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Diketopiperazine Formation During Investigations of Amino Acid Racemization in Dipeptides

Abstract. *The formation of diketopiperazines from the dipeptides isoleucylglycine and glycylisoleucine was investigated at 132°C over the* p*H range ~ 2 to 10. At* p*H 6.2, ~ 50 percent of the original dipeptides were converted to the diketopiperazines during the heating experiments. Hydrolysis of the diketopiperazines can yield either the original dipetide or an inverted dipeptide product. The isoleucine in the diketopiperazines was the most highly epimerized component in the system. Previous racemization and epimerization studies with dipeptides have not taken into account the formation of diketopiperazines and, as a result, the conclusions about the mechanism and geochemical implications of amino acid racemization in dipeptides will require revision.*

Investigations of the kinetics of racemization of dipeptides have been suggested to provide new insights into the mechanism of amino acid racemization in peptides and in calcareous fossils. On the basis of the studies of epimerization of isoleucine in several di- and tripeptides at ~ *p*H 6 and 152°C Kriausakul and Mitterer (*1*) reported that NH_2-terminal isoleucine epimerizes faster than COOH-terminal isoleucine. Smith and de Sol (*2*) studied the racemization of 37 dipeptides at *p*H 7.6 and 122°C. Their results indicate that in general COOH-terminal amino acids racemize faster than NH_2-terminal amino acids. De Sol and Smith explained these results by invoking inductive effects, by neighboring group stabilization of the carbanion intermediate, and by steric effects on the solvation of the carbanion.

We felt that the mechanistic conclusions or geochemical implications of these dipeptide results were premature because of the necessity of first investigating *p*H effects on amino acid racemization rates in various dipeptides. These studies are important in order to deduce which ionic species of the dipeptide is undergoing racemization at a particular *p*H and temperature. For example, elevated temperature experiments (*3*) with free amino acids buffered at various *p*H values indicate that at *p*H 7.6 there is a mixture of ionic species racemizing and that these various ionic species have greatly differing racemization rates. In order to demonstrate which factors control the relative racemization rates of free amino acids, it was necessary to determine and compare the rates for a particular ionic species; comparisons using the observed racemization rates at a particular *p*H give misleading conclusions (*3*).

Besides the dipeptide ionic species problem, there is also the complication that dipeptides may cyclize to form diketopiperazines. Hydrolysis of the diketopiperazine may either regenerate the original dipeptide or create an inverted dipeptide. It is well known that dipeptide esters and amides easily cyclize to diketopiperazines. At 25°C the rate of cyclization competes favorably with ester or amide hydrolysis (*4*). Brewerton *et al.* (*5*) observed extensive sequence inversion in some dipeptides in 2*M* HCl at 84° to 114°C. Long *et al.* (*6*) observed that divalent metal ions catalyzed cyclization of glycylglycine at *p*H 3.8 to 6.0 at 91° to 114°C. These investigations demonstrate the quantitative importance of diketopiperazine formation and dipeptide sequence inversion relative to peptide bond hydrolysis. Furthermore, amino acid residues in diketopiperazines are rapidly racemized at room temperature in dilute alkali (*7*).

In order to investigate the effects that *p*H has on the racemization of dipeptides and the possible formation of diketopiperazines we have conducted heating experiments with glycylisoleucine and isoleucylglycine in various buffered solutions ranging from *p*H ~ 2 to 10 (ionic

Table 1. Diketopiperazine formation, dipeptide inversion, and epimerization in glycylisoleucine and isoleucylglycine at 131°C in the *p*H range ~ 2 to 10. Under the experimental conditions, the extent of hydrolysis of the dipeptides to free amino acids was less than 5 percent in all cases. Abbreviations: allo/iso, alloisoleucine/isoleucine; diketo, 3-isobutyl-2,5-piperazinedione.

*p*H at 131°C*	Heating time (hours)	Original dipeptide allo/iso	Inversion (%)†	Inverted dipeptide allo/iso	Diketo (%)†	Diketo allo/iso	Total allo/iso‡
			Glycylisoleucine				
2.11	72	0.10	71	0			
3.42	72	0.12	62	0	80	< 0.05	~ 0.08
4.85	72	0.15	63	0	77	~ 0.08	~ 0.05
5.55	72	0.16	49	0.06	48	0.21	0.14(0.15)
6.36	24	0.09	49	0.26			
6.36	48	0.19	76	0.59	58	0.79	0.59(0.66)
7.79	24	0.04	60	1.3			
7.79	48	0.07	74	1.4	11	1.23	0.81(0.81)
8.66	24	0.02	13	1.4			
8.66	48	0.03	30	1.4	0.01	1.4	0.30(0.25)
9.56	24	0.02	7	1.4			
			Isoleucylglycine				
2.11	12	0.01	48	0.03			
3.42	12	0.02	34	0.32	28	~ 0	< 0.05
4.85	12	0.02	4	0.64			
5.55	8	0.02	14	1.0	36	< 0.05	< 0.05
6.36	12	0.15	17	0.22	61	0.28	0.28(0.23)
7.79	8	0.36	4	0.15			
7.79	12	0.58	6	0.14	18	1.0	0.73(0.69)
8.66	12	0.16	3	0.20	12	1.4	0.29(0.24)
9.56	8	0.11	3	§			

*The *p*H values of the buffers at 131°C were extrapolated with the use of the equations given by Bates (*12*). †Percent inversion is tabulated relative to the total amount of dipeptide recovered from the heated samples [inverted/(original + inverted)] excluding the diketopiperazine fraction. The percent diketo is tabulated relative to the total amount of dipeptide and diketopiperazine recovered from the heated sample [diketo/(diketo + original + inverted)]. ‡The allo/iso values given in parentheses were calculated from the allo/iso ratios determined for the dipeptide and diketopiperazine fractions and the relative abundances of each component analyzed. §No inverted product was detected.

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strength = 0.5). After the buffered solutions were heated at 131°C for various lengths of time, the dipeptides were separated from the buffer salts by desalting on cation exchange resin (Dowex 50 × 8), and volatile *N*-TFA-L-prolylmethyl esters of glycylisoleucine, glycylalloisoleucine, isoleucylglycine, and alloisoleucylglycine were synthesized (*8*). The volatile diastereomeric tripeptide derivatives were then separated by gas-liquid chromatography (GLC) (*9*) on a glass column (3.6 m by 2 mm) packed with 10 percent SP2250 on Chromosorb WHP (100/120). This analytical scheme enabled us to analyze the extent of isoleucine epimerization (that is, the ratio of allo to iso) directly in the original, and in any inverted dipeptide, and the stereochemistry of any hydrolysis products; the relative abundances of the various components was determined by measurement of the areas of the peaks corresponding to each diasteromeric tripeptide derivative. The formation of diketopiperazines was investigated by collecting the water wash from the Dowex 50 desalting column. The water was removed by rotary evaporation and the remaining residue was hydrolyzed in 6*M* HCl for 24 hours. The amount of diketopiperazine produced and the extent of epimerization in the diketopiperazine were determined by quantifying the hydrolyzed water wash residues (*10*). The total extent of isoleucine epimerization in the heated samples was determined by hydrolysis of the buffered solutions and quantifying the free amino acids produced (*10*) (Table 1).

Our results (Table 1) indicate that there is extensive inversion of glycylisoleucine into isoleucylglycine at all *p*H's, with the exception of *p*H > 9. For isoleucylglycine, the extent of inversion was less than that observed for glycylisoleucine. This inversion indicates that some of the original dipeptide was converted to the diketopiperazine, which subsequently hydrolyzed to yield the inverted dipeptide. At *p*H 6.2, more than 50 percent of the original dipeptide was converted into the diketopiperazine for both isoleucylglycine and glycylisoleucine. The fact that isoleucylglycine is less inverted during these heating experiments probably indicates that in the hydrolysis reactions of the diketopiperazine thermodynamic equilibrium favors isoleucylglycine over glycylisoleucine.

The data in Table 1 also indicate that in the range *p*H 6 to 8, the isoleucine in the diketopiperazines is the most extensively epimerized component in the system. Since the isoleucine in the diketopiperazine undergoes rapid epimerization at neutral *p*H, the inverted dipeptide produced by hydrolysis of the diketopiperazine is also highly epimerized. Only at *p*H values of ~ 5 or less is the extent of epimerization of the inverted dipeptides produced from isoleucylglycine more highly epimerized than the diketopiperazine.

Although we have carried out measurements on only two dipeptides, the cyclization reaction of dipeptides to diketopiperazines is quite general (*4*). Thus, in previous studies (*1, 2*) of amino acid racemization in dipeptides, cyclization to diketopiperazines and the subsequent racemization of the amino acids in this component is likely to be the principal reaction pathway. Also, in all previous dipeptide studies (*1, 2*) the extent of racemization or epimerization of the free amino acids produced from dipeptide hydrolysis during sample heating and the hydrolyzed total (free and dipeptide-bound) amino acids in the sample was determined. Measurements of the hydrolyzed total sample include epimerization and racemization in the original dipeptide, the inverted dipeptide, the diketopiperazine, and any free amino acids produced from dipeptide hydrolysis (Table 1) (*11*). Therefore, any conclusions as to whether amino acids in the NH_2-terminal for COOH-terminal positions are more rapidly epimerized or racemized is not valid if they are based on total D/L ratios. Moreover, the activation parameter (E_a values) reported by Kriausakul and Mitterer (*1*) for the epimerization of isoleucylglycine are actually a combination of activation parameters for the cyclization of isoleucylglycine and the epimerization of isoleucine in the diketopiperazine.

We suggest that even our measurements of the extent of epimerization of isoleucine in the original dipeptides does not actually represent the inherent rate of epimerization of isoleucine at either COOH- or NH_2-terminal positions of peptides. Because of the rapid reversible formation of the diketopiperazine, it is very difficult to evaluate the actual rate of epimerization of isoleucine at either COOH- or NH_2-terminal positions. Since the rate of both formation and epimerization of the diketopiperazine is sufficiently rapid, it is likely that, in these elevated temperature experiments, isoleucine epimerization occurs mainly in the diketopiperazine and not in the actual dipeptides.

The complex *p*H dependence of the extent of epimerization of the dipeptide, diketopiperazine, and the total fractions is not simply a result of changes in the ionic species undergoing epimerization. Rather, the apparent *p*H dependence in each fraction reflects a combination of *p*H effects on the epimerization and cyclization of the dipeptides and on the epimerization and hydrolysis of the diketopiperazines.

Our results indicate that the formation of diketopiperazine is important in determining the extent of racemization of dipeptides in the neutral *p*H region. Thus, any conclusions about mechanisms of racemization of amino acids in dipeptides or the implications which these dipeptide investigations may have to geochemical systems are unjustified unless the formation of diketopiperazines in the elevated temperature heating experiments is taken into account.

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11. Using the total allo/iso ratios that we determined at *p*H 6.36 and 7.79, we obtain ratios for the rate of epimerization of isoleucine in isoleucylglycine and glycylisoleucine that are in close agreement with those reported in the previous studies (*1, 2*).
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