BIOCATALYSIS

Fundamentals of Enzyme Deactivation Kinetics

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AJIT SADANA
Chemical Engineering Department
University of Mississippi

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Preface

Enzyme deactivation is one of the constraints in the rapid development of biotechnological processes. This book provides a kinetic basis and framework for comparison of enzyme deactivations encountered in a wide variety of different areas. The book emphasizes the mathematical description of enzyme deactivations rather than the traditional chemical approach based on structure and function. The kinetic models presented, when applied to representative deactivation data from the biochemical, biomedical, pharmacological, and ontogenetic areas, among others, provide a better understanding of enzyme deactivations in general. This is within the present thrust of molecular biology—the rational interpretations of enzyme structure and function. The volume of data presented with the models provides refreshing physical insights into enzyme structure and function. The model and the data presented provide a neat framework within which workers from different areas can compare their often disparate findings. This would help in the development of guiding principles for the use of enzymes in biotechnology.

Modeling of complex enzyme deactivations is a difficult task. The more complex the deactivation, the greater the number of models that may be appropriate to fit selected data. An overall strategy is required in the selection and application of enzyme deactivation models to experimental data. The book presents strategies that will contribute toward a better utilization of enzymes and enzyme-catalyzed processes.

This text is primarily intended to provide graduate-level instruction on enzyme deactivation kinetics and its effect on biological processes in reactors as well as in the natural environment. The generalized data analysis and material presented will interest chemical, biochemical, and biomedical engineers; chemists; bio-

chemists; the medical profession; and scientists in general who wish to better understand enzyme structure, activity, and stability. With the ever-increasing importance of the multidisciplinary area of biotechnology, and the significant role that enzymes play in bioprocesses, the chapters on enzyme deactivation kinetics can form an effective nucleus even for an undergraduate biochemical course and should prove useful in a wide range of scientific disciplines.

The material in the original manuscript was used to teach a graduate course at the University of Mississippi. Student comments have been incorporated. I wish to thank Professor Octave Levenspiel for teaching me to seek and analyze the solutions to problems on a simple level. My graduate students contributed significantly to the development of the ideas presented. Ms. Judy Wood patiently typed the different versions of the manuscript. I do wish to acknowledge the help provided by the Engineering School and the Graduate School at the University of Mississippi. My parents, Jai and Jinder Sadana, and Krishna and Trilok Tondon deserve a lot of credit. Many thanks to my wife, Lopa and my two girls, Neeti and Richa for their understanding and patience.

Ajit Sadana

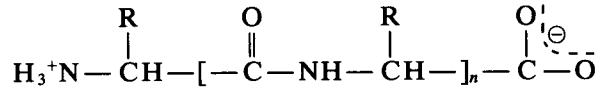
Contents

1

Introduction

1.1 PROTEIN STRUCTURE, FUNCTION, AND DYNAMICS

It is universally accepted that all enzymes are proteins. Since enzymes are proteins, it is worthwhile describing the general structure of proteins. In general, proteins are made of many amino acids linked together by an amide bond (the peptide bond) between the carboxyl group of one amino acid and the α-amino group of another. Consider, for example, the structure



Here R is an amino-acid side chain, and n may be between 40 and 75. Protein structural representation proceeds from the amino terminus at the left to the carboxyl terminus at the right.

The sequential arrangement of amino acids in a protein molecule is called the *primary structure* of the protein molecule. This primary structure may fold to yield the *native* (or active) structure of the protein molecule. The unfolding of the protein molecule leads, in general, to the inactive, or *denatured,* state. Denaturation involves the disruption of the normal conformation without rupture of the covalent main-chain bonds. In other words, denaturation involves a major change from the original native structure without alteration of the amino-acid sequence. Joly (1965) defined the denaturation of a protein as involving "modification of the secondary, tertiary, or quaternary structures of the protein molecule excluding any breaking of covalent bonds."

Two sets of noncovalent interactions are primarily responsible for the stability of the protein molecule in its folded state. The first is hydrogen bonding, which

takes place primarily between the proton on the α-amino nitrogen atom in the peptide bond and the oxygen atom of a carbonyl carbon. There may also be significant hydrogen bonding between side-chain proton donors and acceptors. In general, one H-bond of the type $R-C=O\cdots H-N$ will contribute about 800 cal/g-mol to the stabilization of a protein structure (Jencks, 1969). Some examples of hydrogen-bonded protein structures are the α-helix; the "pleated sheet," or β-structure; and a structure that is "random" in nature. The hydrogen-bonded structure—in any of these forms—is the protein's *secondary structure.* The α-helix involves the formation of hydrogen bonds between the carbonyl and imino groups of nonadjacent peptide links in the primary structure. Chothia (1984) indicates that the principle underlying the structure of helices, sheets, and turns is the simultaneous formation of hydrogen bonds by buried peptide groups and the retention of conformations close to those of minimum energy.

The second set of noncovalent interactions that are important in determining the three-dimensional structure of proteins includes the van der Waals attractions and hydrophobic bonds. A hydrophobic bond in a protein may be thought of as an attractive interaction between the apolar side chains of the protein's constituent amino acids. This interaction reflects the preference of these hydrocarbonlike groups for a medium of lower dielectric constant than water. Earlier protein structure studies by X-ray crystallography showed the nonpolar amino acids buried in the interior of the molecule and the majority of the polar amino acids on the surface exposed to the solvent. Recently, though, a more detailed quantitative examination of the amino acids on the protein surface indicated that this was an oversimplification. About one-half of the exposed surface groups may be apolar. This is in accord with the evidence that no single force or interaction is primarily responsible for determining the three-dimensional structure of proteins (Finney et al., 1980; Stellwagen, 1984).

Examples of the more polar side chains are lysine, arginine, glutamic acid, and aspartic acid. These may be found on the surface of the molecule. The less polar, hydrocarbonlike side chains such as valine, leucine, phenylalanine, and tryptophan are located in the interior of the molecule. This, of course, as indicated earlier, is a generalization, and exceptions do exist.

Figure 1.1 is a schematic diagram that shows the changes in interactions involved in a conformation change leading to partial unfolding (Gray, 1985). Here, "I" represents the ionic groups in the polypeptide, usually side-chain groups of aspartame, glutamic acid, lysine, arginine, and histidine. "B" represents the hydrophobic groups. "H" represents the hydrogen bonds. "S" represents the solvent molecules, most commonly water, that interact with the ionic or hydrophilic groups usually on the outer surface of the protein. "A" represents the groups which accept hydrogen in hydrogen bonds. Note that the interior of the folded protein molecule is not solvated by water and that electrostatic interactions, hydrogen bonds, and nonpolar forces participate in forming the folded, native form. The majority of the amino acids in the unfolded denatured protein are completely solvated by water.

Assume that an equilibrium exists between the active and the inactive forms of the enzyme:

$$E \rightleftharpoons E_d$$

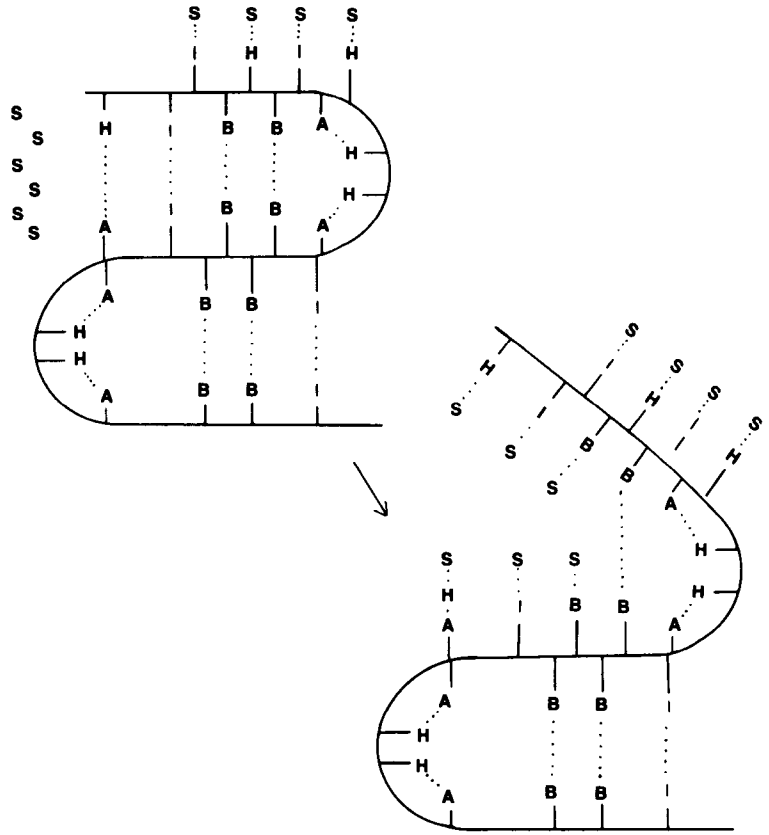


Figure 1.1 Schematic diagram showing changes in interactions involved in a conformational change leading to partial unfolding (Gray, 1985).

The compounds that promote solvation of the amino-acid residues by water shift the equilibrium of the reaction toward the inactive form. Similarly, compounds or conditions that reduce the tendency of water to solvate the amino-acid residues shift the equilibrium toward the folded, biologically active form. Chothia (1984) indicates that the stability of protein structures arises from the reduction in the surface accessible to solvent that occurs on folding and the formation of intramolecular hydrogen bonds. He further indicates that the secondary structures—α-helices and β-sheets—in proteins close-pack. In other words, these α-helices and β-sheets usually pack in one of a small number of relative orientations. Thus, there are families or classes of proteins that have a similar *tertiary structure* but no evolutionary or functional relationship.

The tertiary structure of the protein is determined by the hydrophobic interactions. One may compare a protein to automobile coil springs linked end to end by wires; imagine the structure folded further into a compact tertiary structure. Sternberg and his colleagues (1978, 1983) have shown that packing modes seem to be controlled by patterns of hydrophobic residues on the α-helices and β-sheets. Some proteins can exist as aggregates of a small number of identical subunits (from two to

six) or as aggregates of sets of subunits. The interactions that are responsible for holding the subunits together are of the hydrogen-bonding and van der Waals noncovalent type, and they determine the *quaternary* structure of the protein.

It is also now clear that domains are a common, if not universal, feature of longer peptide chains. A structural domain can be defined as a folded structure looking like a complete small protein molecule (Blake and Johnson, 1984). In many proteins, domains are associated with particular binding functions, and domain enzymes nearly always have their active sites located at the interface between two or more domains. A further consequence of domain structure that has become quite evident recently is the ability of domains to move relative to one another during the biological action of the protein. Bennett and Huber (1984) have studied examples of proteins that exhibit "domain dynamics."

It is well known now that individual atoms in the crystal lattice of proteins possess distinct mobility, which in some cases appears to correlate with biological activity. The protein molecules are not rigid, but have mobility that may extend from simple movements resulting from rotation about single bonds, through "breathing" motions of the whole molecule. Wuthrich and Wagner (1984) indicate that protein conformations are the result of a multitude of weak, nonbinding interactions between different atoms of the polypeptide chain and between the polypeptide and the surrounding medium. With the thermal energy at ambient temperatures, these nonbinding interactions may be frequently opened and re-formed. Wuthrich and Wagner (1984) further emphasize that the occurrence of "breathing motions" was discussed even before protein structures could be determined by crystallographic methods (Linderstrom-Lang, 1955). Karplus and McCammon (1981) indicate that for the interpretation of the structural and functional properties of proteins, it is essential to have a knowledge not only of the average positions of the atoms, but also of the magnitudes and time scales of the fluctuations about the average positions. X-ray diffraction studies of temperature factors for proteins have provided estimates of the magnitude of the fluctuations, expressed in terms of an isotropic and harmonic model (Frauenfelder et al., 1979; Artimiuk et al., 1979). Though the packing densities of atoms or groups within the molecules of globular proteins are as high as those found for crystalline amino acids and small organic compounds (Richards, 1977), the data of Frauenfelder et al. (1979) and Artimiuk et al. (1979) suggest that some packing defects or cavities may exist that permit sizable internal motions and flexibility in response to thermal or mechanical forces.

Koshland (1976) indicated that when protein structure became large enough to accommodate conformational flexibility, a major achievement in the evolution of enzyme function must have been realized. The way was paved for cooperativity as well as for the basis for other molecular means of metabolic regulation (for example, isomerization and oligomerization). Conformational flexibility also provides the basis for allosterism and hysteresis, for example. Hydrogen-exchange experiments as well as experiments involving other relaxational techniques have demonstrated that a protein molecule does undergo substantial fluctuations with regard to the relative positions of its constituent atoms. Gekko and Hasegawa (1986) indicate that a full understanding of the role of the fluctuation in protein function and biochemical phenomena will require further detailed information on the magnitude of the flexi-

bility or rigidity of protein molecules in solution and on the flexibility-structure relationships.

A few years ago, Cooper (1976) indicated that the flexibility of proteins should be reflected in their compressibility, since it is directly related to volume fluctuation. Since then, considerable data have accumulated on the adiabatic compressibility of proteins, ever since the accurate measurement of sound velocity became possible in dilute solutions (Millero et al., 1976; Sarvazyan and Hemmes, 1979; Gekko and Noguchi, 1979; Eden et al., 1982; Gavish et al., 1983; Gekko, 1984). Note that globular proteins exhibit positive compressibility, indicating the large internal compressibility of their molecules. Recently, Gekko and Hasegawa (1986) reported the results of adiabatic compressibility measurements of 11 proteins and discussed the compressibility-structure relationships of globular proteins in terms of some molecular parameters on the basis of the results of statistical analysis of compressibility data for 25 proteins. This included data for 14 proteins reported earlier (Gekko and Noguchi, 1979). Gekko and Hasegawa (1986) concluded that compressibility may be considerably correlated with the structural characteristics and amino-acid composition of proteins. Empirical equations developed by these authors permitted them to determine the compressibilities or volume fluctuations of unknown proteins from their amino-acid compositions.

Measurement of the hydrogen-exchange rate is an important method for revealing fluctuations in the native conformation. The exchange reaction of peptide NH hydrogens is especially suitable to the study of fluctuations in the backbone structure. Segawa and Kume (1986) recently made a comparison between the unfolding rate and the structural fluctuations in native lysozyme. It is of interest to compare the change in fluctuations with the change in the stability of the native structure of protein. These authors noted that although the exchange rate through major unfolding is greatly decreased by intrachain cross-linking between glutamine 35 and tryptophan 108 (1/22,000), the exchange rate through local unfolding is only slightly decreased (1/20). Cross-linking neither appreciably decreases the unfolding rate nor suppresses the fluctuations in the relatively flexible H-bonded structure. The increase in stability of cross-linked lysozyme is due exclusively to the increase in the folding-rate constant.

A major objective of biochemists continues to be the elucidation of the structural basis of enzyme function and activity. The understanding of the relation between enzyme structure and activity has been facilitated by X-ray crystallographic studies, chemical modification experiments, kinetic investigations, and other related techniques. In the next section we analyze briefly the chemical modification of enzymes and its influence on the enzyme's activity and inactivation characteristics.

1.2 CHEMICAL MODIFICATION OF ENZYME STRUCTURE AND ENZYME ACTIVITY CHANGES

Experiments on the chemical modification of enzymes yield considerable information regarding substrate binding and the subsequent intracomplex catalysis. A substantial amount of information is available in the literature which involves the

chemical modification of proteins (Hirs, 1967; Cohen, 1968; Glazer, 1970; Glazer et al., 1975). A large fraction of this effort has been directed, and correctly so, toward the elucidation of amino-acid sequences and toward the catalytic and binding sites of proteins. Chemical modifications of the reactive groups of enzymes have been used in protein studies as a means of identifying those groups responsible for catalytic activity (Barns and Keech, 1968; Ohta et al., 1972; Ferguson et al., 1975; Sugiyama and Mukohata, 1978; Riordan, 1973; Fahrney and Gold, 1963).

For example, it has been reported that cysteine and histidine residues are implicated in the active site of the cytosolic 5′-nucleotidase (Worku et al., 1984; Montero and Fes, 1982), and it seems that thiol groups are not essential for the plasma-membrane 5′-nucleotidase activity (Dornand et al., 1978; Naito and Lowenstein, 1985). Recently, Harb et al. (1986) investigated the modification of plasma-membrane 5′-nucleotidase by the carboxyl-modifying agent *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinone (EEDQ) (Belleau et al., 1968; Pougeois et al., 1978) and the histidyl reagent diethylpyrocarbonate (Miles, 1977). These agents produced an inactivation of the enzyme, indicating that both carboxyl and imidazole groups are probably located inside the active site.

Consider now the modification of D-β-hydroxybutyrate dehydrogenase by cyclohexanedione. D-β-hydroxybutyrate dehydrogenase (EC 1.1.1.30) is a lipid-requiring enzyme that is inlaid in the matrix face of the inner membrane of the mitochondrion. Nickle et al. (1982) initiated chemical derivatization studies on the purified enzyme in order to pinpoint the amino acids that are important for catalysis. These authors demonstrated the importance of a single arginine residue by modification of this enzyme by 1,2-cyclohexanedione. These authors indicate that the D-β-hydroxybutyrate dehydrogenase contains a single essential arginine that is accessible for modification with the arginine reagent. Note that "essential" is used to indicate that there is complete inactivation by derivatization of a single arginine residue and is not meant to infer that arginine is in the active center. Many such examples of enzyme modification are available in the literature.

An interesting aspect of chemical treatment of enzymes is "chemical mutation." This is an apparent breakthrough in the problem of enzyme design. Chemical mutation involves the creation of new enzyme sites (Levine et al., 1977; Levine and Kaiser, 1978, 1980; Fried and Kaiser, 1981; Slama et al., 1981; Kaiser et al., 1980). In the chemical mutation approach, the starting material is a natural protein that has folded to a stable conformation. Groups at or on the periphery of the active site are chemically modified to produce a "semisynthetic" enzyme having catalytic activity different from that of the original enzyme. Kaiser and Lawrence (1984) indicate that an attractive feature of the chemical mutation process is the wealth of X-ray structural information available for relatively simple enzymes. This permits a considerable degree of flexibility in the choice of the natural system in which the new catalytic group is introduced. The resultant semisynthetic enzymes can have catalytic activities very different from those of the corresponding native enzymes. For example, Kaiser and Lawrence (1984) further state that papain has been converted into a highly effective oxidoreductase by covalent modification of the sulfhydryl group of the active-site residue (Cys^{25}) with flavins such as 8-bromoacetyl-10-methylisoalloxazine. Other researchers, too, have studied the

development of these semisynthetic enzymes (Wilson and Whitesides, 1978; Royer, 1980, 1984).

Protein-modifying agents exhibit overlapping specificities (Means and Feeney, 1971; Glazer et al., 1975). The group specificity of a protein-modifying agent is usually determined by amino-acid analysis of the products of protein modification. A casual connection between protein modification and enzyme inactivation is usually sought by a comparison of the rates of protein modification with rates of enzyme activity loss (Ray and Koshland, 1961), or by an examination of the dependence of fractional enzyme activity on the extent of protein modification (Tsou, 1962). A casual connection between protein modification and enzyme inactivation may be established by a comparison of the enzyme-inactivation and protein-modification stoichiometries (Gomi and Fujioka, 1982). Recently, Rakitzis and Malliopoulou (1985) presented procedures for establishing (1) the dependence of enzyme activity loss on the numbers and reactivities of the groups essential for enzyme catalytic function, and (2) the involvement of a particular kind of reactive group in the enzyme-inactivation event. These authors provided examples from the modification of rhodanese by 2,4,6-trinitrobenzene-sulfonic acid. Furthermore, Rakitzis and Malliopoulou (1985) state that protein-modification reactions are described by second-order nonlinear differential equations, with concentration of the protein reactive groups being modified as the dependent variable, and reaction time as the independent variable. Recently, Rakitzis (1984) has presented the correct interpretation of reaction order involved in the kinetics of protein-modification and enzyme-inactivation reactions. In cases of protein modification, or of modification-induced enzyme inactivation, reaction order with respect to concentration of the modifying agent is mainly determined by the application of a simplified form of the Hill (1913) equation:

$$k_{app} = k[C_M]^h \tag{1.1}$$

where k_{app} is the experimentally determined first-order rate constant of the protein-modification or enzyme-inactivation reaction, C_M is the concentration of the modifying agent, h is the Hill coefficient, and k is a proportionality constant (Levy et al., 1963). Reaction order has been assumed to be identical with the number, per enzyme active site, of groups essential for enzyme catalytic activity (Reinsch and Dunlap, 1980; Kaminski and Jezewska, 1982; Ceccarelli and Vallejos, 1983). Rakitzis (1978, 1980) indicates that this number may be determined only by a comparison of enzyme-inactivation and protein-modification constants, or by a juxtaposition of the $(1/i)$th power of fractional enzyme activity (where i is the number, per enzyme active site, of groups essential for activity) with the extent of protein modification (Horiike and McCormick, 1979; Stevens and Colman, 1980; Horiike et al., 1984).

A large proportion of chemical modification studies has been directed, and correctly so, toward the elucidation of residues at the catalytic and binding sites of proteins. However, little effort has been spent on stability tests in these investigations. The chemical modification of enzymes leading to enhanced stability includes monofunctionally substituted proteins, reticulation by glutaraldehyde and other bifunctional agents, and grafting to polysaccharides and synthetic polymers. The in-

troduction of cross links leads to a modification of the tertiary structure and to a general stabilization of the native conformation.

One should consider both the activity and the stability (or longevity) of the enzyme in the economics of biochemical processes. In general, enzyme activity and stability are conflicting properties, and the trade-off between these factors must be considered in the selection and design of enzymes (Wiseman, 1983). This is particularly applicable if one is studying the effect of chemical modification of enzymes. For example, mechanisms that increase enzyme reaction rates may result in adverse conformational or electronic changes that decrease enzyme stability or residual activity. This is not necessarily true for all enzymes; chemical modification of *some* enzymes may increase both activity and stability (Sadana and Henley, 1986). Also, in the case of some enzymes, only the activity or only the stability is affected upon chemical modification. In most cases, enhancing enzyme stability—generally at the expense of enzyme activity—under normal and favorable reaction operating conditions may significantly improve the economics of a process. The longevity or stability is often more important than initial activity. The economic benefit is especially great if the trade-off is well matched to one's needs. For example, it may even be acceptable in some cases to lose as much as 90 percent of the initial activity on immobilization, provided that the immobilized enzyme has a long lifetime (Bucke, 1983).

More specifically, what is needed is a better understanding of the mechanisms of enzyme action, and the changes in structure and function that are involved in altering enzyme activity and stability. For example, biochemists have so far focused primarily on the relationships of structure, function, and stability in thermophilic enzymes, with the general aim of determining the molecular mechanisms responsible for their unusual stability. Fontana (1988) indicates that the molecular mechanisms responsible for the unusual stability of enzymes isolated from thermophilic microorganisms are much more complex and subtle than was originally thought.

The next section briefly discusses the thermodynamics of enzyme unfolding and inactivation. This is a necessary component in understanding enzyme inactivations, since it provides an estimate of the extent or degree of the "driving forces."

1.3 THERMODYNAMICS OF ENZYME INACTIVATION/FOLDING

Since folding and unfolding of proteins is a complex process, investigators have always been attracted to methods that bring to light stable intermediates in folding or unfolding reactions. Thermodynamics plays a central role in determining unambiguously whether the behavior of conformational transitions undergone by polypeptide chains is of a two-state or multistate nature. For example, a physically unambiguous criterion for two-state behavior (a transition of the type $E \rightarrow E_d$) is the equality of van't Hoff and calorimetric enthalpy (Lumry et al., 1966). However, Privalov and Khechinashivili (1974) indicate that this criterion is fulfilled, as a rule, for thermal unfolding of compact proteins having a molecular weight of less than 25,000; that is, a cooperative folding mechanism seems to be a privilege of small, globular proteins.

Brandts (1964a) initially obtained thermodynamic measurements on the denaturation of chymotrypsinogen which included nearly the entire range of temperature, ionic strength, and pH where the $E \rightleftharpoons E_d$ transition could be made to occur reversibly. Brandts stated that the reversible denaturation reaction involves a transition between only a single native and a single denatured state:

$$E \underset{}{\overset{K}{\rightleftharpoons}} E_d$$

$$K = \frac{[E_d]}{[E]} \tag{1.2}$$

where K is the thermodynamic equilibrium constant. He presented results calculated from spectrophotometric data obtained by the difference spectrum technique. Beer's law is obeyed by both the native and the denatured protein over the narrow concentration ranges used in most experiments. Thus, the equilibrium constant can be calculated from:

$$K = \frac{\epsilon - \epsilon_E}{\epsilon_{E_d} - \epsilon_E} \tag{1.3a}$$

where ϵ_E is the extinction coefficient for the native enzyme, ϵ_{E_d} that of the denatured enzyme, and ϵ that of the solution in the transition region. Since the difference spectrum technique yields raw data suitable only for obtaining differences in the extinction coefficient, the calculation of the equilibrium constant is ordinarily made from the equivalent relation

$$K = \frac{\Delta\epsilon - \Delta\epsilon_E}{\Delta\epsilon_{E_d} - \Delta\epsilon_E} \tag{1.3b}$$

where all the extinction coefficients have been reduced by the same amount.

The standard free energy of denaturation is given by

$$\Delta F^\circ = -RT \ln K \tag{1.4a}$$

while the enthalpy change may be obtained from a van't Hoff plot using the relation

$$\Delta H^\circ = -R\frac{d \ln K}{d(1/T)} \tag{1.4b}$$

Brandts (1964a) emphasizes that if the thermodynamic constants are to have validity, it must be established that the equilibrium is completely reversible. Figure 1.2a shows the temperature dependence of the extinction coefficient for chymotrypsinogen at different pH. Figure 1.2b shows the van't Hoff plots obtained from the data given in Figure 1.2a. Brandts (1964a) states that, within experimental error, ΔH° is independent of pH when compared at the same temperature.