Reactivity of the Imino Acids Formed in the Amino Acid Oxidase Reaction†

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ABSTRACT: The reactivity of the imino acids formed in the D- or L-amino acid oxidase reaction was studied. It was found that: (1) When imino acids reacted with the α-amino group of glycine or other amino acids, transimination yielded derivatives less stable to hydrolysis than the parent imino acids. In contrast, when imino acids reacted with the ϵ-amino group of lysine or other primary amines, transimination yielded derivatives more stable to hydrolysis than the parent imino acids. (2) Imino acids react rapidly with hydrazine and semicarbazide, forming stable hydrazones and semicarbazones. At pH 7.7, the rate of reaction of the imino acid analogue of leucine with semicarbazide was 10^4 times greater than that of the corresponding keto acid. The reaction of imino acids with these reagents is rapid enough to permit one to follow spectrophotometrically the amino acid oxidase reaction. Imino acids also reacted with cyanide to yield stable adducts. (3) The rate of hydrolysis of the imino acid analogue of leucine was independent of pH above pH 8.5. At lower pH values, the rate of hydrolysis increased with decreasing pH. At 25 °C and in the absence of added amino compounds, this imino acid had a half-life of 22 s at pH 8.5. Its half-life was 9.9 s at pH 7.9.

Imino acids have been shown to be the products of the reactions catalyzed by D- and L-amino acid oxidase (Hafner & Wellner, 1971). This was deduced from the finding that an inversion of configuration takes place when an optically pure amino acid is oxidized by the appropriate amino acid oxidase in the presence of sodium borohydride. Yagi et al. (1970), based on their observation of a pH drop during turnover in the D-amino acid oxidase reaction, also concluded that unprotonated imino acids are the products of these reactions. This was confirmed, in part, by the demonstration that substantial amounts of imino acid accumulate under steady-state conditions in reaction mixtures containing D-phenylalanine and D-amino acid oxidase (Porter & Bright, 1972b).

We now report that the proton release observed by Yagi et al. (1970) can be accounted for only partially by the amount of enzymatically formed imino acid present in solution. The remainder of the proton release is shown to be due to the formation of N-substituted imino acids arising by transimination reactions between the imino acid product of the oxidation and the parent amino acid. We studied the formation and stability of a variety of N-substituted imino acids. The results of these studies indicated that these imino acids are extremely reactive molecules. We also found that imino acids react rapidly with hydrazine, semicarbazide, or cyanide to form stable products.

Experimental Procedures

Materials

Lyophilized *Crotalus adamanteus* venom was purchased from Ross Allen's Reptile Institute, Silver Springs, FL, and crystalline L-amino acid oxidase was prepared from the venom (Wellner & Meister, 1960). Crystalline D-amino acid oxidase was prepared from hog kidney (Massey et al., 1961). [^{3}H]$NaBH_4$ and ^{14}C-labeled amino acids were obtained from New England Nuclear Corp. Sodium phenylpyruvate was kindly provided by Dr. Alton Meister (Cornell University Medical College). Other reagents were of the highest purity available.

Methods

Assay of Amino Acid Oxidase Activity. The amino acid oxidases were assayed in two ways. The first method measures the absorption at 300 nm of a complex of the phenylalanine oxidation product with borate (Wellner, 1966). The second method, to be published in detail elsewhere, measures semicarbazone formation at 248 nm when amino acid oxidation is carried out in the presence of semicarbazide. It is based on the results reported here.

Determination of Amino Acid Isomers in DL *Mixtures.* After acidification with HCl, the reaction mixtures described in Table I were extracted three times with equal volumes of ether to remove benzoic acid, evaporated to dryness, redissolved in water, adjusted to pH 2.2 with NaOH, and applied to a 1 × 7 cm AG-50 column (H^+ form). After the column was washed with water, elution with NH_4OH (3 M) and subsequent evaporation to dryness yielded the amino acid fraction. When [^{3}H]$NaBH_4$ was used, exhaustive treatment of the amino acid fraction with L-amino acid oxidase and measurement of the [^{3}H]H_2O passing through a 1 × 7 cm AG-50 column (H^+ form) revealed the quantity of ^{3}H-labeled L-amino acid formed by [^{3}H]$NaBH_4$ reduction. The [^{3}H]$NaBH_4$ solution was prepared and standardized as described in the next section.

Amino acid isomers were measured as the semicarbazone derivatives formed during enzymatic oxidation in the presence of semicarbazide (see Results). For example, L-leucine was measured in the presence of D-leucine using L-amino acid oxidase. L-Alanine, a poor substrate for L-amino acid oxidase, was determined separately by measuring DPNH oxidation at 340 nm when 15 μmol of α-ketoglutarate, 0.16 μmol of DPNH, 5 μg of lactate dehydrogenase, and 60 μg of glutamate–pyruvate transaminase were mixed with the alanine in 1.0 mL of 0.05 M potassium phosphate, pH 7.6. A standard curve was made for known concentrations of L-alanine.

Preparation and Standardization of [^{3}*H*]*NaBH*$_4$ *Solution.* A solution of [^{3}H]$NaBH_4$ was prepared by mixing small amounts of [^{3}H]$NaBH_4$ (3.44 mCi/mg) with unlabeled $NaBH_4$ (15 mg/mL) to give an approximate specific radioactivity of 1×10^5 cpm/μmol of BH_4^-. This solution was used in experiments 8 and 9 of Table I and was standardized by

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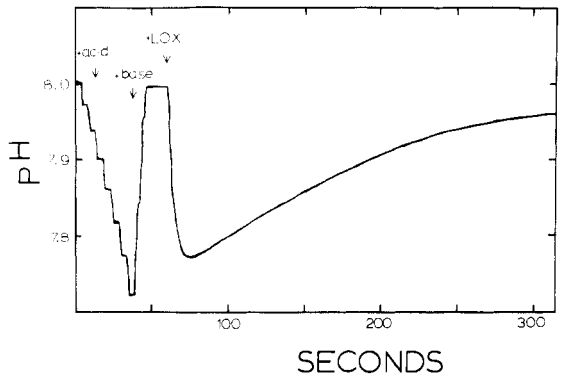


FIGURE 1: pH changes during L-methionine oxidation. Before L-amino acid oxidase (LOX) addition, the buffering capacity of the solution was measured by the addition of several 0.05-mL aliquots of 2 mM acetic acid. The pH was restored to near 8 with NaOH, and the addition of 0.47 mg of enzyme started the reaction. The reaction was carried out at 25 °C in the presence of catalase (0.23 mg), KCl (0.1 M), and L-methionine (4.7 mM), in a final volume of 5.3 mL.

reducing the stable imino acid analogue of proline, Δ^1-pyrroline-2-carboxylate. [The latter compound was prepared by incubating D-amino acid oxidase (approximately 0.2 μg) with 5 μmol of D-ornithine and 230 μg of catalase in 1 mL of a 0.05 M sodium pyrophosphate–HCl buffer, pH 8.3, at 37 °C for approximately 1 h.] An excess of [^{3}H]$NaBH_4$ was used. After isolation of the [^{3}H]proline from an AG-50 column as described above, an aliquot was placed in scintillation fluid (Jeffay & Alvarez, 1961) and counted for radioactivity, while another aliquot was analyzed for proline with an amino acid analyzer. Controls receiving no $NaBH_4$ showed formation of negligible amounts of proline. It is assumed that the reduction of different imino acids by [^{3}H]$NaBH_4$ exhibits kinetic isotope effects of similar magnitude.

pH Monitoring. Experiments were monitored for pH changes during amino acid oxidation in a manner similar to that employed by Yagi et al. (1970). A Radiometer titrator (pH meter), type TTTIa, outfitted with a type C glass electrode, GK-2021, connected to a Sargent recorder (Model SRL) to give a full-scale deflection per pH unit, was used. The reactions were carried out in a water-jacketed 10-mL vessel and stirred with a small magnetic stirring bar.

Determination of Rate Constant for α-Ketoisocaproic Acid Reaction with Semicarbazide. Reactions were carried out at 25 °C in 1-mL volumes with 10–40 μmol of neutralized semicarbazide hydrochloride and 20 μmol of sodium pyrophosphate buffer, brought to the desired pH with HCl. The reactions were initiated by addition of at least 0.4 μmol of keto acid dissolved in a small volume of water. The reactions were followed at 248 nm with a Cary 11 recording spectrophotometer, and initial linear velocities were computed using the extinction coefficient (10 300 M^{-1} cm^{-1}) of the resulting semicarbazone.

Amino Acid Analysis. Analyses were made on a Beckman 120C amino acid analyzer according to the method of Spackman et al. (1958).

Preparation of Semicarbazone Derivative of Sodium Phenylpyruvate. The preparation of the semicarbazone derivative of phenylpyruvate was carried out following the procedure of Shriner et al. (1956).

Results

pH Changes and the Accumulation of Imino Acids during the Amino Acid Oxidase Reaction. The pH changes that occur during the oxidation of D-amino acids catalyzed by D-amino acid oxidase have been described by Yagi et al. (1970). Figure 1 shows the results of an analogous experiment in which the pH was monitored in the course of oxidation of L-methionine by L-amino acid oxidase. Yagi et al. (1970) concluded from such experiments that the enzymatic reaction yields an imino acid, unprotonated near pH 8, from the protonated amino acid substrate. The subsequent rise in pH was ascribed to the hydrolysis of the unstable imino acid and formation of α-keto acid and ammonium ion, resulting in the uptake of protons. We wished to test this conclusion. We therefore measured the amount of imino acid present in amino acid reaction mixtures by two independent methods. The first was based on pH changes. Since the pK values for the α-amino group of most amino acids are near 9.5 while those of the corresponding imino acids are much lower [Cabello et al. (1964) report a pK of 5.99 for the stable imino acid analogue of proline, Δ^1-pyrroline-2-carboxylate], one would expect that, at pH 8, one proton should be released for each molecule of amino acid converted to imino acid (see Scheme I). The second method was based on our previous finding that it is possible to trap imino acids with $NaBH_4$ with an efficiency of about 97% (Hafner & Wellner, 1971). The amino acid resulting from this reduction may be measured because it is racemic, unlike the optically active substrate. For example, after $NaBH_4$ is added to a solution of D-leucine in the presence of D-amino acid oxidase, the amount of imino acid trapped may be calculated as twice the amount of L-leucine formed, as measured with L-amino acid oxidase. When we simultaneously measured the quantity of protons released and enzymatically formed imino acid trapped by $NaBH_4$, we found that the accumulation of the imino acid analogue of the substrate was not sufficient to account for the total amount of protons released (Table I). Some other unprotonated species is therefore being formed in these reaction mixtures. The results indicate either that this new species is not reduced by $NaBH_4$ or that its reduction product is not a substrate for the amino acid oxidase used to identify the optical isomers.

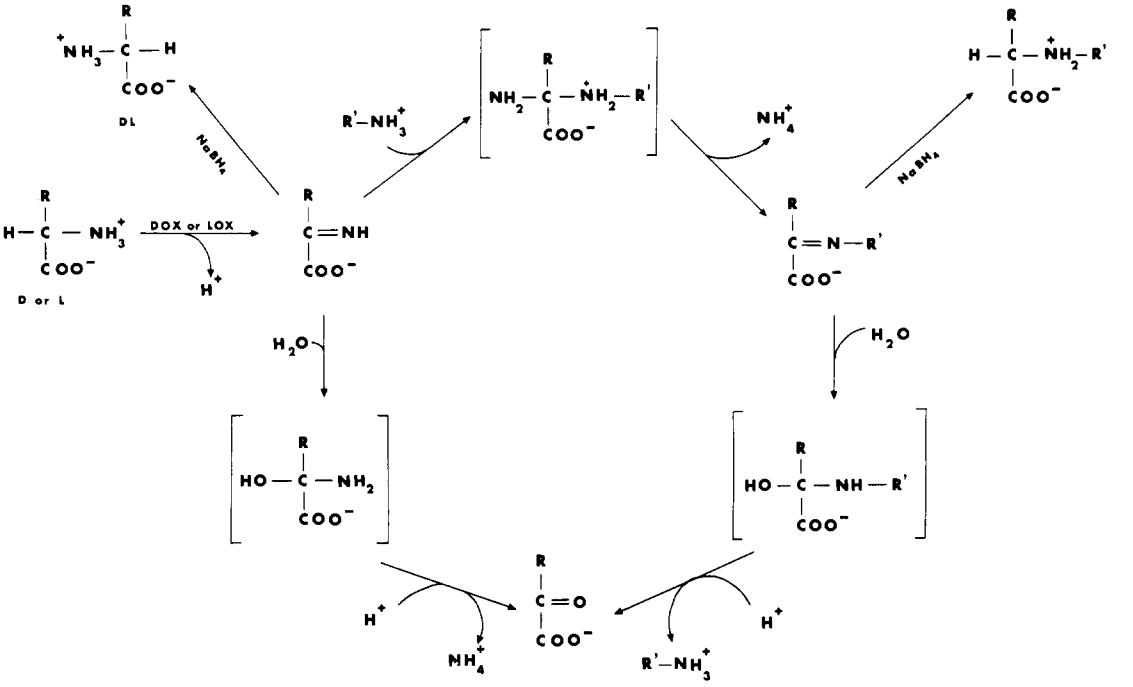
We have shown that transimination reactions occur between the ε-amino groups of lysine (whether free or in polypeptide chains) and the imino acid analogue of alanine (Hafner & Wellner, 1971). Thus, we reasoned that the results in Table

Table I: Comparison of H^+ Production with Imino Acid Production during Amino Acid Oxidation[a]

expt	substrate	concn (mM)	H^+ released (μmol)	imino acid found[b] (μmol)	imino acid found/ H^+ released	av
1	D-Ala	9.4	0.33	0.084	0.25	0.29
2	D-Ala	9.4	0.15	0.049	0.33	
3	D-Met	4.7	0.39	0.22	0.56	0.53
4	D-Met	4.7	0.44	0.22	0.50	
5	D-Leu	4.7	0.24	0.23	0.96	0.91
6	D-Leu	4.7	0.22	0.19	0.86	
7	D-Leu	56.6	0.19	0.056	0.29	
8	L-Met[c]	4.7	0.55	0.37	0.67	
9	L-Met[c,d]	4.7	1.30	0.11	0.085	

[a] The reactions were carried out at 25 °C under O_2, with rapid stirring. When D-amino acid oxidase was used (experiments 1–7), FAD (0.12 μmol) was added. In all cases, KCl (500 μmol), catalase (230 mg), and amino acid oxidase (0.1–1 mg) were present, in addition to the substrate, in a volume of 5.3 mL. Changes in pH were monitored as in Figure 1. After 30 s of reaction, the pH change was noted, and 0.8 mL of a reagent containing $NaBH_4$ (15 mg/mL) and sodium benzoate (1 M) was added, followed in 5–6 s by 0.5 mL of HCl (3 N). [b] Calculated as twice the amount of amino acid of inverted configuration determined as described under Methods. [c] [^{3}H]$NaBH_4$ was used in these experiments. [d] D-Lysine, 14.1 mM, was also present.

Scheme I: Proton Release and Uptake Associated with Reactions Involving Imino Acids[a]



[a] DOX and LOX represent D-amino acid oxidase and L-amino acid oxidase, respectively.

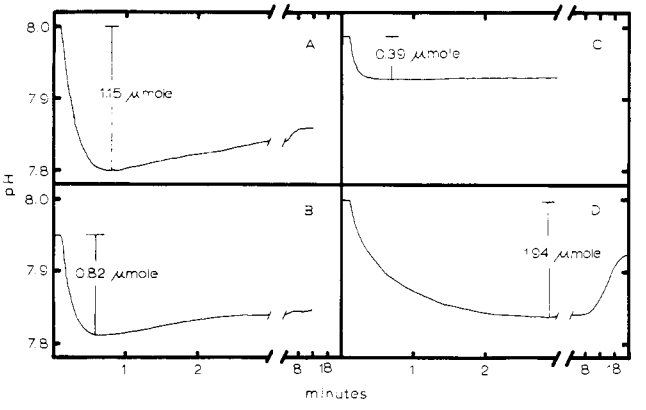


FIGURE 2: pH changes during L-leucine oxidation, with and without added amino compounds. The conditions were as described in Figure 1, except that L-leucine was the substrate. The maximum proton release observed is given for each experiment, calculated from the titration data (not shown). Additions to the reaction system were (A) none; (B) glycine, 0.94 mM; (C) glycine, 28 mM; and (D) D-lysine, 1.9 mM.

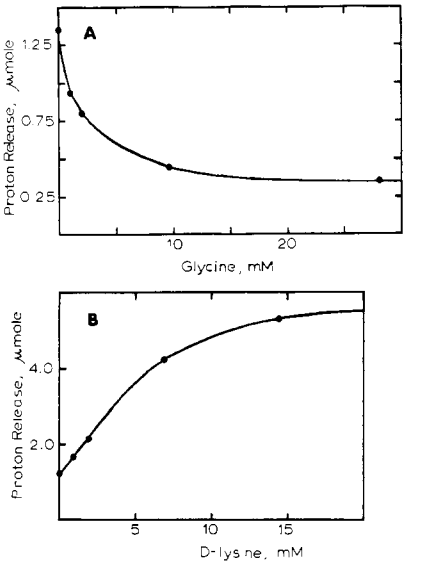


FIGURE 3: Changes in the maximum proton release observed during L-leucine oxidation as a function of increasing glycine concentration (A) or lysine concentration (B). Experiments, represented by the points, were carried out similarly to those presented in Figure 2. The initial pH was chosen in such a way that the minimum pH reached, when the proton release was at a maximum, was about 7.9. One-half the maximal change in proton release is caused by either 1.6 mM glycine or 4.6 mM D-lysine.

I could be explained if transimination reactions of this type between the imino acid formed enzymatically and the substrate yielded substantial amounts of unprotonated transimination products. It would be expected that such products would be reduced by $NaBH_4$ to N-alkylated amino acids which are not oxidized by L-amino acid oxidase. In order to study this possibility further, the pH changes occurring in L-leucine oxidation mixtures containing various added amino compounds were monitored (Figure 2). It can be seen that glycine causes a decrease, while D-lysine causes an increase, in the maximum proton release accompanying substrate oxidation. [D-Lysine is not a substrate for L-amino acid oxidase, and glycine is oxidized only extremely slowly (Lichtenberg & Wellner, 1968). Furthermore, neither of these amino acids inhibits or activates L-amino acid oxidase at the concentrations used (data not shown). Therefore, these results cannot be accounted for by a change in the rate of enzymatic turnover.] A measure of the maximum proton release as a function of the concentration of the added amino compound is given in Figure 3. The maximum proton release represents the total amount of imino compound (the sum of the substrate analogue and the transimination product) present under steady-state conditions, when these imino compounds are being formed and hydrolyzed at the same rate. Such steady-state conditions prevail when the curves of pH against time (Figures 1 and 2) are at a minimum. The results of Figure 3 support the conclusion that transimination with the added amino acid is taking place to form a new, N-substituted imino acid that differs in its hydrolysis rate from the imino acid analogue of leucine. The smaller proton release when glycine is present shows that the

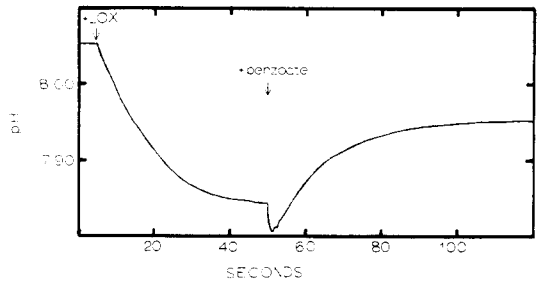


FIGURE 4: pH changes during the hydrolysis of the imino acid produced from L-leucine oxidation. Reaction conditions were as described in Figure 1, with L-leucine (4.7 mM) as substrate. After 50 s of reaction, sodium benzoate (1 M) was added to give a final concentration of 100 mM. The pH of the sodium benzoate solution was adjusted so that its addition did not substantially alter the pH of the reaction mixture.

transimination product formed with glycine is more rapidly hydrolyzed than the parent imino acid, while the much larger proton release in the presence of lysine indicates that the N-substituted imino acid which is formed in this case is more slowly hydrolyzed than the parent imino acid. These reactions are summarized in Scheme I.

Further support for this interpretation is our previous finding that ϵ-*N*-(1-carboxyethyl)-L-lysine is formed following $NaBH_4$ reduction of reaction mixtures containing D-alanine, D-amino acid oxidase, and L-lysine (Hafner & Wellner, 1971). By amino acid analysis, we have also found the analogous compound, ϵ-*N*-(1-carboxy-3-methylthiopropyl)-D-lysine, in experiment 9, Table I. About 1 μmol of this compound was present (assuming a color value equal to that of leucine). When this amount is added to the amount of imino acid (substrate analogue) trapped by $NaBH_4$, about 85% of the proton release in that experiment is accounted for.

Other amino compounds were also tested for their effects on the proton release accompanying amino acid oxidation. It was found that β-alanine, glycine ethyl ester, and ethylamine mimicked the effect of D-lysine, whereas D-alanine and D-methionine mimicked the effect of glycine (see Figure 2). Triethylamine, which cannot form a Schiff base, had no effect.

Imino Acid Hydrolysis. When low concentrations of leucine were oxidized, a very close correspondence existed between the observed proton release and the amount of trapped imino acid (substrate analogue), as indicated in Table I. It is concluded that this imino acid does not readily undergo transimination with its parent amino acid and that, under these conditions, hydrolysis constitutes its major fate. Therefore, this substrate is most suitable for measuring the rate of imino acid hydrolysis without complications from the transimination reaction. This was done by adding sodium benzoate, an oxidase inhibitor, to leucine oxidation mixtures after significant amounts of the imino acid had accumulated as judged by the decrease in pH. A typical experiment is shown in Figure 4. The subsequent rate of decrease in proton concentration, which is equal to the rate of hydrolysis of the imino acid, was found to follow first-order kinetics, provided that the pH changes were kept small (0.05–0.15 pH unit). Over such small pH intervals, the variation in buffering capacity and hydrolysis rate as a function of pH may be neglected. By conducting several of these experiments in different pH ranges, the rate constant for hydrolysis as a function of the pH was determined (Figure 5). It can be seen from Figure 5 that, while the rate of hydrolysis of the imino acid analogue of leucine was constant at high pH values (>8.5), lowering the pH below 8.5 caused a significant increase in the first-order rate constant.

Reaction of Imino Acid with Cyanide, Hydrazine, and

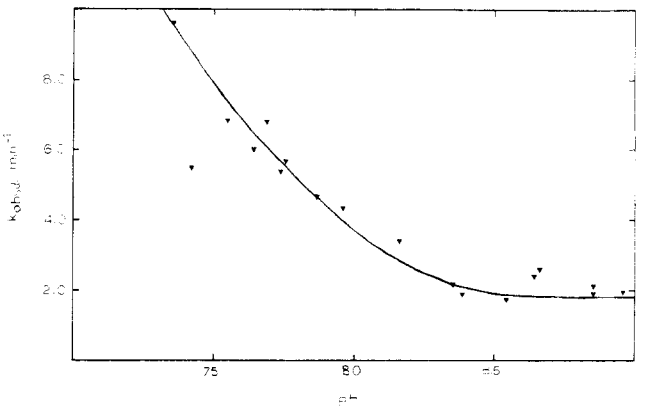
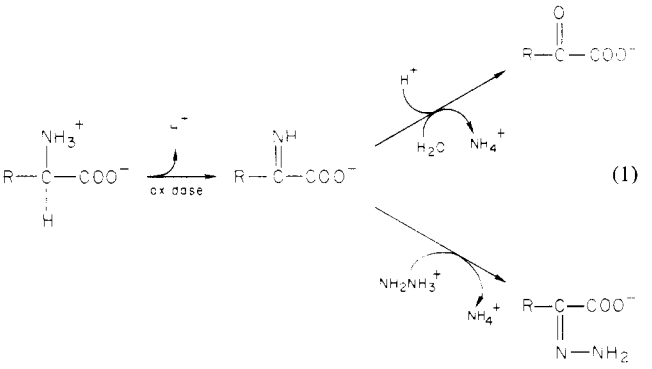


FIGURE 5: First-order rate constant for the hydrolysis of the imino acid analogue of leucine as a function of pH. Each point represents an experiment performed similarly to that shown in Figure 4. Conditions were such that the pH changes in all experiments were small (0.05–0.15 pH unit). Values on the abscissa represent the midpoint of the pH range covered in each experiment.

Semicarbazide. The high reactivity of the imino acids in the experiments presented above suggested to us that these compounds might react rapidly with other nucleophilic reagents. This was found to be the case. Thus, when L-phenylalanine was oxidized under conditions similar to those of Figure 1 and in the presence of 10 mM NaCN, the pH dropped continuously and did not rise again. This suggests the formation of unprotonated α-cyanophenylalanine by an addition reaction between the imino acid and cyanide.

When amino acid oxidase reactions were carried out near pH 8 in the presence of 10 mM hydrazine, a continuously falling pH was observed, similar to that seen in the presence of cyanide. This is accounted for by the fact that, at pH 8, hydrazine (pK = 8.5) is more than 75% protonated, while hydrazones are unprotonated. Therefore, the overall reaction in the presence of hydrazine is accompanied by a net release of approximately 0.75 mol of protons per mol of substrate oxidized. We conclude that the reactions in eq 1 take place.



(1)

With semicarbazide, no pH changes were observed under similar conditions, since semicarbazide is a much weaker base than hydrazine and is unprotonated at pH 8. However, the reaction of imino acids with semicarbazide (and also hydrazine) was followed spectrophotometrically by taking advantage of the high ultraviolet absorption at 248 nm of the semicarbazone (or hydrazone) reaction product. In order to show that it was indeed the imino acid, and not the keto acid, that was reacting with these reagents, amino acid oxidation was carried to completion in the absence of hydrazine or semicarbazide. When either reagent was then added, only a very slow development of absorbance at 248 nm was seen, in contrast to the rapid reaction seen when the reagents were present during enzyme turnover. The rate constant for the

Table II: Rate Constants for the Reaction of Semicarbazide with Imino Acid and Keto Acid Analogues

pH	semicarbazide concn required for 50% trapping of imino acid[a] (mM)	k(hydrolysis) (min^{-1}) imino acid[b]	k(semicarbazide) (min^{-1} M^{-1}) imino acid[c]	keto acid[d]
7.7	4.24	6.0	1400	0.120
8.7	11.60	1.8	150	0.012

[a] The reaction mixtures contained L-leucine (4 mM), catalase (2.3 μg), sodium pyrophosphate buffer (60 mM), and semicarbazide hydrochloride (1–30 mM), neutralized with NaOH, in a final volume of 1 mL. The reaction, carried out at 25 °C, was started by the addition of crystalline L-amino acid oxidase (0.4–2 μg) in 0.1 M KCl (0.04 mL or less). The larger amounts of enzyme were used at low semicarbazide concentrations to increase the accuracy of the measurements. Appropriate corrections were made for dilution and for enzyme inhibition by high salt concentrations. The values are obtained from double-reciprocal plots of the data, as described in the text. [b] Calculated from data of Figure 5. [c] Calculated using eq 2. [d] Determined from initial velocity measurements as described in Methods.

reaction with semicarbazide was deduced as follows. The reciprocal of the rate of change of absorbancy at 248 nm was plotted against the reciprocal of the semicarbazide concentration. The ordinate intercept corresponds to the reciprocal of the rate at infinite semicarbazide concentration and represents 100% trapping of the imino acid. The concentrations of semicarbazide required for 50% trapping of the imino acid were calculated from the data and are given in Table II. At these semicarbazide concentrations, the rate of hydrolysis of the imino acid equals the rate of its reaction with semicarbazide. Since the first-order constants for hydrolysis have been determined (Figure 5), the second-order rate constants for reaction with semicarbazide can be calculated from the following equation, where (semicarbazide$_{50}$) represents semicarbazide concentration required for 50% trapping of the imino acid.

$$k_{\text{semicarbazide}} = \frac{k_{\text{hydrolysis}}}{(\text{semicarbazide}_{50})} \qquad (2)$$

Table II compares these rate constants with the analogous constants for the reaction of semicarbazide with the corresponding keto acid. It can be seen that the imino acid reacts about four orders of magnitude more rapidly with semicarbazide than does the keto acid, both at pH 7.7 and 8.7.

Discussion

The results presented here show that the imino acid products of the amino acid oxidase reactions are extremely reactive molecules capable of undergoing a variety of rapid reactions.

Transimination Reactions of Imino Acids. Several lines of evidence indicate that the imino acids formed in the amino acid oxidase reaction react with amino compounds to form new, N-substituted imino acids through a transimination reaction. Thus, when the amount of proton release accompanying amino acid oxidation, catalyzed by the amino acid oxidases, was compared with the amount of unsubstituted imino acid trapped by $NaBH_4$, the former quantity was always larger than the latter (Table I).

The existence of these transimination reactions is also shown by changes in the maximum proton release accompanying amino acid oxidation in the presence of added amino compounds (Figure 2). Also, when D-lysine was added to a mixture of L-methionine and L-amino acid oxidase, the maximum proton release observed was more than double that in the reaction without added D-lysine, while the amount of imino acid (substrate analogue) trapped was reduced by more than two-thirds (experiments 8 and 9, Table I). A similar increase in the proton release was obtained when L-leucine was the substrate (Figure 3B).

The reactions involved in imino acid production and imino acid transimination, along with the proton release and uptake associated with these reactions, are illustrated in Scheme I. The carbinolamine and *gem*-diamino structures, represented in brackets, are assumed to have a very short half-life relative to the other structures shown (Jencks, 1969). Therefore, because of their low concentrations in solution, their formation or disappearance is not expected to produce measurable pH changes in the experiments described here. Isolation and identification of the $NaBH_4$-reduced Schiff base products of some transimination reactions of the imino acids produced by the amino acid oxidase reaction provides additional evidence for the existence of these transimination reactions (see Results). Evidence for similar enzymatic imine formation and transimination reactions of imines has been described for mitochondrial monamine oxidase (Patek et al., 1972).

Imino Acid Hydrolysis. The dependency of the first-order rate constant for the hydrolysis of the imino acid analogue of leucine on pH (Figure 5), showing a pH-independent region above pH 8.5 and a rise in the rate as the pH is lowered below 8.5, is similar to the pH profile for the hydrolysis of *p*-nitrobenzylideneethylamine and also resembles the pH dependency of the hydrolysis of *p*-nitrobenzylidene-1,1-dimethylethylamine, studied by Cordes & Jencks (1963).

The explanation for the increase in rate when the pH is lowered below 8.5 is that the attack of water in comparison with the attack of OH^- on the protonated imine becomes more significant. The maximal rate of hydrolysis is expected when essentially all of the imine is protonated, that is, at pH values well below the pK of the imine. This was observed by Cordes & Jencks (1963) for *p*-nitrobenzylidene-1,1-dimethylethylamine.

Rate Constants for the Transimination of Glycine and D-*Lysine with the Imino Acid Analogue of Leucine and for the Hydrolysis of These Transimination Products.* Under the conditions of a steady-state reaction, we can equate the enzymatic reaction velocity with the velocity of the reaction (transimination or hydrolysis) that causes the disappearance of the unstable, enzymatically produced imino acid. The first-order rate constant for the hydrolysis of the N-substituted imino acid derived from the reaction of glycine with the imino acid analogue of leucine can be calculated as follows from the data presented in Figures 3A and 5. It can be concluded that when the enzymatic oxidation of L-leucine was carried out at glycine concentrations higher than 20 mM, essentially all of the imino acid present at the steady state was the N-substituted one. This is true because glycine concentrations higher than 20 mM no longer diminished the maximum proton release attending the oxidation (Figure 3A). For this steady-state condition, we can write

$$v = k_2(\text{N-sub. IA}) \qquad (3)$$

where v is the oxidation reaction velocity, (N-sub. IA) represents the concentration of the N-substituted imino acid formed by the transimination reaction at infinite glycine concentration, and k_2 is the first-order hydrolysis constant for this N-substituted compound. Since the enzymatic reaction velocity is the same when glycine is not present, we can also write for this steady-state condition

$$v = k_1(\text{IA})_0 \qquad (4)$$

where k_1 is the first order hydrolysis constant for the imino acid analogue of leucine and $(IA)_0$ is the steady-state concentration of this imino acid when no glycine is present. Combining the two equations above, we have

$$k_2 = k_1 \frac{(IA)_0}{(\text{N-sub. IA})} \quad (5)$$

The value for k_1 is determined from Figure 5 as 4.2 min^{-1} at pH 7.9. From Figure 3A, the ratio $(IA)_0/(\text{N-sub. IA})$ is found to be 1.35/0.35, where the micromoles of protons released are equal to the micromoles of imino acid (essentially all unprotonated at this pH) present. (At high, saturating glycine concentrations, the only imino acid present in significant quantity is the N-substituted one.) Thus, from the above equation

$$k_2 = 4.2\frac{1.35}{0.35}\ min^{-1} = 16.2\ min^{-1} \qquad (t_{1/2} = 2.6\ s) \quad (6)$$

The comparison of the first-order rate constant for the hydrolysis of the Schiff base product of the reaction between glycine and the imino acid analogue of leucine (16.2 min^{-1}) with the analogous constant for the hydrolysis of the latter compound (4.2 min^{-1}) indicates that N substitution of the imino acid by a carboxymethyl group brings about a fourfold increase in the rate constant of hydrolysis. Thus, glycine actually catalyzes the hydrolysis of the imino acid formed enzymatically.

From the data in Figure 3A, it is also possible to calculate the second-order rate constant (k_3) for the reaction between imino acid (leucine analogue) and glycine. This reaction must involve an attack by the unprotonated (free base) form of glycine on the electrophilic carbon of the protonated imino acid. (Only the forms predominating at pH 8 are shown in Scheme I.) From Figure 3A, it can be seen that 1.6 mM total glycine was required to cause one-half of the maximum effect on the proton release at pH 7.9. At this glycine concentration, the rate of the transimination reaction equals the rate of imino acid hydrolysis, since the rate of each reaction represents one-half of the enzymatic reaction rate. Thus

$$k_1(IA) = k_3(IA)(R\text{-}NH_2) \quad (7)$$

or

$$k_3 = \frac{4.2\ min^{-1}}{1.6\ mM} = 2.6 \times 10^3\ min^{-1}\ M^{-1}$$

It should be noted that this value of k_3 was calculated in terms of the total glycine and imino acid concentrations.

When D-lysine was used in place of glycine in the above system, the pH course of the reaction indicated that the new, N-substituted Schiff base was more stable to hydrolysis than the parent imino acid. A calculation analogous to that presented for the glycine reaction gives the following value

$$k_2 = 0.95\ min^{-1} \qquad (t_{1/2} = 44\ s)$$

This calculation was based on the values of 5.5 and 1.25 μmol for the proton release observed at "infinite" and zero D-lysine concentration, respectively (Figure 3B). Thus, N substitution of the imino acid analogue of leucine by an alkyl group resulted in an approximately fourfold decrease in its rate of hydrolysis.

The calculation of k_2 for lysine refers to the hydrolysis rate of the transimination product with the ϵ-amino group rather than the α-NH_2 group. This interpretation is supported by the isolation of the $NaBH_4$-reduced Schiff base formed by this reaction when alanine and methionine were the substrates (see Hafner & Wellner, 1971, and Results), by the finding that ethylamine and methylamine react with the imino acid analogue of leucine to yield N-substituted imino acids that are more stable to hydrolysis than the original imino acid, and by the finding that alanine and methionine reacted like glycine, and not like lysine, in forming short-lived N-substituted imino acids in the leucine oxidation system. The value of k_3 was not calculated in the case of lysine, however, since it is possible that the ϵ-amino group reacts indirectly with the enzymatically formed imino acid following an initial attack by the α-amino group. The α-amino group of lysine would be expected to react faster than the ϵ-amino group because, due to its lower pK, a greater fraction of it is unprotonated at pH 7.9. However, because of its considerably greater stability, the product of reaction of the ϵ-amino group predominates at the steady state.

Reaction of Imino Acids with Cyanide. The addition of cyanide to the carbon–nitrogen double bond of imino acids appears to yield a stable adduct. Porter & Bright (1972a) have postulated this reaction to explain the effect of cyanide in reducing, by mass action effects, the concentration of the 550 nm absorbing intermediate (identified as a reduced enzyme–imino acid complex) observed during L-amino acid oxidase turnover experiments with L-phenylalanine as substrate. Our results are in agreement with this interpretation. In the same study, they also reported that, when high concentrations of L-phenylalanine (75 mM) were used, addition of cyanide to the reaction mixtures no longer reduced the already lowered levels of the 550-nm absorbing intermediate. A possible explanation of this result consistent with the results presented here is that phenylalanine at high concentrations decreases the level of the 550-nm absorbing intermediate by substantially reducing the steady-state concentration of the imino acid through a transimination reaction.

Reaction of Imino Acids with Hydrazine and Semicarbazide. The imino acids produced in the oxidase reaction react rapidly with semicarbazide and hydrazine to yield stable products. The kinetics of the reaction of semicarbazide with the imino acid analogue of leucine, showing a tenfold greater rate at pH 7.7 than at pH 8.7, suggests that the protonated form of the imino acid is the reactive species (Scheme I and Table II). The rapid reaction of imino acids with these reagents provides a convenient method to follow spectrophotometrically the course of the enzymatic reaction.

There are many other naturally occurring amine oxidation reactions in which carbonyl compounds are ultimately formed. It is likely that at least some of these proceed via an imine product. The present study suggests that the use of semicarbazide or hydrazine may be an easy and convenient method for demonstrating the existence of these imines.

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Role of Peripheral Side Chains of Vitamin B_{12} Coenzymes in the Reaction Catalyzed by Dioldehydrase†

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ABSTRACT: Isomers of adenosylcobalamin in which one of the three amide groups of the propionamide side chain of the corrin ring was converted to $-COOH$, $-COOCH_3$, or $-CONHCH_3$ were tested for coenzyme activity with dioldehydrase. The coenzyme activity of these nine isomers ranged from 86 to 7% that of the normal coenzyme. The rate-limiting step with the monocarboxylic acid analogues was the same as with the normal coenzyme as indicated by a 12-fold deuterium isotope effect on the maximal velocity of the reaction with [1,1-^{2}H]-1,2-propanediol. With the *b* site modifications of the coenzyme, the maximal velocities decrease in the order $-COO^-$ $\gg$ $CONHCH_3^-$, $COOCH_3$ suggesting inhibition by steric effects, while at the diagonal *e* site the maximal velocities decrease in the order $-CONHCH_3$, $-COO^-$, $-COOCH_3$ suggesting the importance of H-bond donation from coenzyme to enzyme. The binding of the adenosyl and cyano forms of the analogues to apoenzyme was reversible, although the hydroxo forms bind essentially irreversibly. This is in contrast to the adenosylcobalamin and cyanocobalamin which bind essentially irreversibly. In the presence of propanediol, the *e*-carboxylic acid, *e*-methyl ester, and *b*- and *e*-methylamide coenzymes brought about irreversible inactivation of the enzyme. Optical and EPR spectroscopy demonstrated that except for the complexes with the inactivating analogues, the holoenzymes of the other analogues generate an organic radical and cob(II)alamin during catalysis, whose steady-state concentrations correlated with their activity. The concentrations of the cob(II)alamin species and the radical species are equal. EPR spectra of the complexes with the four inactivating analogues show the accumulation of a disproportionally higher concentration of cob(II)alamin relative to the organic radical. These findings indicate the quenching of the organic radical intermediates in side reactions presumably due to their improper positioning. All of the four binary complexes of the inactivating analogues with the apoenzyme were relatively stable in the absence of substrate. In contrast, the complexes with the normal coenzyme and with the analogues which do not inactivate are unstable in the absence of substrate, presumably due to reaction of the radical species with O_2. This stability of the inactivating complexes suggests insufficient activation of the coenzyme, i.e., lower levels of the dissociated form. Since the modifications of the propionamide side chains used do not affect the intrinsic chemical properties of the coenzyme, all of the observed structural and kinetic effects result from alterations of the coenzyme–protein interaction. The present data thus indicate that the interactions of the coenzyme propionamide side chains with the apoprotein facilitate the homolytic cleavage of the C–Co bond and contribute to the stabilization of the radical intermediates.

Dioldehydrase (DL-1,2-propanediol hydrolyase, EC 4.2.1.28), an enzyme which requires a B_{12} coenzyme (adenosylcobalamin), catalyzes the conversion of several glycols to corresponding aldehydes, for instance, D- or L-1,2-propanediol to propionaldehyde and 1,2-ethanediol to acetaldehyde. A mechanism has been proposed for this reaction (see Scheme I) which is accepted by many, although not all, investigators (Schrauzer, 1971; Corey et al., 1977). An early event in the proposed mechanism is the homolytic cleavage of the C–Co bond of the coenzymes. How does the enzyme catalyze this cleavage? Consideration of the structural features of the coenzyme suggested a possible answer to this question. There are six amide groups, three propionamide groups, and three acetamide groups around the periphery of the corrin ring of the coenzyme. The possibility was considered that these amide groups interact through hydrogen bonds with amide groups of the protein. This interaction could lead to distortion of the corrin ring and possibly facilitate C–Co bond cleavage. These considerations led us to examine the role of the peripheral amide groups in the catalytic process. We prepared analogues of adenosylcobalamin in which each one of the propionamide groups was individually replaced by either $-COOH$, $-COOCH_3$, or $-CONHCH_3$. We investigated the interaction of these nine analogues with dioldehydrase. Our results are reported here.

Previously, some of the above-mentioned analogues have been prepared and some properties reported. It was reported

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0006-2960/79/0418-0417$01.00/0