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The Racemization of Free and Peptide-Bound Serine and Aspartic Acid at 100°C as a Function of pH: Implications for *in Vivo* Racemization

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The relative racemization rates of free and peptide-bound serine and aspartic acid have been studied as a function of pH at 100°C. These results have been used to explain the observed relative *in vivo* racemization rates of serine and aspartic acid in human tooth dentin and human ocular lens proteins.

INTRODUCTION

In vivo racemization of aspartyl residues occurs at a measurable rate in several mammalian proteins, such as those present in dentin, the ocular lens nucleus, myelin, and erythrocyte membranes (*1–4*). This post-translational modification can be used as an age indicator for both terrestrial and marine mammals, as well as for humans whose ages are not well documented (*5*). In addition, since *in vivo* aspartyl racemization may alter protein structure and function, this reaction could be important in regulating protein turnover, and may contribute to the overall aging process in mammals (*5, 6*).

It is of biological interest to determine whether amino acids other than aspartic acid might be prone to *in vivo* racemization. It has been found that phenylalanine, alanine, and leucine are not susceptible to *in vivo* racemization (*1, 2*), which is consistent with the slow racemization rates (relative to aspartic acid) of these amino acids in aqueous solution (*5, 7*). Serine, however, has been found to have a racemization rate at pH 7.6 and 122°C that is 9–10 times that of aspartic acid (*8*). This result suggests that serine might have an *in vivo* racemization rate that exceeds aspartic acid.

In this study, we have measured the racemization rates of serine and aspartic acid as a function of pH at 100°C using both the free and peptide-bound amino acids. We also present some measurements of the extent of *in vivo* racemization of serine and aspartic acid in human dentin and the ocular lens nucleus.

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EXPERIMENTAL PROCEDURES

Serine and aspartic acid were obtained from Calbiochem. Poly-L-serine (M_r 1720) was obtained from Miles-Yeda, and poly-L-aspartic (M_r 7700) was from Sigma. All other chemicals used were of the purest grade commercially available.

Sodium succinate (pH 3.6 and 4.6) and sodium phosphate (pH 5.8, 6.6, and 7.6) buffers were used in the 100°C heating experiments. The buffer concentrations were 0.1 M, and the final ionic strength was adjusted to 0.5 by addition of NaCl. The pH values at 100°C for each buffer were calculated using the equations given in Bates (*9*).

Serine and aspartic acid were added to the various buffers to obtain a final concentration of 1.0 μM for each amino acid. Aliquots of these solutions were then sealed in Pyrex glass test tubes and heated for various lengths of time at 100°C in an oil bath. After heating, the samples were concentrated by evaporation and then processed for their extent of racemization as described below.

Poly-L-serine and poly-L-aspartic acid were added to the buffers so that the final concentration of each amino acid was 10 μM. Aliquots were then sealed in glass tubes and heated at 100°C. After heating, the samples were chromatographed on a 1 × 50-cm column of Sephadex G-10 (in 0.1 M acetic acid) in order to isolate peptides which had a molecular weight greater than 700. The isolated peptides were then hydrolyzed in 6 M HCl for 24 hr at 100°C, and then derivatized for enantiomeric analysis as described below. Unheated samples of both polyamino acids were processed in a similar manner in order to determine the amount of racemization which takes place during the acid hydrolysis step.

Samples of known age human dentin and the ocular lens nucleus were isolated as previously described (*1, 2*). These samples were hydrolyzed for 6 hr at 100°C in 6 M HCl, and then desalted by cation-exchange chromatography. Aspartic acid was isolated from the protein hydrolysate by anion-exchange chromatography (*10*), and serine was isolated by collecting serine-containing fractions eluting from the buffered column of a Beckman Model 119C amino acid analyzer. The pure serine fractions were identified by reversed-phase high-performance liquid chromatography using the *o*-phthaldialdehyde (OPT) technique (*11*). The fractions must contain only serine since other amino acids were found to interfere with serine enantiomeric analyses. Following isolation, aspartic acid and serine obtained from the metabolically stable proteins were analyzed as described below to determine their enantiomeric composition.

The extent of racemization of serine and aspartic acid was determined by synthesizing (*5, 12*) the L-leucine-X dipeptide, where X is either serine or aspartic acid. The diastereomeric dipeptides are prepared by reaction of the free amino acid with L-leucine-*N*-carboxyanhydride (NCA) in a saturated (pH 10.3) borate buffer solution. The excess L-leucine which results from hydrolysis of the NCA was removed by chromatography on a Cu(II)-NH_3-saturated column (1 × 5 cm) of Chelex-100.

The diastereomeric dipeptides from the heating experiments as well as the serine dipeptides from the human tissues were resolved by reversed-phase HPLC on an Altex Model 310 HPLC using a 25 × 0.46-cm Altex Ultrasphere (5 μm) C_{18}

column. The mobile phase was 99.5% 0.2 M KH_2PO_4 (titrated to pH 3 with H_3PO_4) and 0.5% acetonitrile. The flow rate was 1.5 ml/min. The column effluent was monitored at 200 nm on a Hitachi Model 100-10 uv–visible spectrophotometer. A representative chromatogram showing the separation of the diastereomeric dipeptides L-leucyl–D-serine and L-leucyl–L-serine has been published in Ref. (*5*). The D/L aspartic acid ratios in the human tissues used in the studies reported here have been published elsewhere (*1, 2*), and were determined by separating the leucyl–aspartic acid diastereomeric dipeptides on a Beckman Model 119C amino acid analyzer (*5*).

RESULTS

The racemization of free amino acids obeys reversible first-order kinetics (*7*). Thus, the kinetic equation describing the racemization reaction is

$$\ln\left[\frac{1 + \text{D/L}}{1 - K \cdot \text{D/L}}\right] - \ln\left[\frac{1 + \text{D/L}}{1 - K \cdot \text{D/L}}\right]_{t=0} = k_i \cdot (1 + K) \cdot t, \qquad [1]$$

where D/L is the amino acid enantiomeric ratio at a time t, k_i is the first-order rate constant for interconversion of enantiomers, and $K = 1/(\text{D/L})_{eq}$. The racemization rate constant is equal to $2k_i$. For amino acids with more than one center of asymmetry (i.e., isoleucine, threonine, and hydroxyproline) the equilibrium ratio is different from unity, while for monoasymmetric amino acids $K = 1.0$. The $t = 0$ term is required to account for racemization which occurs when an acid hydrolysis step is used in sample processing (*5*).

Free aspartic acid and serine were heated at 100°C for various lengths of time at pH values between 3 and 8. The racemization kinetics of both amino acids at each pH value were found to obey the reversible first-order expression given in Eq. [1].

TABLE 1

VALUES OF k_i (sec^{-1}) FOR FREE AND PEPTIDE-BOUND ASPARTIC ACID AND SERINE AT 100°C BETWEEN pH 3.6 AND 7.6 CALCULATED FROM EQ. [1]

$pH_{100°C}$	Asp	Ser	Poly-Asp	Poly-Ser
3.60	1.4×10^{-6}	3.9×10^{-7}	4.4×10^{-6}	9.7×10^{-9}
4.66	1.5×10^{-7}	2.5×10^{-7}	1.1×10^{-5}	5.5×10^{-7}
5.77	—	—	5.8×10^{-6}	1.1×10^{-6}
6.65	1.5×10^{-7}	2.6×10^{-7}	5.0×10^{-6}	5.8×10^{-6}
7.65	1.2×10^{-7}	1.2×10^{-6}	4.4×10^{-6}	9.9×10^{-6}

Note. The $t = 0$ terms for the polyamino acids, determined by processing aliquots of the unheated solutions, were found to be small in comparison to the extent of racemization measured in the heated samples. The uncertainties of the k_i values are estimated to be ±15–20%.

Values of k_{asp} and k_{ser}, obtained from a least-squares fit of the data, are given in Table 1. The k_{asp} values as a function of pH are similar to those reported previously (*13*) at 100°C. At pH 7.6 k_{ser} is about ten times greater than k_{asp}, which is consistent with previous measurements (*8*) made at 122°C and neutral pH.

The buffered solutions of poly-L-serine and poly-L-aspartic acid were heated for 20 hr at 100°C. We realize that reversible first-order racemization kinetics have not been demonstrated for either of these polyamino acids. However, we chose a short heating time in order to minimize effects on the k_i values if reversible first-order kinetics are not obeyed. The k_{asp} and k_{ser} values determined for poly-L-aspartic acid and poly-L-serine are also given in Table 1.

Results of the analyses of the extent of aspartic acid and serine racemization in known age tooth dentin and ocular lens nucleus proteins are presented in Table 2. Although the extent of aspartic acid racemization increases with the age of the sample, serine racemization shows no such relationship. Apparently, *in vivo* serine racemization does not occur in metabolically stable mammalian proteins.

DISCUSSION

The rate-determining step in the racemization reaction of amino acids in the neutral pH region is the abstraction of the α-proton by a basic species such as OH^- producing a tertiary carbanion intermediate (*5, 7, 13, 14*). Thus, the rate of racemization is controlled by the stability of the carbanion intermediate. For free amino acids, the carbanion stability is dependent upon the degree of protonation of the α-carboxyl and α-amino groups, as well as the electron-withdrawing capac-

TABLE 2

In Vivo RACEMIZATION OF SERINE AND ASPARTIC ACID IN DENTIN AND THE OCULAR LENS NUCLEUS IN HUMANS

Age (years)	D/L Ser	D/L Asp[a]
Dentin		
8	0.06	0.020
30	<0.01	0.033
42	0.03	0.045
60	<0.03	0.060
Lens nucleus		
16	0.07	0.079
41	0.08	0.109

Note. The estimated uncertainty of the D/L Ser ratios is ~±15–20% while for the D/L Asp ratios it is ~±5–10%.

[a] The D/L Asp ratios were previously reported in Refs. (*1*) and (*2*).

ity of the R-group of the amino acid (*5, 7*). Thus, rates of racemization of free amino acids may change as a function of pH due to changes in the ionization of the —COOH and $—NH_3^+$ groups of the racemizing species (*5, 7*), as well as from changes in ionization of functional groups on the R-group of the amino acid.

Rate equations (*7, 13*) for the OH^--catalyzed racemization of free aspartic acid and serine as a function of pH are given as

$$k_{obs}[Asp]_T = k^{0+0}[HOOCCH_2CH(NH_3^+)COOH][OH^-] + k^{0+-}[HOOCH_2CH(NH_3^+)COO^-][OH^-] + k^{-+-}[^-OOCCH_2CH(NH_3^+)COO^-][OH^-] \quad [2]$$

and

$$k_{obs}[Ser]_T = k^{+0}[CH_2(OH)CH(NH_3^+)COOH][OH^-] + k^{+-}[CH_2(OH)CH(NH_3^+)COO^-][OH^-], \quad [3]$$

where the rate constants for racemization of various ionic species of aspartic acid and serine are represented by k values with the superscripts (i.e., 0+0, 0+−, etc.), k_{obs} is the total observed racemization rate constant, and the subscript T indicates total.

Because of the rapid rate of racemization of the aspartic acid species possessing a protonated β-carboxylic acid group (β-COOH), the maximum rate of racemization of aspartic acid is at pH 2–3 (*13*). Our data indicate that, at pH 3.6, aspartic acid racemizes about three to four times faster than serine. At pH 7.6, the β-COOH of aspartic acid is predominantly in an ionized state so that the protonated β-COOH ionic species makes little or no contribution to the observed aspartic acid racemization rate (*13*). Thus, at pH 7.6, the β-OH of serine is a more effective electron-withdrawing group than the ionized β-COOH of aspartic acid. As a result, serine racemizes 9–10 times faster than aspartic acid at pH 7.6.

Since the ionization of α-COOH and α-NH_3^+ groups is prevented in peptide-bound amino acids, the relative rates of racemization of the various amino acids are expected to be determined predominantly by the electron-stabilizing effects of their R-groups. Thus, the ionization state of the β-COOH of aspartic acid would be critical in determining the racemizaion rate of peptide-bound aspartic acid relative to other amino acid residues. In the experiments with poly-L-serine and poly-L-aspartic acid, it was found that, at pH values less than 6.65, aspartic acid racemizes more rapidly than serine. Apparently, for peptide-bound aspartic acid, there is a shift in the pK_a of the β-COOH to more basic pH values as a result of peptide bond formation. This allows the aspartic acid species with a protonated β-COOH group to contribute to the observed racemization of aspartic acid at higher pH values than is the case with free aspartic acid racemization. The maximum rate of aspartic acid racemization in polyaspartic acid is in the pH range 4–5, which is apparently the pK_a of the β-COOH group in polyaspartic acid. The racemization rate of bound serine increases with increasing pH, indicating a dependence on OH^- concentration. The racemization rate maximum of bound serine is observed at the highest pH we used in our series.

The results in Table 1 indicate that, at physiological pH, aspartyl residues should racemize at a rate which is either roughly equal to or somewhat less than that of serine. The extent of *in vivo* aspartic acid and serine racemization measured in two tissues from living humans are given in Table 2. Only aspartic acid is observed to racemize; there is no age-related increase in the extent of serine racemization. The results indicate that, at physiological pH, the serine *in vivo* racemization rate is less than that of aspartic acid.

One explanation for the slow *in vivo* racemization rate of serine is that physiological factors are acting to elevate the pK_a of the β-COOH of aspartyl residues. The protonated species then is contributing extensively to the racemization rate even at physiological pH.

Another possible explanation for the observed slow *in vivo* racemization rate of serine is that the carbanion intermediate of serine undergoes decomposition by a β-elimination reaction to form dehydroalanine (*15*). In proteins, serine is frequently associated with phosphate through an ester linkage or with carbohydrates through an ether linkage. Substituted serine undergoes facile β-elimination (*15*). Thus, the apparent lack of *in vivo* serine racemization may be the result of the rapid β-elimination reaction preventing the addition of a proton to the carbanion intermediate.

As yet, we have not been successful in detecting dehydroalanine or one of its products, pyruvate, in tooth dentin. However, some preliminary measurements suggests that the serine content of human (*16*) and bovine (*17*) tooth dentin decreases with age. Furthermore, the crosslinked amino acids lysinoalanine and histidinoalanine (*18*) have been reported in bovine dentin, and these are products from the reaction of dehydroalanine with lysine and histidine. Evidence for the β-elimination reaction of serine in proteins of calcareous fossils has also been presented (*19, 20*). Additional efforts are required in order to elucidate the importance of this pathway in preventing the accumulation of D-serine in metabolically stable mammalian proteins.

CONCLUSIONS

In this study, despite predictions based upon heating experiments with free amino acids, we have found that serine has an *in vivo* racemization rate much slower than aspartic acid. This is because in peptides serine has a racemization rate which is either equivalent to or less than that of aspartic acid at physiological pH. However, it is possible that decomposition reactions affecting serine or substituted seryl residues prevent *in vivo* serine racemization.

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