for that of free benzoate. These rate constants are in agreement with those expected from the Debye relationship for the bimolecular reaction of +2, 0, and −1 charged species, respectively, in aqueous solution. The direct electron transfer from Co(III) to OH to generate Co(IV) can be ruled out since such a step would result in a +3 charged transient, the second-order disappearance of which would be expected to have a rate constant of $\sim 10^6\ M^{-1}\ \text{sec}^{-1}$. If the observed decay of the complex radical transient contained a substantial first-order component, $t_{1/2}$ would be greater than 200 μsec in agreement with Taube's conclusion on the basis of his kinetic data[1] that internal electron transfer between the ligand radical and the metal center is not a rapid process. It should be noted that we find that the free benzoate OH adduct is unreactive toward $Co(NH_3)_6^{3+}$.

On the basis of all the evidence we conclude that the OH radical adds to the benzoato ligand to form $(NH_3)_5Co^{III}(O_2CC_6H_5OH)^{2+}$. The absorption spectrum of this transient species, resulting from transitions localized on the benzoato OH radical, is intermediate between those for the protonated and anionic forms of the OH adduct to free benzoate in keeping with the intermediate nature of the carboxylate group coordinated to the metal center.

It is of interest to note that $(NH_3)_5Co(O_2CPh)^{2+}$ reacts readily with H atoms (at pH 1 in the presence of 1 *M tert*-butyl alcohol as an OH scavenger) to form an H atom adduct (λ_{max} 350 nm) which appears to be identical with that formed from H atom attack on benzoic acid.[7,8] On the other hand, reaction of the complex with e_{aq}^- does not give the transient with λ_{max} 310 nm obtained from e_{aq}^- addition to free benzoate at pH 7–11;[8] e_{aq}^- evidently reduces Co(III) to Co(II), directly or indirectly, in less than 1 μsec. Substitution of an NO_2 group para on the ligand ring increases the lifetime of the e_{aq}^- adduct sufficiently for its spectrum to be recorded.

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The Use of Proflavin as an Indicator in Temperature-Jump Studies of the Binding of a Competitive Inhibitor to Trypsin

Sir:

The acridine dye proflavin is a strong competitive inhibitor for specific substrates of trypsin[1] and chymotrypsin.[1-4] The binding of dye to either enzyme is accompanied by a substantial shift in the visible absorption spectrum[1,3,5] which serves as a convenient means of detecting complexes between trypsin or chymotrypsin and nonchromophoric substrates and competitive inhibitors. In particular, the displacement of proflavin has been employed to follow the appearance and disappearance of transient intermediates in enzymic catalysis. To date such studies have been limited to stopped-flow measurements far from chemical equilibrium.[1,3,6,7]

It would also be of interest to use proflavin as an indicator for investigating systems in a state of true or pseudoequilibrium by relaxation techniques. No applications of this kind have yet appeared in the literature, however. In the present communication we report thermodynamic and kinetic parameters for the binding of proflavin to trypsin as measured with the temperature-jump technique.[8] In addition, proflavin has been employed as an indicator to estimate thermodynamic and kinetic parameters for the binding to trypsin of the nonchromophoric competitive inhibitor benzamidine.

Kinetic Relationships. By means of the temperature-jump technique, formation and dissociation rate constants for the enzyme–proflavin complex can in principle be evaluated from the linear relationship of eq 2.[8]

$$E + P \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} EP \quad K_{21} = \bar{c}_P\bar{c}_E/\bar{c}_{EP} \tag{1}$$

$$1/\tau_{12} = k_{12}(\bar{c}_E + \bar{c}_P) + k_{21} \tag{2}$$

In the presence of a second competitive inhibitor, I, the minimal reaction scheme which must be considered is

$$\begin{array}{ccc} & I & \\ & + & \\ P + E & \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} & EP \quad K_{31} = \bar{c}_E\bar{c}_I/\bar{c}_{EI} \\ & k_{21}\uparrow\downarrow k_{13} & \\ & EI & \end{array} \tag{3}$$

Mechanism 3 is characterized by two relaxation times

$$1/\tau_{1,2} = S/2(1 \mp \sqrt{1 - 4P/S^2}) \tag{4}$$

S and P are the trace and determinant of the matrix[8] associated with the linearized rate equations: $S = a_{11} + a_{22}$, $P = a_{11}a_{22} - a_{12}a_{21}$. Choosing δc_{EP} and δc_{EB} as variables x_1 and x_2, respectively,[8] the a_{ij} for mechanism **3** are: $a_{11} = k_{12}(\bar{c}_E + \bar{c}_P) + k_{21}$, $a_{12} = k_{12}\bar{c}_P$, $a_{21} = k_{13}\bar{c}_I$, $a_{22} = k_{13}(\bar{c}_E + \bar{c}_I) + k_{31}$. If proflavin binding is rapid enough to equilibrate independently (*i.e.*, $a_{11} = 1/\tau_1 \gg a_{22}$), eq 4 may be expanded,[9] yielding expression 2 for $1/\tau_1$ and

$$1/\tau_2 = k_{13}(\bar{c}_E + \alpha\bar{c}_I) + k_{31}$$
$$\alpha = (\bar{c}_E + K_{21})/(\bar{c}_E + \bar{c}_P + K_{21}) \tag{5}$$

In a practical application the $\bar{c}$'s would be calculated using K_{21} and K_{31}, and then k_{13} and k_{31} could be obtained graphically from the plot of $1/\tau_2$ *vs.* $(\bar{c}_E + \alpha\bar{c}_I)$.

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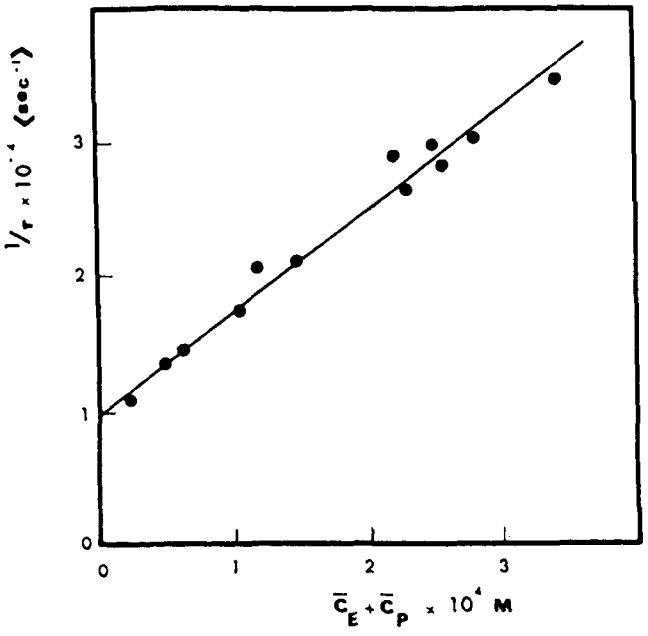

Figure 1. Concentration dependence of the reciprocal relaxation times for the binding of proflavin to trypsin; pH 7.7, 0.07 M Tris chloride, 0.020 M $CaCl_2$, 17°.

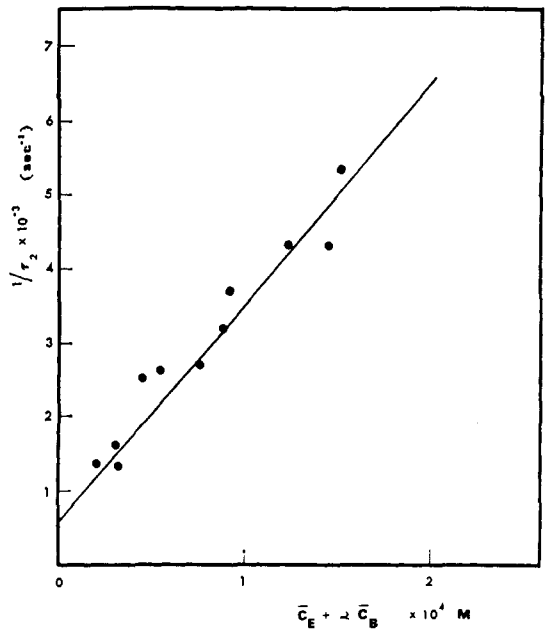

Figure 2. Concentration dependence of the reciprocal relaxation times for the binding of benzamidine to trypsin (refer to Figure 1 for experimental conditions).

Thermodynamic Relationships. Although it has been recognized for some time that temperature-jump measurements can yield information on reaction enthalpies,[8] there exist only a few examples of practical applications.[10] For the mechanism of eq 1 it can be shown that

$$\delta I_{12}^0/I^0 = -(2.30\Delta\epsilon_{12}\Delta H_{12}dT/RT^2)\Gamma_{12} \quad (6)$$

where $\delta I_{12}^0/I^0$ (I^0 = light intensity at time zero) is the measured relaxation amplitude, $\Delta\epsilon_{12} = (\epsilon_{EP} - \epsilon_P)$, $\Delta H_{12} = H_{EP} - H_E - H_P$, and dT = temperature change (defined as a positive quantity). The Γ function for a bimolecular reaction is[8] $\Gamma_{12} = \bar{c}_E\bar{c}_P/(\bar{c}_E + \bar{c}_P + K_{21})$. If K_{21} and $\Delta\epsilon_{12}$ are known, the enthalpy can be calculated from the slope of $\delta I_{12}^0/I^0$ *vs.* Γ_{12}.

The enthalpy of EI formation (mechanism 3) can also be determined from relaxation amplitude data. In the general case, the amplitudes of the two relaxation effects may be expressed as linear combinations of ΔH_{12} and ΔH_{13}[11]

$$\frac{\delta I_1^0}{I^0} = \left[\frac{(a_{11}(1 - \Gamma_{12}\Gamma_{13}/\bar{c}_E^2) - \tau_2^{-1})\Delta H_{12} + (\Gamma_{13}\tau_2^{-1}/\bar{c}_E)\Delta H_{13}}{(\tau_2^{-1} - \tau_1^{-1})(1 - \Gamma_{12}\Gamma_{13}/\bar{c}_E^2)}\right](F)\Gamma_{12} \quad (7)$$

$$\frac{\delta I_2^0}{I^0} = \left[\frac{-(a_{11}(1 - \Gamma_{12}\Gamma_{13}/\bar{c}_E^2) - \tau_1^{-1})\Delta H_{12} - (\Gamma_{13}\tau_1^{-1}/\bar{c}_E)\Delta H_{13}}{(\tau_2^{-1} - \tau_1^{-1})(1 - \Gamma_{12}\Gamma_{13}/\bar{c}_E^2)}\right](F)\Gamma_{12} \quad (8)$$

where $F = (2.30\Delta\epsilon_{12}dT)/RT^2$, $1/\tau_{1,2}$ and a_{11} refer to eq 4, $\Delta H_{13} = H_{EI} - H_E - H_I$, and $\Gamma_{13} = \bar{c}_E\bar{c}_I/(\bar{c}_E + \bar{c}_I + K_{31})$. Under conditions where the proflavin equilibration is decoupled from the binding of I, eq 7 and 8 simplify to $\delta I_1^0/I^0 = \delta I_{12}^0/I^0$ (see eq 6), and

$$\delta I_2^0/I^0 = \left[\frac{2.30\Delta\epsilon_{12}\Delta H_{13}dT}{RT^2}\right]\left[\frac{1 - \beta\Gamma_{12}/\bar{c}_E}{1 - \Gamma_{12}\Gamma_{13}/\bar{c}_E^2}\right]\frac{\Gamma_{12}\Gamma_{13}}{\bar{c}_E} \quad (9)$$

where $\beta = \Delta H_{12}/\Delta H_{13}$. When $\beta\Gamma_{12}/\bar{c}_E$ is negligible, the resulting linear concentration dependence of the amplitude gives ΔH_{13} directly. If $\beta\Gamma_{12}/\bar{c}_E$ is not negligible, ΔH_{13} could be evaluated by fitting the data to eq 9 using trial values of β.

Results. The stoichiometry of eq 1 was verified by spectrophotometric titration of proflavin by trypsin at 469 nm. An iterative fitting of the data gave $K_{21} = 1.1 \pm 0.1 \times 10^{-4}$ M and $\Delta\epsilon_{469} = 2.62 \pm 0.08 \times 10^4$ OD M^{-1} cm^{-1} (pH 7.7, 0.06 M Tris chloride, 0.02 M $CaCl_2$, 16°).[12]

Most temperature-jump measurements of the trypsin–proflavin reaction were made in the concentration range $\bar{c}_P = 0.6\text{–}2.0 \times 10^{-5}$ M and $\bar{c}_E = 0.9\text{–}34 \times 10^{-5}$ M. A single relaxation effect was observed, corresponding to a decrease in OD_{469}. A least-squares fitting of the kinetic results to eq 1 gave $k_{12} = 7.6 \times 10^7$ M^{-1} sec^{-1} and $k_{21} = 9500$ sec^{-1} (Figure 1). A plot of the relaxation amplitudes *vs.* Γ_{12} (eq 6) was reasonably linear and consistent with a ΔH_{12} of −3.7 kcal/mol. The error in ΔH_{12} is about ± 1 kcal/mol.

Benzamidine is known to be a strong competitive inhibitor of trypsin.[15] Using the above $\Delta\epsilon_{469}$ and K_{21} values, K_{31} was estimated to be $1.2 \pm 0.1 \times 10^{-5}$ M from a titration of the trypsin–proflavin complex with benzamidine.[1,3,6]

(10) See, for example, G. H. Czerlinski, and J. Malkewitz, *Biochemistry*, 4, 1127 (1965); R. Winkler, Doctoral Thesis, Göttingen, 1969.

(11) D. Thusius, unpublished.

(12) These parameters are in poor agreement with the results of Glazer.[6] In the present work, inactive protein, known to be present in commercial trypsin,[13] was removed by precipitation in 1 M NaCl.[14] Without this purification, our titration data approach those of Glazer, Figure 4.[5]

(13) D. D. Schroeder and E. Shaw, *J. Biol. Chem.*, 243, 2943 (1968).

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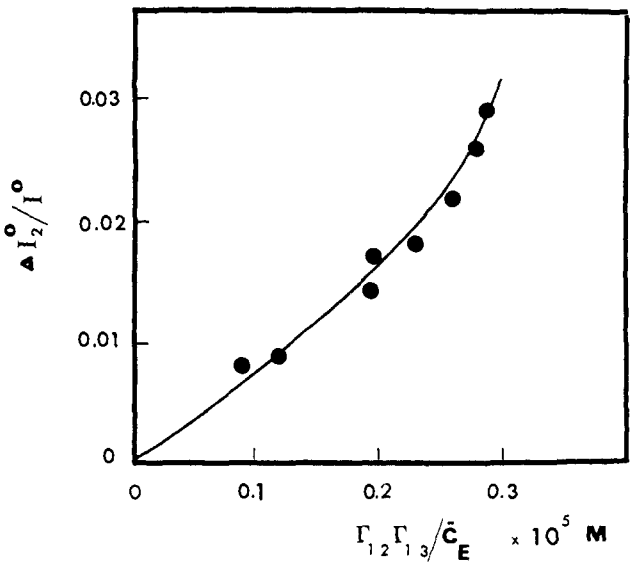

Figure 3. Concentration dependence of the relaxation amplitudes for the binding of benzamidine to trypsin. The solid line was calculated from eq 8 using values for the specific rate constants, equilibrium constants, $\Delta\epsilon_{12}$, and ΔH_{12} given in the text, and assuming $\Delta H_{13} = -4.5$ kcal/mol. Calculations were facilitated by programming the procedure for a Wang electronic calculator.

Addition of benzamidine to solutions of proflavin and trypsin resulted in the appearance of a second, slower relaxation effect, associated with an increase in OD_{469}. In Figure 2 the reciprocal relaxation times of the slow effect are plotted according to eq 5. From the best straight line through the data points we calculate $k_{13} = 2.9 \times 10^7\ M^{-1}\ \text{sec}^{-1}$ and $k_{31} = 6 \times 10^2\ \text{sec}^{-1}$. Although the experimental τ's were the same order of magnitude, substitution of the $\bar{c}$'s and four experimental k's into eq 4 reproduced the relaxation times to ± 10–20%, justifying the use of eq 5 as a first approximation.

The results of the amplitude analysis for the slow relaxation process are presented in Figure 3. In the present work, equilibrium concentrations were such that the quantity $(1 - \beta\Gamma_{12}/\bar{c}_E)/(1 - \Gamma_{12}\Gamma_{13}/\bar{c}_E{}^2)$ was close to unity (assuming $\beta \lesssim 1$). From eq 9 a plot of $\delta I_2^0/I^0$ *vs.* $\Gamma_{12}\Gamma_{13}/\bar{c}_E$ should then be approximately linear. However, calculations suggest that the upward trend in Figure 3 is genuine and arises from coupling between the two steps. A good fit to the data was obtained using the *general* expression of eq 8 and assuming $\Delta H_{13} = -4.5$ kcal/mol. The uncertainty in this figure is probably about ± 1.5 kcal/mol.[16]

The enthalpies[19] and specific rate constants[20] evaluated here are consistent with simple ligand binding processes. It is emphasized, however, that we have assumed the simplest mechanism consistent with the majority of the data. In particular, the path I + EP $\rightleftarrows$ EI + P could play a minor, but significant role. Experiments dealing with this point and applications of the method to other systems are now under way.

(16) Coupling to rapid protolytic equilibria must be considered when interpreting amplitude data for enzyme reactions. However, the fact that the equilibrium constants for the proflavin–α-chymotrypsin[17] and benzamidine–trypsin[18] complexes are constant between pH 7 and 8 suggests that protolytic reactions are not important in the present study.

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A Remarkably High Stereoselectivity in the Addition of Acetic Acid and Trifluoroacetic Acid to Norbornene. Evidence for the Capture of the Norbornyl Cation in an Unsymmetrical State

Sir:

The addition of acetic acid at 100° and of trifluoroacetic acid at 0° proceeds to give the *exo*-norbornyl esters in stereochemical purities of 99.98%. Such exceptionally high stereoselectivities have been considered to require the symmetrically σ-bridged nonclassical norbornyl cation as an intermediate. Yet the distribution of the deuterium atom in the addition of the respective deuterio acids does not correspond to that required by a reaction proceeding through such a symmetrical intermediate.

The addition of acetic acid to norbornene proceeds slowly at 100° to give 2-norbornyl acetate with a remarkably high exo stereoselectivity: 99.97% exo in the absence of sodium acetate and 99.99% exo in the presence of sodium acetate.[1,2] This stereoselectivity is comparable to (actually slightly higher than) the value realized, 99.95% exo in the acetolysis of *exo*-norbornyl tosylate at 100° in the presence of 0.6 *M* sodium acetate.[4–6]

Such an extraordinarily high stereoselectivity has been considered previously to require the intermediacy of a σ-bridged cation.[7] On this basis, the addition of deuterioacetic acid to norbornene should proceed with an equal distribution of the deuterium tag between the exo-3 and syn-7 positions (eq 1). However, this does not occur.

Under the conditions of the previous study acetic acid-d_4 adds to norbornene to yield 2-norbornyl-*d* acetate-d_3 of the same high exo stereoselectivity.[8] The product was converted to *exo*-norbornanol-*d* (1) with

(1) Both esters are stable to the reaction conditions.

(2) The rate of addition was 2.8 times faster than that of the corresponding addition to 7,7-dimethylnorbornene.[3]

(3) H. C. Brown, J. H. Kawakami, and K.-T. Liu, *J. Amer. Chem. Soc.*, 92, 3816 (1970).

(4) Analyzed by glpc using 150 ft × 0.01 in. TCEP column at 70° on a Perkin-Elmer Model 226 gas chromatograph.

(5) Dr. Charles B. Schewene carried out independent analyses at the University of Wisconsin. Excellent agreement was realized between the two sets of analysis. We are grateful for his generous assistance.

(6) See also H. L. Goering and C. B. Schewene, *J. Amer. Chem. Soc.*, 87, 3516 (1965).

(7) S. Winstein, E. Clippinger, R. Howe, and E. Vogelfanger, *ibid.*, 87, 376 (1965).

(8) The rate of addition is approximately one-half that observed for normal acetic acid.