

# **Enzyme Structure and Mechanism**

*Second Edition*

ALAN FERSHT

# **Enzyme Structure and Mechanism**

*Second Edition*

**ALAN FERSHT**  
*Imperial College of Science  
and Technology, London*



**W. H. FREEMAN AND COMPANY**  
New York

Cover: Phosphofructokinase, R state, from *Bacillus stearothermophilus*. Structure determination by P. R. Evans and P. J. Hudson. Photo by Arthur M. Lesk, Medical Research Council, University Medical School, Cambridge.

Library of Congress Cataloging in Publication Data

Fersht, Alan.

Enzyme structure and mechanism.

Includes bibliographical references and index

1. Enzymes. I. Title. [DNLM: 1. Enzymes. QU 135

F399e]

QP601.F42 1984 574.19'25 84-4172

ISBN 0-7167-1614-3

ISBN 0-7167-1615-1 (pbk.)

Copyright © 1977, 1985 by W. H. Freeman and Company

No part of this book may be reproduced by any mechanical, photographic, or electronic process, or in the form of a phonographic recording, nor may it be stored in a retrieval system, transmitted, or otherwise copied for public or private use, without the written permission of the publisher.

Printed in the United States of America

1 2 3 4 5 6 7 8 9 0 MP 2 1 0 8 9 8 7 6 5 4

## Preface

We are now entering a new golden age of enzymology. Just as x-ray protein crystallography transformed the 1960s and 1970s, so recombinant DNA technology is changing the whole perspective in the 1980s. The cloning of enzyme genes for overproduction has facilitated the study of well-known enzymes and has enabled previously inaccessible enzymes to be characterized. Even more exciting are the prospects opened up by our ability to tailor the structure and activity of enzymes by manipulation of their genes. Specific amino acid residues may be changed by site-directed mutagenesis. Even whole genomes may be synthesized and their gene products expressed.

The second edition contains a chapter on protein engineering (Chapter 14) to summarize these developments. Two further new chapters (8 and 9) have been added to cover the recent advances in stereochemical methods and the continued progress in the design of highly specific irreversible inhibitors. Elsewhere, the book has been generally brought up to date, and certain chapters have been reorganized. Chapter 1, in particular, has been expanded to include discussion of the dynamic aspects of protein structure and recent ideas on protein evolution. Also, the chapter on allosteric proteins (Chapter 10) has been expanded to include specific examples of enzymes that regulate metabolic pathways.

I am indebted to several colleagues for their useful comments, especially those of Sir John Cornforth on Chapter 8.

A. F.  
London, 1984

## Preface to the First Edition

During the past two decades the advances in x-ray crystallography, transient kinetics, and the study of chemical catalysis have revolutionized our ideas on enzyme catalysis and mechanism. It is the intention of this text to provide a brief account of these developments for senior undergraduate students and postgraduates who have attended courses in chemistry and biochemistry. The philosophical and theoretical aspects of this book center upon how the interactions of an enzyme with its substrates lead to enzyme catalysis and specificity, and upon the relationship between structure and mechanism. The experimental approaches emphasized are those involving the direct study of enzymes as molecules. As such, there is a strong emphasis on pre-steady state kinetics where enzymes are handled in substrate quantities and enzyme-bound intermediates observed directly. The steady state kinetics of multisubstrate enzymes and the detailed chemistry of coenzymes and cofactors are discussed only in a cursory manner.

There have been two guiding rules in the preparation of this book. The first is to discuss general principles and ideas using specific enzymes as examples. [Although to avoid overloading the more theoretical chapters on kinetics, most of the illustrative examples are presented in a separate chapter (7).] The second is to stick closely to examples where hard evidence is available and to avoid speculation and woolly evidence. In consequence, the discussion of detailed chemical mechanisms is generally restricted to enzymes whose tertiary structures have been solved by x-ray crystallography. Similarly, the discussion of the theoretical aspects of allosteric proteins is very much restricted to hemoglobin because it is the only example where good (or any) evidence is available on the nature of the interactions of positive cooperativity.

The references cited tend to be those of the most recent reviews or papers where more extensive bibliographies are given, and also those of the original papers in order to maintain a historical perspective. Illustrative examples have been taken where possible from the files of the MRC Laboratory of Molecular Biology because of their ready availability and uniform quality of presentation. In this context I must thank Annette Lenton both for the illustrations she has prepared especially for this book and also for those prepared for other members of the laboratory whose files I have shamelessly raided.

I am particularly indebted to W. P. Jencks, H. B. F. Dixon, H. Gutfreund, K. F. Tipton, and R. S. Mulvey for their critical comments on the entire manuscript, and also M. F. Perutz and D. M. Blow for their comments on individual chapters. I wish to thank The Royal Society, the American Chemical Society, the Cornell University Press, Academic Press, John Wiley, and Alan R. Liss for permission to reproduce illustrations.

A. F.  
Cambridge 1977

# Contents

1. THE THREE-DIMENSIONAL STRUCTURE OF ENZYMES	1
A. The primary structure of proteins	2
B. The three-dimensional structure of enzymes	5
1. X-ray diffraction methods	5
2. The structural building blocks	7
3. Assembly of proteins from the building blocks	12
4. Prediction of three-dimensional structure from primary structure	15
C. Enzyme diversity	17
1. Divergent evolution of families of enzymes	17
2. Convergent evolution	22
3. Convergence or divergence?	22
4. Dehydrogenases and domains	23
5. Evolution of proteins by fusion of gene fragments?	23
D. The structure of enzyme-substrate complexes	25
1. Methods for examining enzyme-substrate complexes	26
2. Example 1: The serine proteases	27
3. Example 2: Lysozyme	30
E. Flexibility and conformational mobility of proteins	32
1. Are the crystal and solution structures of an enzyme essentially identical?	32
2. Modes of motion and flexibility observed in proteins	33
F. Higher levels of organization: Multienzyme complexes	39
1. Double-headed enzymes and the noncovalent association of different activities	39
2. The pyruvate dehydrogenase complex	40
	xi

3. Reasons for multiple activities and multienzyme complexes	41
2. CHEMICAL CATALYSIS	47
A. Transition state theory	47
1. The significance and the application of transition state theory	49
2. The Hammond postulate	50
B. Principles of catalysis	51
1. Where, why, and how catalysis is required	51
2. General-acid-base catalysis	53
3. Intramolecular catalysis: The "effective concentration" of a group on an enzyme	56
4. Entropy: The theoretical basis of intramolecular catalysis and effective concentration	59
5. "Orbital steering"	63
6. Electrostatic catalysis	64
7. Metal ion catalysis	67
C. Covalent catalysis	69
1. Electrophilic catalysis by Schiff base formation	69
2. Pyridoxal phosphate—Electrophilic catalysis	72
3. Thiamine pyrophosphate—Electrophilic catalysis	75
4. Nucleophilic catalysis	77
5. A summary of factors responsible for enzyme catalysis	77
D. Structure-reactivity relationships	78
1. Nucleophilic attack at the carbonyl group	79
2. Factors determining nucleophilicity and leaving group ability	83
3. Application of linear free energy relationships to enzyme reactions	87
E. The principle of microscopic reversibility or detailed balance	89
F. The principle of kinetic equivalence	90
G. Kinetic isotope effects	91
1. Primary isotope effects	92
2. Secondary isotope effects	94
3. Solvent isotope effects	94

3. THE BASIC EQUATIONS OF ENZYME KINETICS	98
A. Steady state kinetics	98
1. The experimental basis: The Michaelis-Menten equation	98
2. Interpretation of the kinetic phenomena for single-substrate reactions: The Michaelis-Menten mechanism	99
3. Extensions and modifications of the Michaelis-Menten mechanism	101
B. The significance of the Michaelis-Menten parameters	103
1. The meaning of $k_{\text{cat}}$ : The catalytic constant	103
2. The meaning of $K_M$ : Real and apparent equilibrium constants	104
3. The meaning of $k_{\text{cat}}/K_M$ : The specificity constant	105
C. Graphical representation of data	106
D. Inhibition	107
1. Competitive inhibition	107
2. Noncompetitive, uncompetitive, and mixed inhibition	109
E. Nonproductive binding	109
F. $k_{\text{cat}}/K_M = k_2/K_S$	111
G. Competing substrates	111
1. An alternative formulation of the Michaelis-Menten equation	111
2. Specificity for competing substrates	112
H. Reversibility: The Haldane equation	112
1. Equilibria in solution	112
2. Equilibria on the enzyme surface	113
I. Breakdown of the Michaelis-Menten equation	114
J. Multisubstrate systems	114
1. The random sequential mechanism	115
2. The ordered mechanism	115
3. The Theorell-Chance mechanism	115
4. The ping-pong (or substituted-enzyme or double-displacement) mechanism	115
K. Useful kinetic shortcuts	117
1. Calculation of net rate constants	117
2. Use of transit times instead of rate constants	118



4. MEASUREMENT AND MAGNITUDE OF ENZYMIC RATE CONSTANTS	121
Part 1 Methods for measurement: An introduction to pre-steady state kinetics	121
A. Rapid mixing and sampling techniques	122
1. The continuous-flow method	122
2. The stopped-flow method	122
3. Rapid quenching techniques	123
B. Flash photolysis	125
C. Relaxation methods	126
1. Temperature jump	126
2. Nuclear magnetic resonance	126
D. Analysis of pre-steady state and relaxation kinetics	128
1. Simple exponentials	128
2. Association of enzyme and substrate	131
3. Consecutive reactions	133
4. Parallel reactions	137
5. Derivation of equations for temperature jump	138
6. A general solution of two-step consecutive reversible reactions	139
7. Experimental application of pre-steady state kinetics	141
E. The absolute concentration of enzymes	143
1. Active-site titration and the magnitudes of "bursts"	143
2. The dependence of burst on substrate concentration	145
3. Active-site titration vs. rate assay	146
Part 2 The magnitude of rate constants for enzymatic processes	147
A. Upper limits on rate constants	147
1. Association and dissociation	147
2. Chemical processes	148
3. Proton transfers	148
B. Enzymatic rate constants and rate-determining processes	150
1. Association of enzymes and substrates	150
2. Association can be rate-determining for $k_{\text{cat}}/K_M$	152
3. Dissociation of enzyme-substrate and enzyme-product complexes	153
4. Enzyme-product release can be rate-determining for $k_{\text{cat}}$	153
5. Conformational changes	153

5. THE pH DEPENDENCE OF ENZYME CATALYSIS	155
A. Ionization of simple acids and bases: The basic equations	155
1. Extraction of $pK_a$ 's by inspection of equations	158
B. The effect of ionizations of groups in enzymes on kinetics	159
1. The simple theory: The Michaelis-Menten mechanism	159
2. The pH dependence of $k_{cat}$ , $k_{cat}/K_M$ , $K_M$ , and $1/K_M$	160
3. A simple rule for the prediction and assignment of $pK_a$ 's	161
C. Modifications and breakdown of the simple theory	162
1. Modifications due to additional intermediates	162
2. Breakdown of the simple rules: Briggs-Haldane kinetics and change of rate-determining step with pH: Kinetic $pK_a$ 's	163
3. An experimental distinction between kinetic and equilibrium $pK_a$ 's	164
4. Microscopic and macroscopic $pK_a$ 's	165
D. The influence of surface charge on $pK_a$ 's of groups in enzymes	165
E. Graphical representation of data	166
F. Illustrative examples and experimental evidence	167
1. The $pK_a$ of the active site of chymotrypsin	168
G. Direct titration of groups in enzymes	170
1. The effect of $D_2O$ on pH/pD and $pK_a$ 's	170
2. Methods	170
H. The effect of temperature, polarity of solvent, and ionic strength on $pK_a$ 's of groups in enzymes and in solution	172
I. Highly perturbed $pK_a$ 's in enzymes	174
6. PRACTICAL KINETICS	176
A. Kinetic methods	176
1. Spectrophotometry	176
2. Spectrofluorimetry	177
3. Automated spectrophotometric and spectrofluorimetric procedures	178
4. Coupled assays	179
5. Automatic titration of acid or base	180
6. Radioactive procedures	180

B. Plotting kinetic data	182
1. Exponentials	182
2. Second-order reactions	183
3. Michaelis-Menten kinetics	185
C. Determination of enzyme-ligand dissociation constants	185
1. Kinetics	185
2. Equilibrium dialysis	185
3. Equilibrium gel filtration	186
4. Ultracentrifugation	188
5. Filter assays	189
6. Spectroscopic methods	190
7. Titration procedures	190
D. Plotting binding data	190
1. The single binding site	190
2. Multiple binding sites	191
Appendix: Two convenient scintillants	191
1. BBOT	191
2. PPO/POPOP	192
 7. DETECTION OF INTERMEDIATES IN REACTIONS BY KINETICS	 193
A. Pre-steady state vs. steady state kinetics	193
1. Detection of intermediates: What is "proof"?	194
B. Chymotrypsin: Detection of intermediates by stopped-flow spectrophotometry, steady state kinetics, and product partitioning	195
1. Detection of intermediates from a "burst" of product release	195
2. Proof of formation of an intermediate from pre-steady state kinetics under single-turnover conditions	196
3. Detection of the acylenzyme in the hydrolysis of esters by steady state kinetics and partitioning experiments	200
4. Detection of the acylenzyme in the hydrolysis of amides and peptides	205
5. The validity of partitioning experiments and some possible experimental errors	207
C. Further examples of detection of intermediates by partition and kinetic experiments	208
1. Alkaline phosphates	208
2. Acid phosphatase	209
3. $\beta$ -Galactosidase	209

D. Aminoacyl-tRNA synthetases: Detection of intermediates by quenched flow, steady state kinetics, and isotope exchange	212
1. The reaction mechanism	212
2. The editing mechanism	214
E. Detection of conformational changes	218
 8. STEREOCHEMISTRY OF ENZYMATIC REACTIONS	 221
A. Optical activity and chirality	221
1. Notation	222
2. Differences between the stereochemistries of enzymatic and nonenzymatic reactions	223
3. Conformation and configuration	225
B. Examples of stereospecific enzymatic reactions	225
1. NAD <sup>+</sup> - and NADP <sup>+</sup> -dependent oxidation and reduction	225
2. Stereochemistry of the fumarase-catalyzed hydration of fumarate	227
3. Demonstration that the enediol intermediate in aldose-ketose isomerase reactions is syn	227
4. Use of locked substrates to determine the anomeric specificity of phosphofructokinase	228
C. Detection of intermediates from retention or inversion of configuration at chiral centers	230
1. Stereochemistry of nucleophilic reactions	230
2. The validity of stereochemical arguments	231
3. Intermediates in reactions of lysozyme and $\beta$ -galactosidase	231
D. The chiral methyl group	232
1. The fundamental difference between generating a chiral methyl group from a methylene group and converting a chiral methyl group into methylene	232
2. The chirality assay	233
3. Stereochemistry of the malate synthase reaction	234
E. Chiral phosphate	235
1. A preview of phosphoryl transfer chemistry	236
2. Chirality of phosphoryl derivatives	237
3. Examples of chiral phosphoryl transfer	239
4. Positional isotope exchange	242
5. A summary of the stereochemistry of enzymatic phosphoryl transfers	243

F. Stereoelectronic control of enzymatic reactions	243
1. Pyridoxal phosphate reactivity	244
2. Stereoelectronic effects in reactions of proteases	245
 9. ACTIVE-SITE-DIRECTED AND ENZYME-ACTIVATED IRREVERSIBLE INHIBITORS: "AFFINITY LABELS" AND "SUICIDE INHIBITORS"	 248
A. Chemical modification of proteins	248
1. The chemical reactivity of amino acid side chains	251
B. Active-site-directed irreversible inhibitors	252
C. Enzyme-activated irreversible inhibitors	256
1. Pyridoxal phosphate-linked enzymes	259
2. Monoamine oxidases and flavoproteins	260
 10. COOPERATIVE LIGAND BINDING, ALLOSTERIC INTERACTIONS, AND REGULATION	 263
A. Positive cooperativity	263
B. Mechanisms of allosteric interactions and cooperativity	265
1. The Monod-Wyman-Changeux (MWC) concerted mechanism	265
2. The Koshland-Némethy-Filmer (KNF) sequential model	269
3. The general model	270
C. Negative cooperativity and half-of-the-sites reactivity	271
D. Quantitative analysis of cooperativity	272
1. The Hill equation: A measure of cooperativity	272
2. The MWC binding curve	273
3. The KNF binding curve	277
4. Diagnostic tests for cooperativity, and MWC vs. KNF mechanisms	277
E. Molecular mechanism of cooperative binding to hemoglobin	278
1. The physiological importance of the cooperative binding of oxygen	278
2. Atomic events in the oxygenation of hemoglobin	278
3. Chemical models of hemes	281
F. Regulation of metabolic pathways	283
G. Phosphofructokinase and control of glycolysis	283
1. The structure of the R state	286

H. Glycogen phosphorylase and regulation of glycogenolysis	288
1. The structure of phosphorylase	290
11. FORCES BETWEEN MOLECULES, AND ENZYME-SUBSTRATE BINDING ENERGIES	293
A. Interactions between nonbonded atoms	293
1. Electrostatic interactions	293
2. Nonpolar interactions (van der Waals or dispersion forces)	294
3. The hydrogen bond	296
4. The hydrophobic bond	299
B. The binding energies of enzymes and substrates	301
1. Estimation of increments in binding energy from kinetics	302
2. Why are enzymes more hydrophobic than organic solvents?	306
3. Summary	307
C. Entropy and binding	307
D. Protein-protein interactions	308
12. ENZYME-SUBSTRATE COMPLEMENTARITY AND THE USE OF BINDING ENERGY IN CATALYSIS	311
A. Utilization of enzyme-substrate binding energy in catalysis	311
1. Binding energy lowers the activation energy of $k_{\text{cat}}/K_M$	311
2. Interconversion of binding and chemical activation energies	312
3. Enzyme complementarity to transition state implies that $k_{\text{cat}}/K_M$ is at a maximum	316
B. Experimental evidence for the utilization of binding energy in catalysis and enzyme-transition state complementarity	317
1. Classic experiments: Structure-activity relationships of modified substrates	317
2. Transition state analogues: Probes of complementarity	319
3. The modern approach: Structure-activity experiments with modified enzymes	323
C. Evolution of the maximum rate: Strong binding of the transition state and weak binding of the substrate	324
1. The principle of maximization of $K_M$ at constant $k_{\text{cat}}/K_M$	325
2. Experimental observations on $K_M$ 's	327
3. The perfectly evolved enzyme for maximum rate	330

D. Molecular mechanisms for the utilization of binding energy	331
1. Strain	331
2. Induced fit	331
3. Nonproductive binding	333
4. The unimportance of strain, induced fit, and nonproductive binding in specificity	334
5. Experimental evidence concerning the existence and the nature of strain and induced-fit processes	334
6. Conclusions about the nature of strain: Strain or stress?	341
7. Strain vs. induced fit vs. nonproductive binding	342
E. Effects of rate optimization on accumulation of intermediates and internal equilibria in enzymes	343
1. Accumulation of intermediates	343
2. Balanced internal equilibria	344
13. SPECIFICITY AND EDITING MECHANISMS	347
A. Limits on specificity	348
1. Michaelis-Menten kinetics	350
2. The general case	351
3. Interacting active sites	352
4. The stereochemical origin of specificity	353
B. Editing or proofreading mechanisms	354
1. Editing in protein synthesis	355
2. Editing in DNA replication	358
C. The cost of accuracy	363
1. The cost-selectivity equation for editing mechanisms	363
2. Single-feature recognition: $f = f'f''$	365
3. Double-feature recognition: $f'f'' > f$	367
14. GENETIC ENGINEERING AND ENZYMOLOGY: PROTEIN ENGINEERING	369
A. The structure and properties of DNA	370
1. DNA may be replicated: DNA polymerases	372
2. Gaps in DNA may be sealed: DNA ligases	374
3. Duplex DNA may be cleaved at specific sequences: Restriction endonucleases	374
4. DNA fragments may be joined by using enzymes	375
5. Joining DNA by complementary homopolymeric tails: Terminal transferase	377

6. Processive vs. distributive polymerization	377
B. Cloning enzyme genes for overproduction	378
1. Vectors	379
2. Screening	380
3. Examples	381
C. Site-directed mutagenesis	382
1. Oligodeoxynucleotide-directed mutagenesis	382
15. STRUCTURES AND MECHANISMS OF SELECTED ENZYMES	389
A. The dehydrogenases	390
1. The alcohol dehydrogenases	392
2. L-Lactate dehydrogenase	397
3. Malate dehydrogenase	400
4. Glyceraldehyde 3-phosphate dehydrogenase	400
5. Some generalizations about dehydrogenases	404
B. The proteases	405
1. The serine proteases	405
2. The thiol proteases	413
3. The zinc proteases	416
4. The carboxyl (aspartyl) proteases	422
C. Ribonuclease	426
1. The structure of the enzyme and the enzyme-substrate complexes	428
D. Staphylococcal nuclease	432
E. Lysozyme	433
1. The carbonium ion	434
2. Electrostatic and general-acid catalysis	434
3. Binding energies of the subsites	435
F. Carbonic anhydrase	436
G. Triosephosphate isomerase	439
1. Deuterium and tritium tracer experiments and the mechanism of aldose-ketose isomerases	440
2. The structure of the enzyme-substrate complex	442
H. Epilogue	443
AUTHOR INDEX	453
SUBJECT INDEX	467



## The three-dimensional structure of enzymes

In 1930, J. B. S. Haldane wrote a book on enzymes that is still worth reading today.<sup>1</sup> The most striking feature of this book is that so much was then known about the properties and action of enzymes, yet so little was known about the enzymes themselves: the question of whether or not enzymes are proteins was still the subject of raging controversy. The knowledge was so one-sided because there was no means of studying enzymes directly. All the information had been deduced indirectly from the effects of enzymes on their substrates. Nevertheless, the foundations of modern steady state kinetics had been laid in a little over thirty years, the first cell-free enzyme extract having been prepared by E. Büchner in 1897.

In order to proceed further, it was necessary to isolate purified enzymes in *substrate* quantities and examine them directly. This was accomplished in 1926, when J. B. Sumner crystallized urease from jack bean extracts. Soon afterwards (1930–36), J. H. Northrop and M. Kunitz crystallized pepsin, trypsin, and chymotrypsin. This provided the material to prove finally that enzymes are proteins, and to allow the development of the techniques of modern protein chemistry: the sequencing of proteins pioneered by F. Sanger; the solution of the three-dimensional structure of proteins pioneered by M. F. Perutz and J. C. Kendrew; and the use of rapid-reaction kinetics, which had been initiated by F. J. W. Roughton in 1923.

The major part of the present book deals with the direct study of enzymes in substrate quantities, taking up the story from where Haldane was forced to stop. This first chapter discusses the general features of the most significant advance in our knowledge of enzymes since then—their three-dimensional structure, from the basics of the peptide bond and the various elements of protein folding to macromolecular assemblies. The chapter also describes the evolution of protein structure and function, and considers the dynamic aspects of proteins. To set the scene for later