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Racemization Rates of Asparagine–Aspartic Acid Residues in Lysozyme at 100°C as a Function of pH

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The racemization of asparagine (ASN) and aspartic acid (ASP) in lysozyme at 100°C between pH 4 and 8 has been investigated by comparing the overall ASX (ASP + ASN) racemization rate in lysozyme with that in polyaspartic acid. Between pH 4 and 6, the racemization rate of ASN is roughly similar to that of ASP, while at pH 8 the ASN racemization rate is much faster. The racemization of ASX residues may be a contributor to the thermoinactivation of lysozyme. © 1989 Academic Press, Inc.

INTRODUCTION

Investigations of amino acid racemization reactions and their implications have been an area of active research for nearly 2 decades (*1–4*). Recently, there has been an interest in the possible role that amino acid racemization has in the thermoinactivation of enzymes (*5*). Because racemization is a chemical reaction, rates will increase at the elevated temperatures often employed to increase enzymatic activity (*6*). In general, racemization has been ruled out as contributing to thermoinactivation of enzymes (*5*) since the amount of racemization of aspartic acid (one of the fastest racemizing amino acids) would be predicted to be very small. However, the racemization of asparagine has never been systematically studied. Asparagine is often a major component of ASX,[2] and based on mechanistic considerations should racemize faster than aspartic acid at neutral and basic pH (*1*). Racemization of asparagine could be even more important since recent evidence (*7*) suggests that asparagine deamidation, a major reaction contributing to enzyme thermoinactivation (*6, 8, 9*), takes place via the formation of a five-membered succinimide intermediate which is highly prone to racemization (*7*).

We have used egg white lysozyme, which contains 21 ASX residues, 14 of which are ASN, to study the racemization of ASN and ASP in an enzyme incubated at 100°C.

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[2] Abbreviations used: ASN, asparagine; ASP, aspartic acid; ASX, ASP + ASN; AA, amino acids.

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MATERIALS AND METHODS

The preparation of thermally inactivated lysozyme at 100°C between pH 4 and 8 has been described elsewhere (*8*). The incubated lysozyme samples were hydrolyzed in 6 M HCl (double distilled) for 6 h at 100°C and then desalted using cation exchange chromatography. Unheated lysozyme was processed in a similar manner in order to determine the amount of racemization of ASX during the acid hydrolysis step. The desalted samples were analyzed by reversed-phase HPLC using the *o*-phthaldialdehyde/*N*-acetyl-L-cysteine method to determine the extent of racemization of ASX (*10*). An Altex Model 332 HPLC and an Alltech 250 × 2.6-mm Econosphere C_{18} column were used for the analyses. The mobile phases are 100% MeOH (A) and 92% 50 mM NaAc + 8% MeOH titrated with HAc to pH 5.8 (B). The column was equilibrated with 100% B. A gradient was started 4 min after injection to 63% B with a duration time of 10 min at a flow rate of 1 ml min^{-1}. The column effluent was monitored with a Gilson fluorescent detector and recorded on a DEC PDP-1103 computer. ASX *D*/*L* ratios were determined from peak area integrations and were corrected based on the analyses of standards with known *D*/*L* ASP ratios.

The extent of lysozyme thermoinactivation in the incubated samples was assayed by measuring the lysis of dried *Micrococcus lysodeikticus* cells at pH 6 and 25°C (*8*).

RESULTS AND DISCUSSION

The racemization reaction of amino acids (AA) with one asymmetric center obeys the reversible first order kinetic relationship (*1*):

$$\ln\left[\frac{1 + D/L}{1 - D/L}\right] - \ln\left[\frac{1 + D/L}{1 - D/L}\right]_{t=0} = 2k_i t.$$

D/*L* is the amino acid enantiomeric ratio at a time t, and k_i is the first-order rate constant for interconversion of enantiomers. The $t = 0$ term accounts for racemization which takes place during the acid hydrolysis step. When the extent of racemization is small the above equation simplifies to

$$\ln[1 + D/L] - \ln[1 + D/L]_{t=0} = k_i t.$$

The rate-determining step in the racemization reaction of AA is the production of a tertiary carbanion intermediate (*1*). For free AA, the degree of protonation of the α-carboxyl and amino groups and the electron-withdrawing capacity of the R-groups all affect carbanion intermediate stability. In peptide and proteins, however, except for terminal residues, the protonation of the α-carboxyl and amino groups is prevented by the peptide bond, so the rates of racemization of AA are expected to be dependent mainly upon the electron-withdrawing abilities of the R-groups. For aspartic acid, however, the extent of protonation of the β-COOH is critical in determining the racemization rate of ASP in proteins (*1*, *11*).

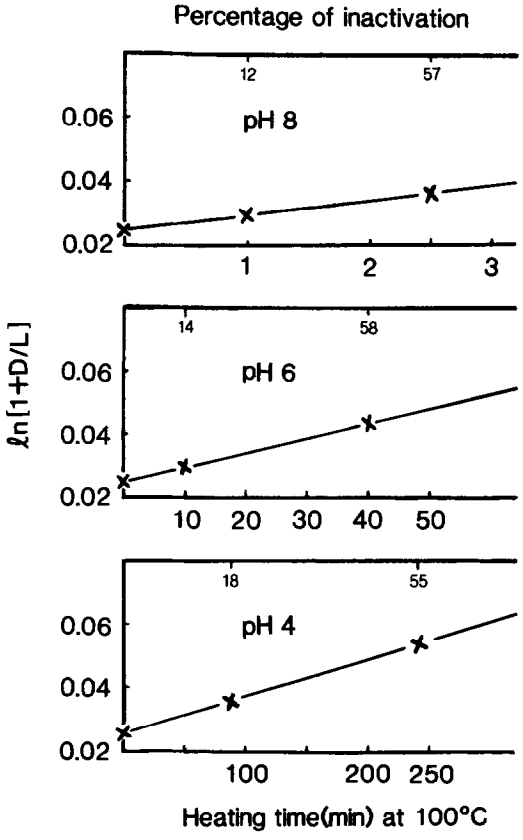

FIG. 1. Racemization kinetics of ASX in lysozyme incubated at 100°C at pH values of 4, 6, and 8. Also shown is the percentage of inactivation (upper horizontal axis) which occurred during incubation.

The racemization kinetics of ASX in heated lysozyme at pH 4, 6, and 8 are shown in Fig. 1. Over the heating times of our experiments, the extent of racemization was found to be very small, but the *D/L* ASX ratio systematically increased with increasing heating time and enzyme inactivation. Although deamidation is one of the major causes of thermal inactivation (*8, 9*), the overall *extent* of deamidation (i.e., the fraction of the 14 ASN residues converted to ASP) is minor during the course of our heating experiments (*8*). Therefore, the relative amounts of ASN : ASP did not change significantly during our incubation experiment.

Figure 2 shows the k_i values for ASX as a function of pH at 100°C for lysozyme, poly-ASP (*11*), and some other proteins (*12*). At pH 4 the racemization rate of ASP in poly-ASP exceeds that of lysozyme while between pH 5 and 6, the rates of racemization in poly-ASP, lysozyme, calmodulin, and ribonuclease are roughly similar. At pH 8, the ASX racemization rate in lysozyme greatly exceeds that in poly-ASP. We interpret these observations as follows.

The electron-stabilizing capacity of $-COOH$ is somewhat greater than that of $-COOH_2$ (*1*). At pH 4, the ASP β-COOH group would be nearly totally proton-

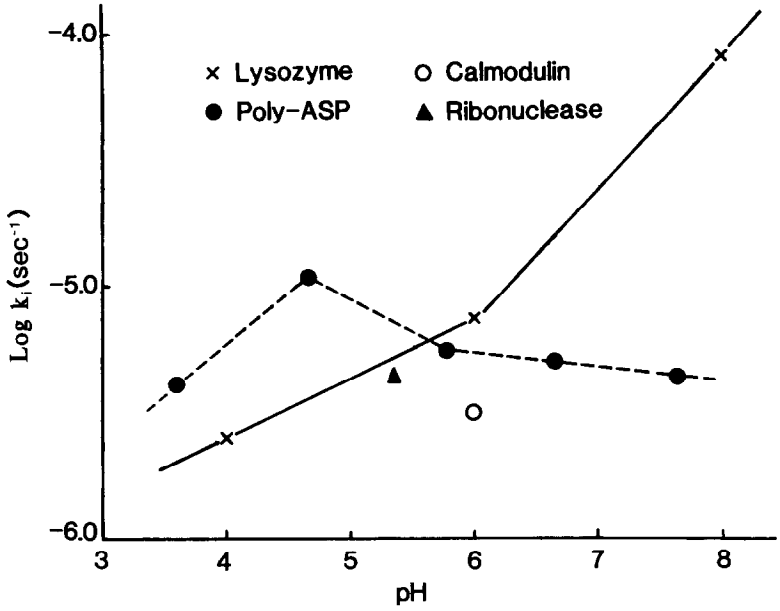

FIG. 2. Racemization rate constant (k_i) of ASX in lysozyme, poly-ASP (*11*), calmodulin (*12*), and ribonuclease (*12*) as a function of pH at 100°C.

ated (estimated pK_a at 100° is ~5) and therefore the racemization rates of ASP should exceed that of ASN. Thus, the overall racemization rate of ASX (ASP + ASN) in lysozyme at this pH would be expected to be somewhat less than the racemization rate in poly-ASP, which is what is observed. In the pH range 5–6, the rates of ASX racemization of poly-ASP, lysozyme, calmodulin, and ribonuclease are about the same, which indicates that it makes no difference whether the racemizing moiety is ASP or ASN. This is because the β-COOH group of ASP is partly deprotonated and thus the overall electron-withdrawing capacity of ASP and ASN are apparently roughly similar. At pH values substantially greater than the β-COOH pK_a, the β-COOH group of ASP would be extensively deprotonated, causing a large decrease in the electron-withdrawing power of the *R*-group of ASP (*1*). With ASN, the $-CONH_2$ group will not change in ionic character, and the racemization rate of ASN would be expected to increase with increasing pH since racemization is a base-catalyzed reaction (*1*). The contribution of ASN to the overall racemization rate at pH values greater than the β-COOH pK_a of ASP would dominate because ASN accounts for 66% of the total ASX residues in lysozyme. Our data indicate that at pH 8, the racemization rate of ASN exceeds that of ASP by more than a factor of 10.

We have demonstrated that the racemization of ASX occurs during enzyme thermoactivation at a measurable rate at 100°C. Since the racemization rate of any particular ASX residue may depend on the neighboring residues, we do not know whether the racemization rate we measured is an overall average or is for only a few selected ASX residues. In lysozyme, 3 ASN residues are components of the

active center (*13*). The racemization of any of these critical ASN residues would change the stereochemical structure of the active center and thus might contribute to the inactivation of lysozyme. As shown in Fig. 1, the extent of ASX racemization increases as the percentage of inactivation increases. If we assume that racemization of any one of the 21 ASX residues can cause inactivation, then a *D/L* ASX ratio of only ~0.05 (1/21) could be associated with complete enzyme inactivation. This *D/L* ratio is in the range at which lysozyme is >50% inactivated at 100°C (see Fig. 1). We do not know if this relationship is a coincidence, or whether ASX racemization, especially if it involves active center ASN residues, is indeed a contributor, albeit possibly minor, to the inactivation of heated lysozyme.

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