a symmetrical bridge a ruthenium–proton distance of 1.90 Å and a Ru–H–Ru angle of 101° is obtained.

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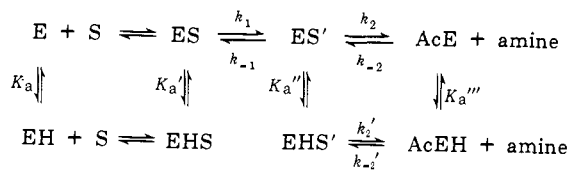
Concerning a Reported Change in Rate-Determining Step in Chymotrypsin Catalysis[1]

Sir:

A pH-induced change in the rate-determining step has been proposed for the chymotrypsin-catalyzed hydrolysis of formylphenylalanine formylhydrazide.[2] The basis of this proposal was a discrepancy between the pK of the Michaelis complex as determined from V_{max} and K_m measurements; values equal to 6.08 and 6.7, respectively, were determined from these kinetic parameters. The asymmetry of the proposed kinetic mechanism (Scheme I, where ES, ES′, and AcE represent

Scheme I



the Michaelis complex, a tetrahedral intermediate and acyl enzyme, respectively, and the site of proton addition is His-57) permits a change in the rate-determining step; at low pH k_2' (EHS′) > k_{-1} (ES′) so that the attack step (k_1) is rate limiting, and at high pH k_{-1} (ES′) > k_2 (ES′) and k_2' (EHS′), so that tetrahedral intermediate breakdown to the acyl enzyme is rate limiting. The change in the rate-determining step presumably makes the pK influencing V_{max} a "kinetic constant" which is lower than that influencing K_m. This interpretation was supported by studies of proton release associated with substrate binding;[3] as is required by the suggested mechanism no protons were found to be rapidly released. In disagreement with the earlier results[2] we have found the same pK (6.0–6.1) for the Michaelis complex from both V_{max} and K_m measurements.[4]

The distinctive feature of the proposed mechanism[2] (Scheme I) is the involvement of both imidazole and imidazolium paths in the formation and breakdown of the tetrahedral intermediate from the acyl enzyme: the k_2, k_{-2} and k_2', k_{-2}' reactions in Scheme I. To test this mechanism we have looked for the imidazolium-catalyzed path (k_{-2}') in the attack of formylhydrazine on a formylphenylalanyl enzyme; *i.e.*, the reverse of the reaction previously studied.[2] We have found that there is no reaction involving the imidazole conjugate acid. This result does not appear to be consistent with Scheme I.

The rate law for formation of formylphenylalanine formylhydrazide from a formylphenylalanyl enzyme (generated from the corresponding methyl ester[5]) is

$$\text{obsd } k_{amine}/(\text{amine}) = \frac{\dfrac{k_{-1}k_{-2}K_a'''K_a''}{(H)^2k_2'} + \dfrac{k_{-1}k_{-2}'K_a''}{(H)k_2'}}{1 + \dfrac{(k_{-1}+k_2)K_a'''K_a''}{(H)^2k_2'} + \dfrac{k_2'K_a''' + (k_{-1}+k_2)K_a''}{(H)k_2'}} \quad (1)$$

At high and low pH this reduces to eq 2 and 3, respectively.

$$\text{obsd } k_{amine}/(\text{amine}) = k_{-1}k_{-2}/(k_{-1}+k_2) \quad (2)$$

$$\text{obsd } k_{amine}/(\text{amine}) = k_{-1}k_{-2}'K_a''/(H)k_2' \quad (3)$$

Hydrolysis of the formylphenylalanyl enzyme follows the rate law

$$\text{obsd } k_{H_2O} = k_{H_2O}K_a'''/(K_a''' + (H)) \quad (4)$$

Measured values of k_{H_2O} (V_{max}) and pK_a''' were found to be equal to 85 sec^{-1} and 6.85, respectively (ionic strength 0.1, 25°). The apparently "normal" value of the pK for acyl enzyme hydrolysis suggests that this reaction does not involve a pH-dependent change in the rate-determining step (as was suggested for acyl enzyme aminolysis[2]) so that the pK obtained from the kinetics of the hydrolysis reaction is likely to be equal to K_a''' in Scheme I, rather than a complex constant, as was proposed for the acyl enzyme aminolysis.[6] We are not aware of any evidence suggesting a pH-dependent change in the rate-determining step in acyl enzyme hydrolysis. The result of the fact that there is only a single path for acyl enzyme hydrolysis (equiva-

(1) This investigation was supported by a Public Health Service Research Career Development Award to M. C. (1-K4-GM-10, 010-02) from the National Institutes for General Medical Studies and by a grant (DE03246) from the National Institutes of Health.

(2) A. R. Fersht and Y. Raquena, *J. Amer. Chem. Soc.*, **93**, 7079 (1971).

(3) A. R. Fersht, *ibid.*, **94**, 293 (1972).

(4) E. C. Lucas, M. Caplow, and K. J. Bush, *ibid.*, **95**, 2670 (1973).

(5) (a) That the reactions of the formylphenylalanine methyl ester and formylhydrazide go by the same acyl enzyme path was demonstrated by studying the effect of formylhydrazine on the hydrolysis of the formylhydrazide (see ref 5b for the rationale here). Formylhydrazine decreases the rate of this reaction and the decrease was equal to that predicted from studies of the partitioning of the acyl enzyme formed from the methyl ester; (b) M. Caplow and W. P. Jencks, *J. Biol. Chem.*, **239**, 1640 (1964).

(6) If identical mechanisms are assumed for acyl enzyme aminolysis and hydrolysis, the rate expression for hydrolysis will resemble eq 1. Both aminolysis and hydrolysis will have two kinetic pK's, equal to $[k_2'K_a''' + (k_{-1}+k_2)K_a'']/k_2'$ and $[(k_{-1}+k_2)K_a'''K_a'']/[k_2'K_a''' + (k_{-1}+k_2)K_a'']$, and these will undoubtedly be different for the two reactions. Also, for eq 1 to generate the observed simple sigmoidal pH–rate profile, it is required that in both hydrolysis and aminolysis $K_a''' = K_a''$, $k_{-1} > k_{-2} + k_{-2}'$ and $k_{-2} = k_{-2}'$. It is virtually inconceivable that all of these requirements are met for both reactions. The observation of an identical pH dependence for acyl enzyme hydrolysis and aminolysis would appear to dispose of the notion that Scheme I holds for *both* reactions.

lent to k_{-2} in Scheme I) and a dual path for acyl enzyme aminolysis (k_{-2} and k_{-2}') will result in a pH dependence for the partitioning of a formylphenylalanyl enzyme between water and formylhydrazine; at high pH there should be relatively less aminolysis than at low pH. Stated quantitatively, from eq 2–4 at high pH

$$\frac{\text{obsd } k_{\text{amine}}/(\text{amine})}{\text{obsd } k_{H_2O}} = \frac{k_{-1}k_{-2}}{(k_{-1} + k_2)k_{H_2O}} \quad (5)$$

and at low pH

$$\frac{\text{obsd } k_{\text{amine}}/(\text{amine})}{\text{obsd } k_{H_2O}} = \frac{k_{-1}k_{-2}'K_a''}{k_{H_2O}k_2'K_a'''} \quad (6)$$

The ratio of the partitioning of the acyl enzyme between amine and water at high and low pH is equal to eq 5/eq 6; this equals $k_2/(k_{-1} + k_2)$. The proposed change in rate-determining step[2] requires that $k_{-1} > k_2$, so that Scheme I predicts an increased yield of amide at lower pH. The results given in Table I are not consistent with Scheme I; acyl enzyme partitioning between water and formylhydrazine is invariant over the range pH 7.5–4.5. Scheme I does not apparently describe the enzyme-catalyzed hydrolysis of formylphenylalanine formylhydrazide.

Table I. Partitioning of a Formylphenylalanylchymotrypsin Intermediate between Water and Formylhydrazine as a Function of pH[a]

pH	[Amine],[b] M	[Enzyme], M	% amide formed[c]	$k_{\text{amine}}/k_{H_2O}$[d]
7.5	0.0505	1×10^{-7}	5.76	1.21
7.5	0.101	1×10^{-7}	10.5	1.16
6.0	0.0505	4×10^{-6}	5.56	1.17
6.0	0.101	4×10^{-6}	10.7	1.19
4.5	0.0493	1×10^{-4}	5.44	1.17
4.5	0.0986	1×10^{-4}	10.2	1.17

[a] Reactions were run at 25° in 0.1 M KCl with an initial concentration of N-formyl-^{14}C-phenylalanine methyl ester (2.18×10^5 cpm/μmol) of 0.7 mM. The pH was controlled with a pH stat and reactions were followed to completion as indicated by a cessation of proton release. [b] Concentration of the amine free base. [c] The yield of amide was determined by an isotope dilution technique. [d] Equal to (% amide)/(% carboxylic acid) (concentration of the amine free base).

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Determination of the Average Singlet–Triplet Splitting in Biradicals by Measurement of the Magnetic Field Dependence of CIDNP[1]

Sir:

We have previously reported the CIDNP spectra of alkenal products resulting from photochemical α cleavage of alicyclic ketones.[2] We now report measurements of the average singlet–triplet splitting in biradicals resulting from α cleavage, based on the magnetic field dependence of the CIDNP aldehyde signal in the alkenal products. To our knowledge, this is the first determination of the average isotropic exchange interaction in highly reactive biradical species.

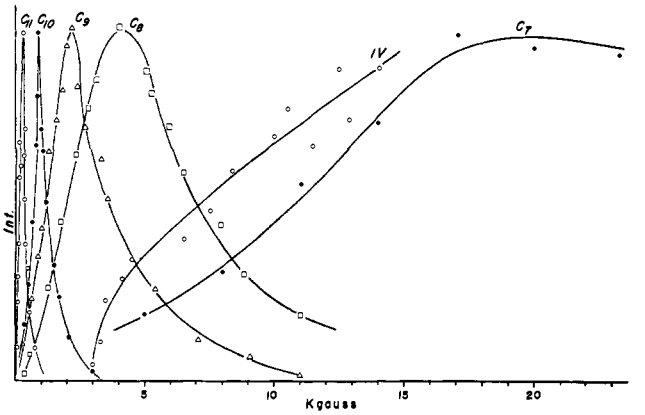
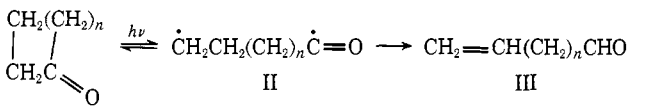
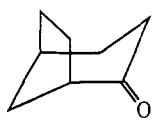


Figure 1. Intensities of aldehyde proton emission signals of IIIa–e (C_7–C_{11}) and of aldehyde derived from IV, as function of magnetic field. The intensities are in arbitrary units and not normalized among the different compounds.

Irradiation of ketones Ia–e and IV in chloroform solution yielded the corresponding alkenals. Each irradiation was carried out for 50 sec on a 0.140 M



a, $n = 4$; b, $n = 5$; c, $n = 6$; d, $n = 7$; e, $n = 8$



IV

sample of ketone in chloroform placed between the pole pieces of a Varian 12-in. magnet at the desired field strength. The sample was then transferred quickly to the probe of an HA-100 spectrometer, and the aldehyde CIDNP signal was immediately recorded on a CAT. For each ketone, the relative integrated intensity of aldehyde CIDNP signal is plotted as a function at the field strength at which the sample was irradiated and is displayed in the curves of Figure 1.

These curves show two striking features. As the length of the biradical increases, the maxima move to lower energies, and the curve widths decrease dramatically. The first of these phenomena is straightforward to interpret.

For simplicity we first consider a fictitious, totally rigid biradical of the type II with a singlet–triplet splitting $2J$, corresponding to an effective exchange Hamiltonian $-J(1/2 + 2\mathbf{S}_1 \cdot \mathbf{S}_2)$. Moreover, the principal hyperfine-induced singlet–triplet mixing will occur between the T– and S states.[2] By varying the magnetic field H_0 in which the biradical is created, one can shift the T– level (with energy $g\beta H_0$ below the T_0 level) to become essentially degenerate with the S level. From

(1) Supported by grants from the Petroleum Research Fund (3965 C-4), administered by the American Chemical Society, and the National Science Foundation (GP 18719X).
(2) G. L. Closs and C. E. Doubleday, *J. Amer. Chem. Soc.*, **94**, 9248 (1972).