

International Union of Microbiological Societies

Developments  
in  
Biological Standardization  
Vol. 74

Edited by  
the  
International Association of Biological Standardization

Acting Editors

Joan C. May (Bethesda, MD) — F. Brown (Guildford)



---

Basel • München • Paris • London • New York • New Delhi • Singapore • Tokyo • Sydney

---

**Bibliographic Indices**

This publication is listed in bibliographic services, including Current Contents® and Index Medicus.

---

**Drug Dosage**

The authors and the publisher have exerted every effort to ensure that drug selection and dosage set forth in this text are in accord with current recommendations and practice at the time of publication. However, in view of ongoing research, changes in government regulations, and the constant flow of information relating to drug therapy and drug reactions, the reader is urged to check the package insert for each drug for any change in indications and dosage and for added warnings and precautions. This is particularly important when the recommended agent is a new and/or infrequently employed drug.

---

**All rights reserved**

No part of this publication may be translated into other languages, reproduced or utilized in any form or by any means, electronic or mechanical, including photocopying, recording, microcopying, or by any information storage and retrieval system, without permission in writing from the publisher.

© Copyright 1992 by S. Karger AG, P.O. Box, CH-4009 Basel (Switzerland).

Printed in Switzerland by Médecine et Hygiène, Genève.

ISBN 3-8055-5466-4

*International Symposium on Biological Product Freeze-Drying and Formulation  
Bethesda, USA, 1990  
Develop. biol. Standard., Vol. 74, pp. 73-81 (Karger, Basel, 1991)*

Department of Chemistry, Massachusetts Institute of Technology,  
Cambridge, MA 02139, USA

## **ALTERATIONS IN THE STRUCTURE OF PROTEINS THAT CAUSE THEIR IRREVERSIBLE INACTIVATION**

*D.B. Volkin and A.M. Klibanov*

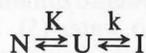
### **ABSTRACT**

A variety of changes in environmental conditions (temperature, pH, salts, solvents, etc.) can cause protein inactivation. However, the mechanisms of irreversible protein inactivation often follow common pathways. Using heat stress as an example, the conformational and covalent processes leading to the irreversible thermo-inactivation of enzymes will be described. In particular, work in our laboratory has identified several chemical reactions which contribute to enzyme thermo-inactivation: cystine destruction, thiol-catalysed disulfide interchange, oxidation of cysteine residues, deamidation of asparagine and glutamine residues, and hydrolysis of peptide bonds at aspartic acid residues. Implications of this work for the stability of proteins during, and following, the lyophilization process will be addressed.

### **INTRODUCTION**

Enzyme thermostability is of interest to the pharmaceutical scientist who uses elevated temperatures to accelerate protein inactivation processes (both in aqueous solution and as lyophilized powders) in order to ascertain their mechanism and to stabilize therapeutic proteins. A basic understanding of the causes and mechanisms of enzyme thermo-inactivation leads to the rational development of formulations to minimize degradation and hence maximize the storage stability of therapeutic proteins.

Thermally-induced enzyme inactivation is classified as either reversible or irreversible depending on whether enzymatic activity is recovered following return to ambient conditions. Irreversible enzyme thermo-inactivation can be represented by the simple scheme (1):



where N is a native, catalytically active enzyme, U is a reversibly thermo-unfolded enzyme, and I is an irreversibly thermo-inactivated enzyme. The first step is the reversible denaturation of the native conformation of an enzyme, and K defines the equilibrium constant between the N and U forms of the enzyme. The subsequent irreversible thermo-inactivation processes, represented by a first order rate constant (k), involve both covalent and conformational changes which are specific for individual enzymes.

### ***Reversible thermal denaturation***

The native, catalytically active conformation of a protein is maintained by a delicate balance of noncovalent forces including hydrogen bonds, hydrophobic, ionic and van der Waals interactions. An increase in temperature affects the strength of these interactions to different extents, thereby distorting this delicate balance and causing protein molecules to unfold. Upon cooling, the non-covalent interactions return to their initial state, and the enzyme regains its native, catalytically active conformation. This reversibility stems from the «thermodynamic hypothesis» which states that the native conformation of a protein in a given environment corresponds to the minimum free energy of the entire system. This conformation is determined by the amino acid sequence. The thermodynamic hypothesis is based on the classic experiments of Anfinsen and coworkers who demonstrated that ribonuclease, once reduced and unfolded in urea, can refold into the native, catalytically active structure by removal of urea and re-oxidation of sulfhydryl groups (2).

The reversible unfolding of proteins has been examined extensively and its mechanisms are well understood (3). The design of protein formulations frequently utilizes additives and solvents which stabilize proteins against reversible denaturation (4). The reversible partial unfolding of a protein is usually the first step, subsequently followed by conformational or covalent processes in irreversible thermo-inactivation.

### ***Irreversible thermal inactivation of proteins***

If a protein solution is heated and then rapidly cooled, but full catalytic activity is not recovered within a reasonable time, then the protein has undergone irreversible thermal inactivation. In this case, conformational or chemical changes (see below) have occurred which either prevent refolding or destroy the integrity of the polypeptide molecule.

#### ***1. Aggregation***

Heat-induced protein aggregation is most commonly described as a two-step process. First, as the temperature rises, the conformational flexibility («breathing») of a protein intensifies until eventually conformational changes (local changes in secondary and tertiary structure) or reversible protein denaturation (cooperative loss of higher ordered structure) take place. This partially exposes the buried, interior hydrophobic amino acid residues to the aqueous solvent. In the second stage of this process, the thermally altered protein molecules associate intermolecularly (primarily via hydrophobic interactions) in order to minimize the unfavorable exposure of hydrophobic amino acid residues to water (5). Subsequent chemical reactions may also occur, especially intermolecular disulfide cross-links (6).

#### ***2. Mechanisms of protein thermo-inactivation***

Intermolecular aggregation can be circumvented by employing dilute protein solutions or by using immobilized enzymes (7). As a result, the actual monomolecular processes that cause irreversible thermal inactivation of enzymes can be determined. In order to elucidate their mechanisms, the relative contribu-

tions of conformational and covalent reactions to the overall thermo-inactivation pathway must be determined.

A monomolecular model for irreversible *conformational* thermal inactivation was proposed based on studies with immobilized trypsin (8). At high temperatures, an enzyme loses its native non-covalent (intramolecular) interactions; subsequently, non-native interactions may form which, although thermodynamically unfavorable, remain for purely kinetic reasons so that the protein cannot spontaneously refold to the native conformation at ambient temperatures.

Two separate criteria were established to identify monomolecular conformational inactivation. First, enzymes can be reactivated after thermo-inactivation has occurred. For example, immobilized trypsin was heated and rapidly cooled (losing virtually all enzymatic activity), then completely unfolded and reduced in urea, and subsequently re-oxidized in the absence of denaturant. Recovery of enzymatic activity was nearly complete, implying that immobilized trypsin inactivated almost entirely via incorrect structure formation (8). A second methodology to measure irreversible conformational inactivation is based on the difference between the rates of thermo-inactivation in the presence and absence of reversible denaturants (9).

Employing these two strategies, some authors (10, 11) quantitatively accounted for the processes causing irreversible thermal inactivation (pH range of 4-8) of hen egg-white lysozyme at 100°C and bovine pancreatic ribonuclease A at 90°C, respectively. First, the overall monomolecular rate constants of irreversible thermal inactivation were determined for both enzymes (protein concentrations were selected so that no aggregation occurred), as shown in the first line of Table I. Second, a comparison of the rate constants of thermo-inactivation in the presence and absence of denaturants, along with reactivation experiments via the method described above for immobilized trypsin, provided the rate constant of incorrect structure formation, as shown in Table I. In the case of ribonuclease, these incorrect structures were found to stem from the thiol-catalysed interchange of disulfide bonds (see next section).

Samples of thermally inactivated lysozyme and ribonuclease were also analysed for peptide chain integrity and amino acid destruction to identify deleterious covalent processes that contribute to inactivation. As can be seen in Table I, the degradative covalent changes in the primary structure of lysozyme and ribonuclease are pH-dependent reactions whose relative contribution to irreversible thermo-inactivation consequently varies with pH. The general nature of these covalent reactions is discussed in the next section. These degradative reactions not only pointed to «weak links» in the primary structure of proteins, but also helped to define the upper limit of protein thermostability.

#### *Hydrolysis of peptide bonds at aspartic acid residues*

In the case of both ribonuclease and lysozyme, the hydrolysis of the polypeptide chain at the carboxyl terminus of aspartic acid at acidic pHs was shown to proceed at rates comparable to thermo-inactivation. A combination of SDS polyacrylamide gel electrophoresis and gel scanning densitometry was used, along with measurements of the appearance of new carboxy and amino termini. By examining a series of Asp-X peptides, it has been shown (12) that the Asp-Pro bond is particularly labile at high temperatures under acidic conditions. Replacement of

# Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

## Real-Time Litigation Alerts



Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

## Advanced Docket Research



With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

## Analytics At Your Fingertips



Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

## API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

## LAW FIRMS

Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

## FINANCIAL INSTITUTIONS

Litigation and bankruptcy checks for companies and debtors.

## E-DISCOVERY AND LEGAL VENDORS

Sync your system to PACER to automate legal marketing.