RATES AND EQUILIBRIUM CONSTANTS OF IMINE FORMATION WITH PYRIDINE-4-ALDEHYDE AND VARIOUS AMINO ACIDS*

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Velick and Vavra (1962) have shown that the maximal velocity of glutamic-aspartic transaminase is independent of pH over the range 6 to 9. The first step in the enzymatic reaction is postulated to involve the formation of an imine from free amino acid ($RCH(NH_2)COO^{\ominus}$) and enzyme-bound cofactor. Hammes and Fasella (1963) have suggested that the same imine may be formed from protonated amino acid ($RCH(NH_3^{\oplus})COO^{\ominus}$) by way of a rapid intramolecular proton transfer that occurs before Schiff base formation. (If the rate of imine formation depended on free amine alone, the maximal velocity would increase markedly from pH 6 to 9.)

Cordes and Jencks (1963) have proposed that the rate-determining step of the over-all process of nitrogen derivative formation in aqueous solutions is the uncatalyzed, base-catalyzed, or acid-catalyzed dehydration of the carbinolamine addition intermediates. The dehydration of carbinolamine addition intermediates formed from weakly basic amines, such as hydroxylamine, is acid- and base-catalyzed, whereas derivatives of stronger bases, such as ethylamine, undergo uncatalyzed dehydration as well.

Here we report that for the reactions:

$$\underset{(AH)}{NH_3^{\oplus}\text{-}CHR\text{-}COO^{\ominus}} \overset{K_a}{\rightleftharpoons} \underset{(A)}{NH_2\text{-}CHR\text{-}COO^{\ominus}} + H^{\oplus} \qquad (1)$$

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$$AH + PCHO \underset{k_{-1}'}{\overset{k_1'}{\rightleftharpoons}} PCH{=}N\text{-}CHR\text{-}COO^{\ominus} + H^{\oplus} + H_2O$$
(I)

$$A + PCHO \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} I + H_2O$$

$$K_H = \frac{k_1'}{k_{-1}'} \quad ; \quad K_o = \frac{k_2}{k_{-2}}$$

(a). The second-order rate constants (k_1' and k_2) for reaction of protonated (AH) and unprotonated (A) amino acid with pyridine-4-aldehyde are of the same order of magnitude for eleven amino acids.

(b). Because k_1' and k_2 are nearly equivalent the apparent second-order rate constant (k_f) for imine formation is insensitive to changes in pH over the range examined ($pK_a' \pm 0.6$). It follows that both the rates of imine formation

$$\frac{d(I)}{dt} = k_f (A_T)(PCHO) \qquad (2)$$

$$A_T = AH + A$$

from AH, and from A, must be taken into account in the enzymatic transamination reaction.

(c). It is found (as anticipated from the derived relationship, $K_o/K_H = 1/K_a$) that plots of the apparent equilibrium constant for imine formation, based on A_T, vs pH provides titration curves that are characteristic of the pK_a' of the amino acid (equation 1). The values of K_o are of the same order of magnitude for all eleven amino acids investigated.

Theoretical considerations and experimental details will appear elsewhere. Further experiments with 3-hydroxypyridine-4-aldehyde, a closer analog of pyridoxal phosphate, are in progress.

EXPERIMENTAL

Methods - All reactions were carried out at 30° in aqueous solutions that were adjusted to one molar ionic strength with KCl.

Equilibrium constants were measured spectrophotometrically (Zeiss PMQ II) in a thermostated polyproplene cell that was fitted with quartz windows, a mechanical stirrer, a combination glass electrode (Radiometer GK 2021C), a nitrogen inlet, and a capillary tube leading from a solution of standard KOH contained in a micrometer buret. The base was added to neutral solutions of 0.1 M amino acid and 8×10^{-5} M aldehyde in the cell, and the optical density at 270 mμ (or 280 mμ for aromatic amino acids) and the pH were recorded 10 minutes after each addition. Equilibrium was reached within this time interval--a fact established from independent kinetic studies. A total of 7 to 12 points were taken in each run, and K_o was then calculated from equation 3:

$$K_o = (D - D_{PCHO}) / (D_I - D)[A] \qquad (3)$$

where D is optical density at 270 (or 280) mμ, between pH 7 and 10, and D_{PCHO} and D_I are the optical densities of aldehyde and imine under the same conditions, all corrected for the absorbance of the amino acid solution alone. [A] was calculated at each pH from the Henderson-Hasselbach equation, and the required pK_a' values were measured at different concentrations of amino acid by half-neutralization. The most accurate value of D_I was obtained by use of the double reciprocal plot of Lucas et al. (1961). The average deviations of duplicate and triplicate runs were generally 2% to 7%.

In the rate studies imine was formed by the addition of amino acid solution ($[A]/[A_T]$ = 0.2 to 0.8) from a thermostated syringe to a small volume of aldehyde solution in a thermostated cuvette. Pseudo first-order rate constants were calculated from plots of log $(D_\infty - D_t)$ vs time. Equations 4 and 5 were used to calculate apparent second-order rate constants

$$k_f = f\, k_{obs}/[A_T] \qquad (4)$$

$$f = K_o[A]/(1 + K_o[A]) \qquad (5)$$

where f is the fraction of complete imine formation at equilibrium; k_{obs} is the observed pseudo first-order rate constant.

RESULTS

Equilibrium constants for imine formation (Table I) - The highest value (leucine) is only three times that for the lowest (phenylglycine). In all cases there was no deviation from a straight line in the plot of Lucas et al. (1961), which indicates that only the free amino group is essential to the overall equilibrium.

TABLE I

EQUILIBRIUM CONSTANTS AND SECOND-ORDER RATE CONSTANTS FOR IMINE FORMATION

Amino Acid	K_o M^{-1}	k_1' M^{-1} sec^{-1}	k_2 M^{-1} sec^{-1}
Leucine	531	0.097	0.298
Valine	459	.051	.24
Arginine	403	.118	.158
Glycine	377	.153	.47
Phenylalanine	376	.138	.165
Serine	319	.081	.27
Aspartic Acid	306	.085	.143
Glutamic Acid	306	.105	.203
Asparagine	300	.185	.039
Alanine	280	.049	.33
Phenylglycine	177	.096	.074

Second-order rate constants for imine formation - The apparent second-order rate constants for imine formation at different

values of pH were nearly constant at low total amino acid concentration. Under these conditions:

$$k_f = k_1' \left[\frac{a_H}{K_a' + a_H} \right] + k_2 \left[\frac{K_a'}{K_a' + a_H} \right] \quad (6)$$

where a_H is the hydrogen ion activity. A plot of $k_f (K_a' + a_H)$ vs a_H yields a straight line (Fig. 1) with slope equal to k_1' and intercept equal to $k_2 K_a'$. The standard error generally lay between 3% and 8% at the 99% confidence level for both k_1' and k_2. Between the highest and lowest rate constants there is only a four-fold difference in k_1' and a twelve-fold difference in k_2 (Table I). For most amino acids, $1 < k_2/k_1' < 3$. Phenylalanine closely approaches the conditions for complete insensitivity of k_f to pH, since $k_1' \simeq k_2$. Asparagine and phenylglycine are the only amino acids for which $k_2/k_1' < 1$.

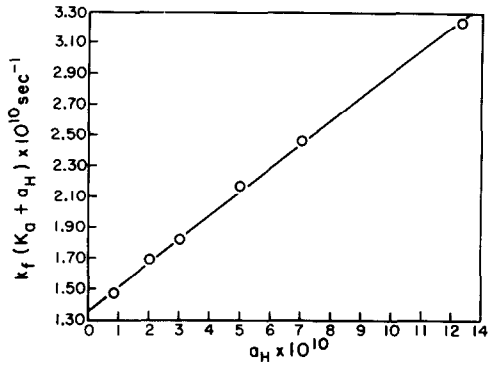

Fig. 1 -- Determination of second-order rate constants for the formation of $PCH{=}NCH_2COO^{\ominus}$ at $[A_T]$ = 0.02 M (see text).

Equation (6), which describes the pH sensitivity of the rate of imine formation may be rewritten in a form that indicates the mechanism of the reaction. By use of the relationship $k_1 = k_1'/K_a'$ equation (7) is obtained from (6).

$$k_f = \frac{(k_1 a_H + k_2) K_a'}{K_a' + a_H} \quad (7)$$

Substitution of equation (7) into equation (2) leads to equation (8).

$$\frac{d(I)}{dt} = (k_1 a_H + k_2)(NH_2\text{-}CHR\text{-}COO^{\ominus})(PCHO) \qquad (8)$$

For all the amino acids $k_1 \cong 10^9\ k_2$.

The near-equivalence of k_1' and k_2 in the model system, pyridine-4-aldehyde and amino acid, explains the observed independence of second-order rates of imine formation with respect to pH. These results may also lead to an explanation of the observed pH-independence of velocity of enzymatic transamination under the same mildly alkaline conditions.

REFERENCES

Cordes, E.H., and Jencks, W.P., J. Am. Chem. Soc., 85, 2843 (1963).
Hammes, G.G., and Fasella, P., J. Am. Chem. Soc., 85, 3833 (1963).
Lucas, N., King, H.K., and Brown, S.J., Biochem. J., 84, 1962 (1961).
Velick, S.F., and Vavra, J., J. Am. Chem. Soc., 85, 3029 (1963).