ENZYMOLOGY IN THE PRACTICE OF LABORATORY MEDICINE

EDITED BY
Philip Blume
Esther F. Freier

ACADEMIC PRESS, INC.
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ACADEMIC PRESS RAPID MANUSCRIPT REPRODUCTION

Proceedings of a Continuation Course
held at the University of Minnesota, Minneapolis, Minnesota,
10-12 May 1972.

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ACADEMIC PRESS, INC. New York and London
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ACADEMIC PRESS, INC.
111 Fifth Avenue, New York, New York 10003

United Kingdom Edition published by
ACADEMIC PRESS, INC. (LONDON) LTD.
24/28 Oval Road, London NW1

Library of Congress Cataloging in Publication Data

Blume, Philip.
Enzymology in the practice of laboratory medicine.

"Proceedings of a continuation course held at the University of Minnesota, Minneapolis, Minnesota, 10-12 May 1972."
1. Clinical enzymology–Congresses. I. Freier, Esther F., joint author. II. Title. [DNLM: 1. Diagnosis, Laboratory–Congresses. 2. Enzyme tests–Congresses. 3. Enzymes Analysis–Congresses. 4. Enzymes –Congresses. QU135 E65 1972]
RB48.B55 616.07 74-613
ISBN 0–12–107950–3

PRINTED IN THE UNITED STATES OF AMERICA

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PREFACE

Within the emerging field which we call laboratory medicine the study of enzymes constitutes a major area of endeavor. We want to know how enzymes function, how we may best determine their presence and activity, and how to interpret the measurements we make when we are confronted with the problem of clinical diagnosis. As we planned the program of the continuation course from which this book arose, it was our intent to focus upon each of these diverse areas.

Laboratory medicine is a discipline whose basic goals deal with the understanding of disease processes. Since enzymatic reactions are at the foundation of all living systems, it is important to enhance our understanding of these reactions. The first section of this book deals, therefore, with topics related to an understanding of enzymes at the most fundamental level.

From a somewhat more pragmatic point of view, it has been found that the measurement of enzyme activities is of great value in the diagnosis and management of disease states. Since one of the contributions that laboratory medicine makes to the practice of medicine is the acquisition of accurate and reliable analytical data, it is natural that we should concern ourselves with the problems of fulfilling that role in the best possible way. The ability to obtain analytical data of superior quality will result in greater understanding of disease states and an enhanced ability of the laboratory to contribute to the overall diagnostic process. Analytical methodology thus constitutes the second section of this book.

After dealing with the topics of basic molecular biology and with the applied technology of the analytical laboratory we come to what is, in a very real way, the intersection of these two areas; the study of the clinical significance of the data obtained in the laboratory. Among the subjects dealt with are the various factors that result in the presence of enzymes in the serum at fairly predictable levels and some of the conditions that result from congential abnormalities of enzyme production. In addition, this final section of the book deals with the interpretation of multiple laboratory analyses in various disease conditions, ultimately using techniques that employ mathematical analyses of multiple variables.

By and large, each chapter follows the presentation made at the continuation course which was held from the 10th to the 12th of May 1972 at the

University of Minnesota. With the passage of time, the authors were given an opportunity to modify their presentations to a greater or lesser extent as they were prepared for publication.

One could not suggest that this book is in any way a comprehensive treatment of the rather imposing topic suggested by its title. In covering subjects ranging from the most fundamental considerations of the mechanisms of enzymatic catalysis to the observations of abnormalities of serum enzyme activity in acutely psychotic individuals, however, it was our intent to suggest to those working in the field of laboratory medicine what vast areas of research exist to be drawn from; and to indicate to those working outside the clinical laboratory, how much their efforts can contribute to laboratory medicine.

MOLECULAR BIOLOGY

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SOME RECENT DEVELOPMENTS IN THE SEARCH FOR MECHANISMS OF ENZYMIC CATALYSIS*

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1. Introduction

The mechanisms of enzymic catalysis remain an enigma not only in the sense that we do not known which of the many suggested mechanisms actually apply but also in the sense that there are such fundamental weaknesses in our understanding of globular proteins as a state of matter that important mechanisms may not yet have been suggested. Just what constitutes understanding of an enzymic process varies from investigator to investigator depending on the orientational biases provided by his previous training and experience. However, it is doubtful that ball and stick descriptions will prove satisfying to many. In a sense the general level of attack on the reaction mechanisms of much simpler molecules has become too sophisticated to make pictorial descriptions satisfying. Of more fundamental importance is the fact that many of the concepts essential even in qualitative discussions of proteins are thermodynamic in nature. Ultimately many of these will be put on the sounder basis of potential functions and statistical mechanics but the time when we can trust these very difficult approaches seems far away. Uses of small-molecule models has, if anything, become less and less satisfactory as problems of protein stability and conformational dy-

*This work was supported by the National Science Foundation, Grant No. GB25795, and the National Institutes of Health through Grants HL 13109 and AM05853. This is paper number 82 from this Laboratory. Please request reprint by this number.

namics are revealed at more subtle levels by improved experiments. It seems probable that for some time to come we must rely heavily on comparisons of such model behavior with protein behavior to provide at least qualitative insight but this is a treacherous undertaking. For example, using small-molecule or polymer models it is possible to argue that hydrogen bonding of the unfolded polypeptide to water confers stability, instability or has a negligible effect. The situation is a bit better with the interaction usually called "the hydrophobic bond" but there may be serious errors in this concept as a result of the use of oily small molecules as models. Kauzmann[1] pointed out that the low solubility of hydrocarbons in water relative to solution in an organic solvent is due to a large negative entropy change. The enthalpy change is favorable for solubility in water and has been attributed to improved hydrogen bonds, increased numbers of hydrogen bonds or van der Waals interactions of the solutes with the walls of the water cavities which enclose them. If this interpretation is correct, emphasis should not be placed on van der Waals interactions among non-polar groups in puddles, patches and layers within the folded protein but in fact this interpretation is becoming increasingly common as often explicitly as implicitly. For example, the basis for the success of the type of correlation of biological activity with solubility originated by Hansch[2] and very generally applied; is implicitly attributed by most users to the van der Waals interaction among non-polar groups. Similarly x-ray studies have shown that globular proteins contain puddles and layers of non-polar side-chains. These have become known as "cores" and often have been assumed to confer special stability and rigidity on the protein conformation. The nondirectional nature of van der Waals dispersion forces and the relative weakness of these forces between small molecules suggests that these regions of hydrophobic groups should be the weakest and the least rigid of the protein[3] except, of course, for the charged groups at the surface which have long tethers and considerable freedom to move in the water-protein interface. Kuntz[3] on examination of these problems in terms of the geometries of the oily regions in carboxypeptidase has favored the "weak" concept but he calculates an effective density of 0.93 g/cm which is greater than that of benzene and considerably higher than the

density of non-aromatic hydrocarbons such as appear as protein side-chains. These ideas are inconsistent but what we might call the "van der Waals" view of the "hydrophobic bond", at least as applied to the folding of proteins is often attractive as it is in the applications of the Hansch solubility method. We should begin to suspect that the use of small molecules as models for proteins fail here and that perhaps the van der Waals interactions in such regions have properties not yet observed in models. The approach of Sinanoglu emphasizes van der Waals interactions somewhat more.[4]

A fundamental source of difficulty in assessing the importance of the factors responsible for the stability of folded proteins is the large number of such factors no one of which at the present time can be said to dominate the others. Each factor makes a small contribution so that small errors in estimates of the contributions from a single group becomes greatly exaggerated when the contributions are summed over the entire protein. The factors include "hydrophobic bonding"; hydrogen bonding relative to hydrogen bonding of the protein polar groups with water; hindered rotation; dipole-dipole interactons primarily between peptide groups; -S-S- bonds; van der Waals interactions; dielectric-constant effects responsible among other things for changes in force constants and thus a major problem in estimating whole-protein entropies since these contain major contributions from the weak vibrations; interactions of unfolded and folded polypeptide with bulk water; interfacial free-energy contributions which bear not only on the nature of the solvation of non-polar surface regions, often numerous, but also on the electrostatic interaction among charge residues and charged residues and the ion atmosphere; ion-pairing at surfaces and within proteins; orbital-overlap effects between π-electron systems and between sigma orbitals of groups pushed to less than normal van der Waals contact distances; local dielectric inhomogeneity due to fixed geometries; and the conversion of hindered or nearly free rotational motions to torsional oscillations. Others could be mentioned but this list is sufficient to illuminate the complexity of the total problem. Even the assessment of the charcteristics which are the result of all these factors is far from satisfactory and would be

much assisted if there were good data on mechanical behavior of proteins as functions of pressure, temperature, solvent composition, charge state and so on. Recently it has become clear that such information, despite the difficulty of the experiments required and their current unpopularity, is essential to make even qualitative decisions about the relative importance of proposals of specific mechanims. For example, there has been a tendency to treat protein interiors as possessing more liquid-like than solid-like character so that models based on the transfer of substrates and inhibitors from liquid solution to water have been much used in describing protein unfolding. However, if, as Page and Jencks[5-7] and Klapper[8] have suggested, the protein regions where specific binding occurs are more like solids, these models are inappropriate. We then require a solid rather than a solution model and must think of the small molecules specifically bound by a protein as having highly restricted motion such as obtains in crystals. Since the updating of all those mechanisms called "propinquity" and "approximation" by Jencks[9] is based on this restricted motion, it has become one of the most important of the new developments in enzyme mechanisms, it is desirable to examine the few facts we have about the physical nature of globular proteins.

2. Physical Nature of Globular Proteins: In Table I are given the available data on compressibility, β, and thermal expansion coefficients, α, for globular proteins. Only one value for β exists, that one estimated as an upper limit by Brandts et al.[10] from the data of Fahey, Kupke and Beams.[11] This value, an upper limit, would appear to be experimentally reliable but its implications are not at all easy to digest and much more work in this area is required. The compressibility is a particularly important parameter for the protein systems since at constant T, $\beta^{-1} = -\left(\frac{\partial P}{\partial V}\right)_T$, and is thus a measure of the rigidity, tightness of packing and free volume. For comparison we find that the sodium chloride crystals have a β value of 4.2×10^{-6} atm^{-1} at 30^{o}.[12] These are strong solids so that the similarity of their β values to that of ribonuclease A, even though RNase may not prove to be typical, indicates very tight packing in which such holes as they exist must be strongly supported by neighboring

Table I

Physical Constants for Proteins in Water Solution

COMPRESSIBILITY $-\frac{1}{V}(\frac{\partial V}{\partial P})_T$

β: ribonuclease A (water solution 25°) $< 5 \times 10^{-6}$ atm^{-1}

THERMAL EXPANSION COEFFICIENT $\frac{1}{V}(\frac{\partial V}{\partial T})_P$

α: Protein	t°C	$\alpha \times 10^{-3}$ deg^{-1}	Ref.
chymotrypsin	3.4	1.51	119
dimethionine sulfoxide chymotrypsin	3.6	1.12	119
denatured chymotrypsin	3.4	1.81	10
ribonuclease A	20	0.65	120
denatured RNase A	20	2.59	120
ribonuclease A	20	1.15	121
bovine plasma albumin	20	0.5	122
bovine mercap. albumin	20	0.5	122
human mercap. albumin	20	0.5	122
egg albumin	20	0.57	124

For $\beta = 5 \times 10^{-6}$ atm^{-1} and $\alpha = 5.7 \times 10^{-4}$ deg^{-1} the internal pressure is about 3×10^{4} atm.

regions. Studies, mostly indirect and usually inadequate to separate true protein volume from changes in the volume of nearby water due to protein-water interactions,[13] suggest changes of several percent in some specific binding reactions when analyzed in terms of protein volume changes only. Using the upper-limit β value for RNase A, and making the weak assumption that β does not depend on volume, the free-energy change in an expansion of 2 percent of the protein would require about 1000 kcal per mole of protein. This is an upper limit for expansion established by our approximation. The use of the β value is not in error insofar as it also was determined on a protein solution thus including water-protein interactions but if the true value of β is actually smaller, the free-energy estimate is of the right order of magnitude for expansion but likely to be considerably larger for compression of 2 percent.[12] There is no reason to believe that the quantitative characteristics of a protien are uniform but any poorly folded regions such as exist behind the hemo plane in myoglobin[13] or may well exist around catalytic sites, will tend to increase the compressibility. More precise values of β under varying experimental conditions would be well worth the considerable difficulty associated with their measurement.

A seemingly different and paradoxical picture is presented by the thermal expansion coefficient, α. The larger of the wide range of protein α values given in Table I would require that the heat capacity at constant volume for the dissolved proteins for which they were determined would be zero or negative if the β value for RNase has any accuracy and generality. As a consequence, we choose $\alpha = 5 \times 10^{-4}$ deg^{-1} as a reasonable value. Since $\alpha = \frac{1}{V}\left(\frac{\partial V}{\partial T}\right)_P = -\frac{1}{V}\left(\frac{\partial S}{\partial P}\right)_T$, it has less direct relationship to the free energy but is as meaningful as β in providing information about the physical nature of dissolved proteins. Weak liquids such as pentane (1.6×10^{-3} deg^{-1} at 20°) and ethyl ether (1.7×10^{-3} deg^{-1} at 20°) and soft solids like paraffin (5.9×10^{-4} deg^{-1} at 20°) have similar large values but tin (6.9×10^{-5} deg^{-1} at 20°), NaCl (1.2×10^{-4} at 50°) and even ice (1.1×10^{-4} at -7°) have much smaller values.[12] Water has a much lower value at ordinary temperatures which may be ratio-

nalized in terms of changes in structure favoring lower structural free volume with increasing temperature, a special situation not yet related in any direct way to protein behavior.

So far as we can determine these observations, which indicate through α that the entropy decrease on increasing pressure is large but the volume change through β is small with increasing pressure under constant-temperature conditions, make proteins a qualitatively different class of materials. The internal pressure calculated from the values of α and β we have used is not a very useful quantity but it is about ten-fold higher than the internal pressure of time or NaCl. In view of the values for the corresponding parameters of water it seems unlikely that the peculiar properties of soluble globular proteins are due to the compressive effect of liquid water on the protein. Ultimately it may be shown that the large thermal expansion is water-based but the available data are quite inconsistent with this interpretation. The latter is the least improbable of the conclusions we could make if forced to ascribe the unusual properties of proteins to the water around them. However, it appears more probable, though far from satisfying, that we should look for a cohesive force in the protein to support the low value of β and that we explain α in terms of high internal force constants, low deformability and low free volume such that a small increase in protein volume produces a large increase in the very low initial entropy. Exploration in this direction receives some support from a very illuminating discussion of the meaning of x-ray diffraction results on carboxypepetidase A given by Kuntz.[3]

Kuntz treated all non-backbone carbons as belonging to "hydrophobic-bonding" groups in his assessment of the importance of hydrogen-bonded networks and hydrophobe interaction on stability. He found in carboxypepetidase A a distinct tendency for hydrophobic carbons to be in large puddles and layers encased in a three-dimensional mosaic formed by backbone through hydrogen bonding to itself and to polar sidechains. Some non-polar regions extend to the protein-water interface and a few lie entirely at this interface. For the hydrophobic regions the estimate density is 0.93 g/ml, considerably higher than that of liquid or solid

hydrocarbon. His polar-region density is 1.55 g/ml giving a mean value of 1.39 g/ml may be a bit high for proteins dissolved in water because of the low density of interfacial water but Klapper[8] using scaled-particle theory has calculated that the density is considerably higher than can be expected for normal van der Waals' radii. It should be pointed out that the van der Waals radii as revised by Pauling and others often may be too short because the covalent component of atom interaction is larger than has been presumed heretofore.[14] What is noteworthy relative to the low value of β is the large difference in density between the two kinds of regions. We can only suggest that the low value of β, if general, is due in part to non-polar groups but primarily to the polar cage surrounded by the low dielectric constant regions which enhance the strength of electrostatic interactions. In a tightly packed protein orientation contributions to the dielectric constant are small and the electrostatic lines of force will tend to pass through liquid water regions as much as possible. Many hydrophobic regions undoubtedly are compressed as Klapper suggests, and thus deformable only to the extent that packing remains good, e.g., as by slipping motions and, oranges-in-a-sack rearrangements. Whether or not the thermal characteristic of the volume can be attributed to the oily regions is unclear but is more probably a result of cooperation of oily and polar groups. Kuntz suggests that slippage and random angular distortion is possible in the oily regions because of the weak angular dependence of dispersions interactions. In polar regions the highly directional nature of hydrogen-bonds and other strong dipole-dipole interactions must tend to limit structural modification. Both polar and non-polar regions are somewhat constrained by bond rotational potentials but in the polypeptides aside from the peptide bond itself rotational potentials are nearly second-order relative to polar interactions. Studies of the effects of bond rotations based on models having non-distortable peptide bonds will have to be reexamined on the basis of the finding by Dunitz et al. that the potential function is flat for these bonds in the intervals -6 to + 6°.[15] Hence the assumption of rigid planarity for peptide bonds is incorrect.

Enhancement of the polar interactions by restricting motions

of polar groups in their local environment and by high polarizability but low permanent polarization of oily regions in local environments undoubtedly can rigidify the structure, the effect increasing as one moves toward the middle of protein from its surface. This stiffening due to reduced dielectric constant does not improve the free energy situation since such a bootstrapping procedure would defy the first and second laws. We must nevertheless bear in mind that both enthalpy and entropy of the folded conformations will be lower as a result of the increased force constants and rotation potentials.

Additional confusion is produced by the low thermodynamic stability of the globular proteins which have been studied. Hemoglobin derives about -40 kcal/mole of free energy from the associations among the four subunits (chains)[16] but single-subunit proteins have maximum stabilities well below -20 kcal/mole in free energy relative to their unfolded states.[17-19] Extension of a good hydrogen bond by 0.4 A, better than the limit of precision in measuring distance between two hydrogen bonded atoms in the protein lattice with present resolution, reduces the stability by perhaps 3 kcal per mole of bonds. Bending also weakens hydrogen bonds though just how much is still a matter of dispute. These considerations suggest that hydrogen bonds must either be formed with best geometry nearly all the time or that there is a large additional source of stability which makes such detail unnecessary. The only apparent source of any large stability is the free-energy decrease when the oily groups move from water to their protein position. Kuntz[3] presents arguments suggesting the latter explanation and suggests that hydrogen-bonding in carboxypeptidase A is far from perfect. Kretsinger and Nockolds[20] find many broken H-bonds in their calcium-binding proteins. These are matters of some fundamental interest in relation to protein function since we shall have to examine swelling and shrinking ("breathing"), slippage, unfolding, folding and refolding and the existence of a distribution of conformational isomers as potentially important in function. A question of major relevance is whether low folded stability is essentially to function thus a result of evolution or is the maximum level of stability that nature has been able to achieve by the type