.1.2	Bacterial Protein Glycosylation	194
8.2	<i>N</i> -Glycosylation	194
8.2.1	Introduction	194
8.2.2	The Acceptor Protein	195
8.2.2.1	Primary Acceptor Consensus	196
8.2.2.2	Conformational Requirements for <i>N</i> -Glycosylation	196
8.2.2.3	Crystal Structures of Bacterial <i>N</i> -Glycoproteins	197
8.2.3	The LLO Substrate and the <i>N</i> -OTase, PglB	197
8.3	<i>O</i> -Glycosylation	199
8.3.1	<i>O</i> -Glycosylation in <i>Pseudomonas aeruginosa</i>	200
8.3.1.1	Introduction	200
8.3.1.2	The Acceptor Protein for <i>O</i> -Glycosylation	200
8.3.1.3	Glycan Structures in <i>P. aeruginosa</i> <i>O</i> -Glycosylation	201
8.3.2	<i>O</i> -Glycosylation in <i>Neisseria</i>	201
8.3.2.1	Introduction	201
8.3.2.2	PglL, the <i>O</i> -OTase of <i>N. meningitidis</i>	201

8.3.2.3	Glycan Substrates for <i>N. meningitidis</i> O-Glycosylation	202
8.4	Exploitation of N- and O-Linked Glycosylation	203
8.4.1	Therapeutic (Human) Proteins	203
8.4.2	Bioconjugate Vaccines	204
8.4.3	Glycoengineering	205
	References	206

Part Two Other Modifications 209

9 Biopharmaceuticals: Post-Translational Modification Carboxylation and Hydroxylation 211

Mark A. Brown and Leisa M. Stenberg

9.1	Introduction	211
9.2	γ -Carboxylation	211
9.2.1	Biological Function of γ -Carboxylation	211
9.2.2	The Gla Domain	214
9.2.3	Biosynthesis of Gla	217
9.2.4	γ -Carboxylated Biopharmaceuticals	219
9.2.4.1	Factor IX	219
9.2.4.2	Factor VIIa	221
9.2.4.3	Protein C/Activated Protein C	222
9.2.4.4	Prothrombin	223
9.2.4.5	Conotoxins	224
9.2.5	Enhancement of Cellular Carboxylation Capacity	224
9.2.5.1	Enhanced Expression of the γ -Carboxylation Machinery	225
9.2.5.2	Inhibition of Calumenin Expression	226
9.2.5.3	Propeptide/Propeptidase Engineering	226
9.2.6	Purification of γ -Carboxylated Proteins	226
9.2.7	Analytical Characterization of γ -Carboxylated Proteins	227
9.2.7.1	Methods for Detecting Gla	227
9.2.7.2	Metal Content	227
9.2.7.3	Metal Binding-Induced Structural Changes	228
9.2.7.4	Phospholipid Membrane Binding Assays	228
9.2.7.5	γ -Carboxylase Enzyme Assays	228
9.3	Post-Translational Hydroxylation	229
9.3.1	Biological Function of Hydroxylation	229
9.3.2	Biosynthesis of Hydroxylated Amino Acids	231
9.3.2.1	Biosynthesis of Hya/Hyn	231
9.3.2.2	Biosynthesis of Hydroxyproline	232
9.3.3	Hydroxylated Biopharmaceuticals	233
9.3.3.1	Factor IX	233
9.3.3.2	Protein C/Activated Protein C	233
9.3.3.3	Conotoxins	234
9.3.4	Analytical Characterization of β -Hydroxylated Proteins	235
9.3.4.1	Methods for Detecting Hya/Hyn	235

9.3.4.2	β -Hydroxylase Enzyme Assays	235
9.4	Conclusions	235
	References	236

10 C-Terminal α -Amidation 253

Nozer M. Mehta, Sarah E. Carpenter, and Angelo P. Consalvo

10.1	Introduction	253
10.2	Substrate Specificity of PAM	253
10.3	Activity of PAM	254
10.3.1	Assays for Measurement of PAM Activity	254
10.3.2	Mechanism of Action	255
10.3.3	Species Distribution of α -Amidated Peptides and PAM	257
10.4	Genomic Structure and Processing of PAM	257
10.4.1	Organization of the PAM Gene	257
10.4.2	Tissue-Specific Forms of PAM	258
10.5	Structure–Activity Relationships (SAR) for Rat PAM Activity	258
10.6	α -Amidation of Glycine-Extended Peptides	260
10.6.1	<i>In Vitro</i> α -Amidation	260
10.6.2	Optimization of the PAM Reaction <i>In Vitro</i>	261
10.7	Cloning and Expression of Various Forms of PAM	263
10.7.1	PHM, PHMcc and PAL	264
10.7.2	Bifunctional PAM	264
10.7.3	Co-expression of PAM with Glycine-Extended Peptides	265
10.8	A Process for Recombinant Production of α -Amidated Peptides	265
10.8.1	Expression of Glycine-Extended Peptides in <i>E. coli</i> by a Direct Expression Process	266
10.8.2	Purification of the Glycine-Extended Peptides	266
10.8.3	Expression of PAM in CHO Cells	267
10.8.4	Purification of Recombinant PAM (rPAM)	267
10.8.5	Post-Amidation Purification	268
10.8.6	Expression Levels of Peptides by Direct Expression Technology	269
10.9	Marketed Peptides	269
10.9.1	Marketed α -Amidated Peptides	269
10.10	Conclusions	271
	References	271

11 Disulfide Bond Formation 277

Hayat El Hajjaji and Jean-François Collet

11.1	Introduction	277
11.2	Disulfide Bonds have a Stabilizing Effect	278
11.3	Disulfide Bond Formation is a Catalyzed Process	278
11.4	Disulfide Bond Formation in the Bacterial Periplasm	278
11.4.1	The Oxidation Pathway: DsbA and DsbB	279
11.4.1.1	DsbA, a very Oxidizing Protein	279
11.4.1.2	DsbB	280