

## METHODS IN ENZYMOLOGY

## New Approaches for Flavin Catalysis

Edited by

## **BRUCE A. PALFEY**

Associate Professor of Biological Chemistry & Associate Director, Program in Chemical Biology, Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, United States







## **Contents**

	ributors	xiii
refo	ace	xix
1.	Methods for determining the reduction potentials of flavin enzymes  Shelbi L. Christgen, Sophia M. Becker, and Donald F. Becker	1
	1. Introduction	2 5
	2. Spectroelectrochemistry method	5 8
	3. Xanthine oxidase method	18
	<ul><li>4. Effects of ligands</li><li>5. Conclusion</li></ul>	19
	References	21
	Hererences	
2.	Anaerobic methods for the transient-state study of	
	flavoproteins: The use of specialized glassware to define the	
	concentration of dioxygen	27
	Graham R. Moran	
	1. Introduction	28
	Controlling the concentration of molecular oxygen	32
	3. Preparing reduced flavoproteins	46
	4. Summary and conclusion	48
	References	48
3.	Performing anaerobic stopped-flow spectrophotometry inside	
	of an anaerobic chamber	51
	Hannah Valentino and Pablo Sobrado	
	1. Introduction	52
	2. Anaerobic chambers	54
	3. Introduction to stopped-flow spectrophotometry	58
	4. Setting up, preparing, and performing stopped-flow spectrophotometry	
	assays inside of an anaerobic chamber	61
	5. Other uses for anaerobic chambers	82
	6. Summary and conclusions	84
	Acknowledgments	85
	References	85

4.	Chemical quenching and identification of intermediates in flavoenzyme-catalyzed reactions	89
	Kalani Karunaratne and Tatiana V. Mishanina	
	1. Introduction	90
	2. Practical considerations	95
	3. Case study: Flavin-dependent thymidylate synthase	98
	4. Concluding remarks	110
	Acknowledgments	111
	References	111
5.	• • • • • • • • • • • • • • • • • • • •	
	investigate flavoenzyme mechanism	115
	Kevin Francis and Giovanni Gadda	
	1. Introduction	116
	2. Steady-state versus rapid kinetic investigations of flavoenzymes	122
	3. Solvent kinetic isotope effects to probe flavoenzyme mechanism	132
	4. Multiple KIE studies of flavin dependent enzyme	133
	5. Conclusions	135
	Acknowledgments	135
	References	135
6.	Isotopically labeled flavoenzymes and their uses in probing reaction mechanisms	145
	Andreea I. lorgu, Matthew J. Cliff, Jonathan P. Waltho, Nigel S. Scrutton,	
	and Sam Hay	
	1. Introduction	146
	2. PETNR as a model flavoenzyme from the OYE family	149
	3. Expression and purification of isotopically labeled PETNR in E. coli	149
	4. Spectral characteristics and quality control of isotopically labeled PETNR	155
	<b>5.</b> Analysis of deuterium-hydrogen exchange of backbone amides in PETNR	157
	<ol><li>Spectroscopic characterization of the equilibrium unfolding behavior of PETNR</li></ol>	157
	7. Differential labeling of PETNR: Exchanging the FMN cofactor	160
	8. Uses of isotopically labeled PETNR in probing biological hydride transfer	, 50
	mechanisms	161
	9. Conclusions	163
	Acknowledgments	163
	References	163

7.	Unraveling flavoenzyme reaction mechanisms using flavin analogues and linear free energy relationships	167
	Christopher J. Thibodeaux, Wei-chen Chang, and Hung-wen Liu	
	1. Introduction	168
	2. Chemoenzymatic synthesis of flavin analogues	169
	3. Mechanistic analysis of flavoenzymes using linear free energy	
	relationships	177
	4. Summary and conclusion	186
	References	186
8.	Vibrational spectroscopy of flavoproteins	189
	James N. Iuliano, Jarrod B. French, and Peter J. Tonge	
	1. Introduction	190
	2. Applications of vibrational spectroscopy to flavoproteins	195
	3. Methods to interpret vibrational spectra	204
	4. General experimental considerations	208
	References	210
9.	Measuring electronic structure properties of flavins and	
	flavoproteins by electronic Stark spectroscopy	215
	Robert J. Stanley and Cornelius J. van Galen	
	1. Introduction	216
	2. Electronic Stark spectroscopy: Materials and methods	230
	3. Calculations	246
	4. Summary	247
	Acknowledgments	247
	References	248
10.	EPR spectroscopy on flavin radicals in flavoproteins	251
	Daniel Nohr, Stefan Weber, and Erik Schleicher	
	1. Introduction	251
	2. The electronic structure of flavin radicals	253
	3. Protein-cofactor interactions in flavin radicals	255
	4. Long-range interactions in flavoproteins	260
	5. Time-resolved methods for investigating radical pairs	261
	6. Outlook	268
	References	270

11.	Applications of molecular modeling to flavoproteins: Insights and challenges	277
	Emil Sjulstok, Ilia A. Solov'yov, and Peter L. Freddolino	
	1. Introduction	278
	2. Quantum mechanical methods	280
	3. QM/MM approximation	284
	4. Atomistic molecular dynamics simulations	291
	5. Coarse-grained and continuum methods	303
	6. Concluding remarks	308
	Acknowledgments	308
	References	309
12.	Exploring the sequence, function, and evolutionary space of protein superfamilies using sequence similarity networks and phylogenetic reconstructions	315
	Janine N. Copp, Dave W. Anderson, Eyal Akiva, Patricia C. Babbitt, and Nobuhiko Tokuriki	
	1. Introduction	316
	2. Curating a sequence dataset	319
	3. Calculation and visualization of sequence similarity relationships	324
	4. Defining appropriate SSN thresholds	329
	5. Generation of "higher resolution" subgroup-specific SSNs	329
	<b>6.</b> Advanced generation and analysis of SSNs using expanded sequence sets	331
	7. Using SSNs to streamline phylogenetic analyses	333
	8. Advanced analysis of evolutionary relationships across a superfamily	339
	9. Discussion	340
	References	343
13.	Structural methods for probing the interaction of flavoenzymes with dioxygen and its surrogates	349
	Raspudin Saleem-Batcha and Robin Teufel	
	1. Introduction	350
	2. Terminology of the interaction between $O_2$ and proteins	352
	<b>3.</b> Brief overview of strategies to study the interaction of O <sub>2</sub> with flavoenzymes	353
	4. O <sub>2</sub> -pressurized X-ray crystallography: Experimental setup	355
	5. O <sub>2</sub> -pressurized X-ray crystallography: Required controls and data analysis	358
	6. Summary and conclusion	361
	Acknowledgments	361
	References	362

14.	Reduction midpoint potentials of bifurcating electron transfer flavoproteins		
	AF. Miller, H.D. Duan, T.A. Varner, and N. Mohamed Raseek		
	1. Introduction to flavin-based bifurcation	366	
	2. Theory associated with measurement of E°s	372	
	3. Basic overview of a measurement	375	
	4. Protocol for E° determination using xanthine/xanthine oxidase to deliver		
	reducing equivalents	378	
	5. Ingredients of success: Tips	382	
	6. Controls and precautions	391	
	7. Concluding remarks	394	
	References	394	
15.	Investigations of two-component flavin-dependent		
	monooxygenase systems	399	
	John M. Robbins and Holly R. Ellis		
	1. Introduction	400	
	2. Expression and purification of the alkanesulfonate monooxygenase		
	enzymes	403	
	3. Steady-state kinetic analyses of the FMN-dependent monooxygenase		
	enzymes	405	
	4. Measuring the substrate affinity of the alkanesulfonate monooxygenase		
	enzymes	407	
	5. Evaluating protein-protein interactions	410	
	<b>6.</b> Monitoring flavin transfer between SsuE and SsuD	416	
	7. Conclusions and future directions	420	
	Acknowledgment	420	
	References	420	
16.	The styrene monooxygenase system	423	
	George T. Gassner		
	1. Introduction	424	
	2. Recovery of recombinant SMOs	426	
	3. Biochemical characterization of SMOs	426	
	4. Steady-state kinetic assay of two-component SMOs	436	
	5. Thermodynamic equilibrium properties of SMOs	439	
	6. Summary and conclusion	451	
	References	452	

17.	Flavin-N5-oxide intermediates in dibenzothiophene, uracil, and hexachlorobenzene catabolism	455
	Sanjoy Adak and Tadhg P. Begley	
	1. Introduction	456
	2. General protocols for enzyme overexpression and purification	456
	3. Analytical methods	458
	4. Chemical methods	459
	5. Enzyme studies	460
	Funding	466
	References	466
	Further reading	468
18.	Prenylated FMN: Biosynthesis, purification, and Fdc1	
	activation	469
	Anna N. Khusnutdinova, Johnny Xiao, Po-Hsiang Wang,	
	Khorcheska A. Batyrova, Robert Flick, Elizabeth A. Edwards, and	
	Alexander F. Yakunin	
	1. Introduction	470
	2. Biosynthesis of prFMN	473
	3. Extraction and quantitation of protein-free prFMN	480
	4. Activation of the ferulic acid decarboxylase Fdc1 by protein-free	
	and UbiX-bound prFMN cofactors	484
	5. Conclusions	486
	Acknowledgments	487
	References	487
19.	Heterologous production, reconstitution and EPR spectroscopic	
	analysis of prFMN dependent enzymes	489
	Stephen A. Marshall, Karl A.P. Payne, Karl Fisher, Deepankar Gahloth,	
	Samuel S. Bailey, Arune Balaikaite, Annica Saaret, Irina Gostimskaya,	
	Godwin Aleku, Huanming Huang, Stephen E.J. Rigby, David Procter, and	
	David Leys	
	1. Introduction	490
	2. In vivo production of holo-UbiD/apo-UbiD	492
	3. In vitro reconstitution of apo-UbiD using prFMN <sup>reduced</sup>	495
	4. In vitro oxidative maturation of prFMN containing UbiD	497
	5. Synthesis of DMAP	499
	6. In vitro reconstitution of apo-UbiD using prFMN <sup>iminium</sup>	501

-	0	n	te	m	tc

vi		
VI		

	7. EPR spectroscopy as a tool to study prFMN <sup>radical</sup> species	503
	8. Summary and conclusion	505
	Acknowledgments	507
	References	507
20.	Physical methods for studying flavoprotein photoreceptors	509
	Estella F. Yee, Siddarth Chandrasekaran, Changfan Lin, and Brian R. Crane	
	1. Introduction	510
	2. Recombinant expression and purification of flavoproteins	514
	3. Photoreduction/photoactivation of flavoprotein photoreceptors	518
	4. Electron paramagnetic resonance spectroscopy	524
	5. Measurement of flavoprotein redox potentials	531
	6. Protein crystallography	533
	7. Small-angle X-ray scattering	534
	Acknowledgments	536
	References	536