A Mechanism-Based Kinetic Analysis of Succinimide-Mediated Deamidation, Racemization, and Covalent Adduct Formation in a Model Peptide in Amorphous Lyophiles

MICHAEL P. DEHART, BRADLEY D. ANDERSON

Department of Pharmaceutical Sciences, College of Pharmacy, University of Kentucky, Lexington, Kentucky 40536-0082

ABSTRACT: The succinimide intermediate generated during deamidation of asparagine-containing peptides and proteins has been implicated as having a role in the formation of multiple types of degradants in addition to hydrolysis products, including racemization products and, more recently, amide-linked, nonreducible protein and peptide aggregates. The formation of alternative degradants may be particularly important in solid-state formulations. This study quantitatively examines the role of the succinimide intermediate in hydrolysis, racemization, and covalent, amide-linked adduct formation in amorphous lyophiles. The degradation of a model peptide, Gly–Phe–L-Asn–Gly, and its L- or D-succinimide intermediates were examined in lyophiles containing hydroxypropyl methylcellulose and varying amounts of excess Gly–Val. Disappearance of the starting reactants and formation of up to 10 degradants were monitored when lyophiles were exposed to either 27°C/40% relative humidity (RH) or 40°C/75 RH using a stability indicating high-performance liquid chromatography method. Terminal degradant profiles were the same when the starting reactant was either Gly–Phe–L-Asn–Gly or its succinimide intermediate. Nucleophilic attack occurred preferentially at the α-carbonyl of the succinimide intermediate at ratios of approximately 2:1 for both water and the N-terminus of Gly–Val as the attacking nucleophiles. A mechanism-based kinetic model analysis indicates that hydrolysis, racemization, and covalent, amide-linked adduct formation all proceed via the succinimide intermediate. © 2012 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 101:3096–3109, 2012

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INTRODUCTION

Proteins and peptides are often formulated as amorphous lyophiles to maximize stability compared with aqueous solutions and to provide solids that can be readily reconstituted prior to administration. Although processes involved in physical and chemical degradation may be significantly slower in the amorphous solid state, they are not completely arrested. The development of reliable quantitative methods to predict long-term stability of drugs in lyophilized and other amorphous solid formulations from kinetic data generated over short periods of time under accelerated conditions continues to be an active area of investigation.

For reactions in solution, mechanism-based kinetic models and the rate equations derived therefrom are often used to describe the influence of various factors on reactivity, including drug concentration, pH, temperature, and formulation components. In contrast, publications in the pharmaceutical literature that attempt to address reaction kinetics in amorphous systems quantitatively typically assume that molecular mobility is an overriding factor, such that reactivity will be coupled to one or more indicators of structural relaxation such as the glass transition temperature (T_g) or relaxation times generated from methods such as dielectric analysis[1] or nuclear magnetic resonance.[2] For example, Sun et al.[3] found that Williams et al.[4] empirical equation relating relaxation processes in amorphous polymers to temperature[4] was useful in describing deviations from the Arrhenius equation observed in the inactivation of glucose-6-phosphate dehydrogenase at

temperatures below or above T_g.[3] Yoshioka et al.[5] observed that the rates of the Maillard reaction and acyl transfer processes in lyophilized formulations containing various polymeric excipients increase with a decrease in T_g of the formulations. Below T_g, the variation in reaction rates with temperature was found to correlate with the temperature dependence of structural relaxation times calculated using the Adam–Gibbs–Vogel equation. Several groups have found that the empirical Kohlrausch–Williams–Watts equation used to describe heterogeneous relaxation in amorphous solids can be applied to chemical degradation in amorphous solids as well as physical processes such as protein aggregation.[6–9]

Although reactant and matrix mobility are clearly important considerations, the degree to which chemical degradation may be coupled to structural relaxation is likely to depend on the nature of the rate-determining step.[10] Such observations serve as a reminder that despite the increased importance of molecular mobility in solid-state formulations, the underlying reaction chemistry and mechanistic details are still critical in determining both the rates of degradation and the degradants formed. Most chemical degradation pathways of pharmaceutical relevance involve the generation of one or more reactive intermediates. Consider, for example, some of the important pathways for peptide and protein degradation. Peptide and protein deamidation occurs through a reactive succinimide intermediate.[11] Covalent protein aggregate formation may involve amide cross-linking mediated by succinimide[12] or anhydride intermediates,[13–15] thiol–disulfide rearrangement involving free thiol intermediates,[16] or lysinoalanine cross-linking via dehydroalanine residues generated during β-elimination of cysteine.[17] The Maillard reaction involves a Schiff base intermediate,[18] ultimately leading to a variety of degradation products.

Although the level of mechanistic understanding of these reactions currently available originates largely from studies in aqueous solution, the same reaction pathways may be operative in the amorphous solid state. Thus, a firm understanding of the mechanism of formation and the ultimate fate of reactive intermediates may prove useful in predicting and explaining differences in reaction kinetics in amorphous solids when compared with solutions. This has been a pursuit in several recent studies published from the authors' laboratories. For example, Luo and Anderson[19] demonstrated that the amino acid cysteine forms a reactive sulfenic acid intermediate in the presence of hydrogen peroxide in aqueous solutions resulting in the ultimate formation of the disulfide cystine when the intermediate reacts with another molecule of cysteine.[20] In amorphous solid formulations containing hydrogen peroxide, additional degradants were observed that could be traced to the same reactive intermediate. Their preferential formation in an amorphous polymer glass was attributed to competing reactions of the sulfenic acid intermediate with additional molecules of hydrogen peroxide, which, due to its small size, has a mobility advantage in amorphous glasses.[19] A detailed mechanism-based kinetic model was successfully employed to account for both the decline in reactant concentrations and formation of products as a function of time in various amorphous formulations.[19] Strickley and Anderson[14] demonstrated that covalent, amide-linked aggregates of insulin formed in certain amorphous lyophiles via nucleophilic attack of a second insulin molecule on a reactive cyclic anhydride intermediate. Despite the fact that covalent aggregate formation was in competition with hydrolysis, increasing water content increasingly favored covalent aggregation, which was attributed to the plasticizing effects of water.[15]

It is now well established that deamidation of asparagine residues in peptides and proteins occurs by way of a cyclic imide intermediate, both in aqueous solutions[11,12] and in amorphous lyophiles,[21,22] resulting in the formation of aspartate- and isoaspartate-containing peptides. In both amorphous solids and solutions, deamidation favors formation of isoaspartate over aspartate in approximately a 3:1 ratio.[11,22] However, this ratio is dependent on several factors including primary sequence,[23] higher order protein structure,[24] solvent choice,[25] and apparent pH in amorphous solids.[22] The results of experiments evaluating the effects of various factors on asparagine deamidation in model peptides have been summarized in several reviews[26,27] and books.[28,29]

Lately, there has been an increase in publications correlating succinimide formation and protein aggregation. Severs and Froland[30] observed succinimide intermediates and dimerization of a pituitary adenylate cyclase-activating peptide in anhydrous dimethyl sulfoxide (DMSO).[30] The authors hypothesized that the dimerization occurs via nucleophilic attack of a free amine (e.g., N-terminus, lysine, and arginine) on the succinimide carbonyl groups, resulting in a myriad of dimers. Using a more quantitative approach, Desfougeres et al.[31] prepared hen egg-white lysozyme with various amounts of succinimide intermediates. They demonstrated a linear dependence between dimer formation and the percent of succinimide intermediate present.[31] Similar to the experiments in DMSO, the authors proposed that a free amine reacts with a carbonyl of a succinimide intermediate on an adjacent protein.

Quite recently, we conducted kinetic studies to describe the decomposition of a model asparagine-containing peptide in amorphous lyophiles containing