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Mechanism of Azide Binding to Chloroperoxidase and Horseradish Peroxidase: Use of an Iodine Laser Temperature-Jump Apparatus†

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ABSTRACT: The kinetics of azide binding to chloroperoxidase have been studied at eight pH values ranging from 3.0 to 6.6 at 9.5 ± 0.2 °C and ionic strength of 0.4 M in H_2O. The same reaction was studied in D_2O at pD 4.36. In addition, results were obtained on azide binding to horseradish peroxidase at pD 4.36 and pH 4.56. Typical relaxation times were in the range 10–40 μs. The value of k_H/k_D(on) for chloroperoxidase is 1.16, and k_H/k_D(off) is 1.7; corresponding values for horseradish peroxidase are 1.10 and 2.4. The H/D solvent isotope effects indicate proton transfer is partially rate controlling and is more important in the dissociation of azide from the enzyme–ligand complex. A mechanism is proposed in which hydrazoic acid binds to chloroperoxidase in a concerted process in which its proton is transferred to a distal basic group. Hydrogen bonding from the newly formed distal acid to the bound azide facilitates formation of hydrazoic acid as the leaving group in the dissociation process. The binding rate constant data, k_{on}, can be fit to the equation $k_{on} = k_3/(1 + K_A/[H^+])$, where $k_3 = 7.6 \times 10^7$ M^{-1} s^{-1} and K_A, the dissociation constant of hydrazoic acid, is 2.5×10^{-5} M. The same mechanism probably is valid for the ligand binding to horseradish peroxidase.

Azide is a well-known inhibitor of many enzyme systems. The study of the kinetics of azide binding to heme enzymes has been hampered because the relaxation time of the reaction falls within the dead time of most instrumental techniques. Thus, the kinetics of azide binding to horseradish peroxidase required the use of an ultrafast (coaxial cable) temperature-jump apparatus (Morishima et al., 1978). In this paper we report on the kinetics of azide binding to chloroperoxidase at eight pH values over the accessible pH range using an ILTJ[1]

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[1] Abbreviations: ILTJ, iodine laser temperature-jump apparatus; R.Z., reinheitzahl (purity number).

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apparatus. Also the solvent deuterium isotope effect is reported for azide binding to both horseradish peroxidase and chloroperoxidase.

Materials and Methods

Chloroperoxidase from *Caldariomyces fumago* was isolated and purified by established procedures (Pickard, 1981; Carmichael et al., 1986; Hager, 1970). A stock solution of purified chloroperoxidase (R.Z. 1.41, final concentration 1.1 mM) was dialyzed against 2 mM phosphate buffer, pH 5.2. Horseradish peroxidase stock solution, final concentration 1.0 mM, was prepared by dialyzing Boehringer-Mannheim grade I horseradish peroxidase (R.Z. 3.0) against deionized water. The stock enzyme solutions were stored at ~4 °C. Citric acid–phosphate buffers were prepared on the basis of literature data (Dawson et al., 1969). According to these data, constant ionic strength buffers could be prepared by adding specified amounts of potassium chloride. Since chloride ion binds weakly in the active site of chloroperoxidase at low pH (Lambeir & Dunford, 1983; Sono et al., 1986), we used potassium sulfate instead to bring all buffers to a constant ionic strength of 0.40 M. pH values were checked on a Mikroprocessor pH meter 741 from Knick, Berlin, in combination with an electrode from Schott, Mainz. A single stock solution of 0.5 M sodium azide was used for all experiments. All chemicals, reagent grade, were from Merck, Darmstadt. Water was triply distilled from a quartz apparatus; D_2O was from Merck. Solutions were prepared approximately 0.5 h before the experiments to a total volume of 2.0 mL with Eppendorf pipets. The solution composition was 0.20 mL of stock enzyme solution, a variable amount of sodium azide, and the remainder buffer. The largest final sodium azide concentration was 75 mM for one solution at pH 6.6 and was normally much smaller; thus, it had negligible effect on the total ionic strength. For the deuterium oxide experiments, buffer was prepared in an identical fashion with that for pH 4.0 in H_2O, and the measured pH was 4.0 with the meter calibrated with buffer made up in H_2O. Enzyme and azide solutions prepared in H_2O were added. The measured pH can be corrected according to the formula

$$pD = pH_{measured} + 0.4 \quad (1)$$

(Glasoe & Long, 1960; Covington et al., 1968). Because of the small amount of H_2O in our D_2O preparation, we add 0.36 to obtain our final pD value of 4.36.

For the chloroperoxidase experiments at pH 3.0 and horseradish peroxidase at pD 4.36, the enzyme was not added to the solution until 5 min before the temperature-jump experiments. Kinetic experiments were usually finished after 20 min. Spectral checks of the Soret region absorption on a Perkin-Elmer Model 555 UV–visible spectrophotometer showed no sign of enzyme decomposition.

Kinetic experiments were performed on our ILTJ equipment. Details of the laser itself are given elsewhere (Holzwarth, 1979a; Holzwarth et al., 1977, 1984; Bannister et al., 1984). Here we describe briefly the laser properties, the experimental arrangement for optical absorption kinetic measurements, and the detection and data processing system.

The ILTJ apparatus produces an increase in the temperature of an aqueous solution by direct optical excitation of vibrational–rotational states of water (H_2O or D_2O) molecules at a wavelength of 1.315 μm. The temperature jump produced in this way is as fast as the laser emission, which may vary from 200 ps to 2.4 μs depending upon the mode of operation. For the experiments reported in this paper we used a laser pulse which produced a temperature jump of 1 °C in 2 μs. The heating occurred in 150 μL of the sample solution contained

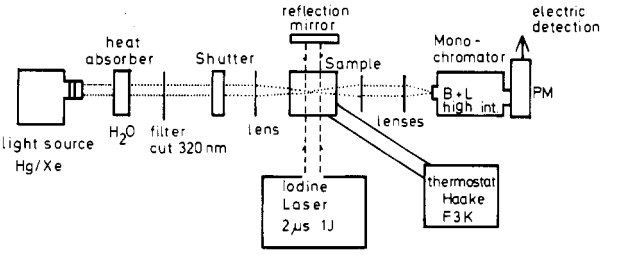

FIGURE 1: Schematic diagram of the arrangement of the iodine laser temperature-jump (ILTJ) apparatus. The transmitted detection light passes through a Bausch and Lomb high-intensity monochromator to the photomultiplier tube from which it is processed and analyzed.

in a quartz cuvette (QI 5 × 10 × 50 mm from Hellma, Mülheim). The laser beam and detection light were perpendicular (Figure 1). The detection light source was a Hg/Xe arc lamp (Oriel and Hannovia 200W). We followed absorption changes caused by the temperature jump at a wavelength of 388 ± 10 nm for horseradish peroxidase and 395 ± 10 nm for chloroperoxidase. To avoid photochemical damage to the enzyme solutions, the detection light beam was limited to a duration of 8 s for each experiment.

The signals from the photomultiplier tube (RCA 1P28) and a homemade electrical circuit (Holzwarth, 1979) were fed into a Tektronix 7904 oscilloscope equipped with a 7A22 amplifier and a 7B92A time base (Tektronix, Portland) allowing for bandwidth and sensitivity selection. The analog signal from the oscilloscope was connected to a Biomation 8100 transient digitizer used in the dual time base mode for digitizing and recording of the signals. The digitizer was linked through an IEEE bus to a Hewlett-Packard HP9816 computer. Further data processing using the computer included sampling, calculation of relaxation times and amplitudes, and data plotting. The program which we used allowed for the calculation of two relaxation times simultaneously (if necessary) and calculated the correct relaxation amplitudes. Typical sensitivity of the instrument was better than $1/10^4$ at a bandwidth of 300 kHz.

The cuvette was placed in a black metal holder which allowed only the desired light to pass through the sample. A Haake F3 thermostat (Berlin) was used to control the initial temperature to 8.5 ± 0.2 °C. Thus, the temperature of the relaxation processes following the temperature jump was 9.5 ± 0.2 °C. For most calculations of relaxation times and amplitudes, we used the average of two temperature-jump experiments.

The laser of the ILTJ was contained in a ~8 ft × 12 ft walk-in Faraday cage, so that the laser and detection electronics were completely isolated. The ILTJ offers the following advantages over commercial Joule heating temperature-jump equipment: (1) high quality of the electrical parts of the detection system; (2) direct optical heating of the water solvent so that electric field effects are eliminated; (3) the size and rise time of the temperature jumps being independent of ionic strength.

Results

All of our results are consistent with 1:1 binding of azide to peroxidase. No indication was ever obtained of a second chemical relaxation process. Therefore, the phenomenological equation

$$E_{tot} + azide_{tot} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} E_{tot}{\cdot}azide_{tot} \quad (2)$$

describes all of the kinetic results. A typical relaxation result is shown in Figure 2, where the measured relaxation time τ_{obsd} is 26.0 μs. For small values of τ_{obsd} we corrected for interaction

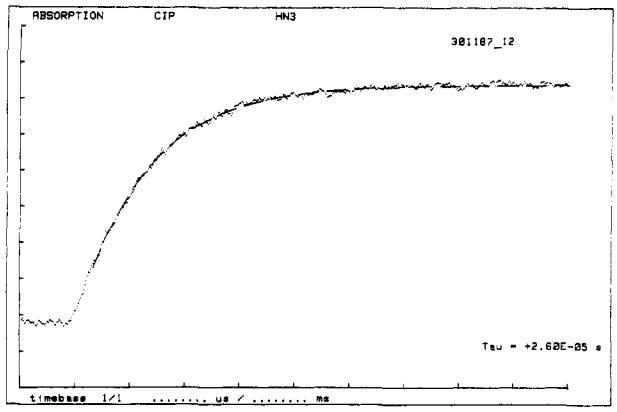

FIGURE 2: Photograph of the printout from the Hewlett-Packard computer for an experiment conducted at pH 3.0, 11 μM chloroperoxidase, 2.5×10^{-4} M sodium azide, final temperature 9.5 °C, and ionic strength of 0.4 M. The digitized printout contains 500 data points. The beginning of the solid line indicates the starting position of the data points used to calculate the best-fit solid curve, for which the relaxation time, τ, is 26.0 μs. Vertical scale 1 mV/division. Overall signal 840 mV. Horizontal scale 20 μs/division. The data are the average of two temperature-jump experiments.

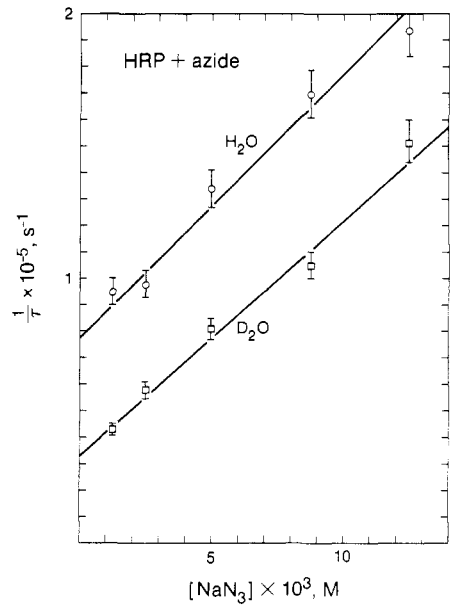

FIGURE 3: Reciprocal relaxation time ($1/\tau$) versus concentration of sodium azide plots for the reaction of sodium azide with horseradish peroxidase in H_2O, pH 4.56, and D_2O, pD 4.36. In this region the reaction is insensitive to small changes in pH/pD. From the ratio of slopes, one obtains k_H/k_D(on) = 1.10, and from the ratio of intercepts, one obtains k_H/k_D(off) = 2.4.

with the 2-μs rise time ($\tau_{instrumental}$) of the temperature jump according to

$$\tau_{obsd} = \sqrt{\tau_{chem}^2 + \tau_{instrumental}^2} \quad (3)$$

(Turner, 1986) where τ_{chem} is the chemical relaxation time for the azide binding and dissociation process. For τ_{obsd} values of 15 μs, the correction falls within the experimental error. For a τ_{obsd} value of 5.0 μs, one obtains τ_{chem} = 4.6 μs. The smallest τ_{obsd} value measured in this study was 6.21 μs; the usual values were in the 10–40-μs range. Henceforth, we shall use the simpler designation τ for the chemical relaxation τ_{chem}.

The relation among τ and the apparent binding and dissociation rate constants k_{on} and k_{off} is

$$1/\tau = k_{on}([E]_{tot} + [azide]_{tot}) + k_{off} \quad (4)$$

where total molar concentrations of enzyme and sodium azide

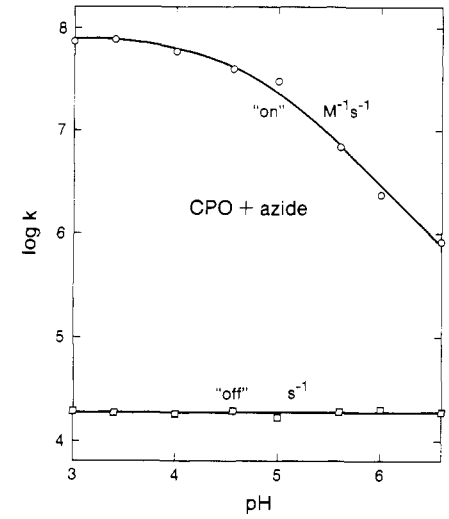

FIGURE 4: Rate constant data for the binding of hydrazoic acid to chloroperoxidase (k_{on}) and the dissociation of hydrazoic acid from chloroperoxidase (k_{off}) as a function of pH. The best-fit line through the log k_{on} data is calculated with the parameters $k_3 = 7.6 \times 10^7$ M^{-1} s^{-1} and $K_A = 2.5 \times 10^{-5}$ M from the equation $k_{on} = k_3/(1 + K_A/[H^+])$, where K_A is the ionization constant of hydrazoic acid. The k_{off} data are pH independent within an average deviation of ±4%.

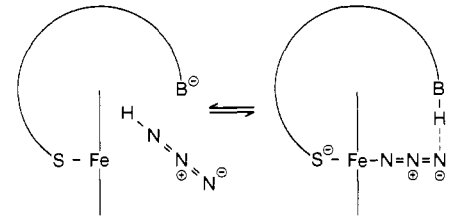

FIGURE 5: Representation of the active site of chloroperoxidase during the binding of hydrazoic acid. The hydrazoic acid proton is transferred to the distal base B^- during the concerted binding process. A hydrogen bond from the newly formed BH acid to the bound azide facilitates the reverse reaction in which azide is converted into the much better leaving group hydrazoic acid.

are indicated. Since $[azide]_{tot} \gg [E]_{tot}$ for all experiments, eq 4 reduces to

$$1/\tau = k_{on}[azide]_{tot} + k_{off} \quad (5)$$

Two plots of $1/\tau$ versus $[azide]_{tot}$ are shown in Figure 3. Slopes are equal to k_{on} and intercepts to k_{off}. The results in Figure 3 summarize the hydrogen/deuterium kinetic isotope effect obtained for azide binding to horseradish peroxidase. All of our $1/\tau$ results and corresponding $[azide]_{tot}$ values for chloroperoxidase are summarized in Table I. The k_{on} and k_{off} values as a function of pH for azide binding to chloroperoxidase in H_2O are displayed in Figure 4. The results obtained in deuterium oxide for azide binding to chloroperoxidase (Table I) lead to values of k_{on} and k_{off} of 4.3×10^7 M^{-1} s^{-1} and 1.1×10^4 s^{-1} at pD 4.36. When compared to the corresponding rate constants at pH 4.36 which can be read from Figure 5, one obtains k_H/k_D(on) = 1.16 and k_H/k_D = 1.7 for chloroperoxidase. These compare to k_H/k_D(on) = 1.10 and k_H/k_D(off) = 2.4 for horseradish peroxidase at pH/pD = 4.56–4.36 (Figure 3). The value for k_H/k_D for compound I formation of horseradish peroxidase is 1.6 at 25 °C and ionic strength of 0.11 M (Dunford et al., 1978).

Discussion

An important consequence of the advantages of the ILTJ experiments is that k_{off} data can be obtained with a degree of accuracy hitherto not obtained in comparable work [see,

Table I: Kinetic Data for the Reaction of Hydrazoic Acid with Chloroperoxidase at 9.5 °C, Ionic Strength of 0.4 M, and [Chloroperoxidase] = 11 μM[a]

$1/\tau$ ($\times 10^{-4}$ s^{-1})	[NaN_3] ($\times 10^4$ M)	$1/\tau$ ($\times 10^{-4}$ s^{-1})	[NaN_3] ($\times 10^4$ M)
pH 3.0		pH 4.56	
2.32	0.625	2.44	1.25
2.76	1.25	2.96	2.50
3.84	2.50	3.94	5.00
5.24	5.00	5.17	7.50
7.30	7.50	6.71	12.5
pH 3.4		pH 5.0	
2.32	0.625	2.08	1.25
2.72	1.25	2.42	2.5
3.83	2.50	3.40	5.0
5.59	5.00	5.43	12.5
7.46	7.50	9.17	25.0
		15.5	50.0
pH 4.0			
2.52	1.25	pD 4.36	
3.20	2.50	1.55	1.25
4.88	5.00	2.26	2.50
6.20	7.50	3.28	5.00
9.16	12.5	4.20	7.50
13.6	18.8	6.45	12.5
$1/\tau$ ($\times 10^{-4}$ s^{-1})	[NaN_3] ($\times 10^3$ M)	$1/\tau$ ($\times 10^{-4}$ s^{-1})	[NaN_3] ($\times 10^3$ M)
pH 5.6		pH 6.6	
1.93	0.250	2.00	2.50
2.32	0.625	2.56	6.25
2.73	1.25	3.10	12.5
3.47	2.50	4.00	25.0
5.46	5.00	6.54	50.0
		8.20	75.0
pH 6.0			
3.33	5.00		
4.61	10.0		
5.20	12.5		
8.20	25.0		
14.1	50.0		

[a] The reciprocals of the relaxation time, $1/\tau$, are listed as a function of sodium azide concentration. All entries are for data obtained in H_2O except for those at pD 4.36 which were obtained in D_2O.

for example, Dunford and Alberty (1967)]. Since k_{off} is obtained by extrapolation to zero ligand concentration, its value usually contains a large experimental error. We can state with certainty that there is no detectable pH dependence in our k_{off} results from pH 3.0 to pH 6.6 for azide binding to chloroperoxidase within an average deviation of ±4%. This has significance in the discussion of mechanisms. See the Appendix for mathematical details.

Mechanisms which clearly can be excluded include the following: mechanism I, binding of azide anion to the native enzyme (Appendix); mechanism II, binding of hydrazoic acid to native enzyme, in which the hydrazoic acid proton is transferred to solvent. This mechanism is excluded by our accurate k_{off} data.

Most plausible is mechanism III, in which hydrazoic acid binds to native chloroperoxidase and the ligand proton is retained within the enzyme–ligand complex. For reasons outlined below, we shall indicate transfer of the proton to the enzyme upon binding as

$$E + HN_3 \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} HE{\cdot}N_3 \qquad (6)$$

During the bind process the acid ligand, dissociation constant K_A, remains in equilibrium with the azide anion. Introduction of this ionization equilibrium leads to

$$k_{on} = \frac{k_3}{1 + K_A/[H^+]} \qquad (7)$$

The value of k_{on} is approaching asymptotically that of k_3 as the pH is decreased to 3. Our experimental results are fit by eq 7, with k_3 and K_A as adjustable parameters. The line in Figure 4, for the k_{on} data, is calculated from eq 7 and the best-fit values for k_3 of 7.6×10^7 M^{-1} s^{-1} and for K_A of 2.5×10^{-5} M ($pK_a = 4.60$). The latter result provides a simple example of the determination of a thermodynamic quantity from kinetic data. (The converse is not possible.) The value of 4.60 for our experimental conditions can be compared to literature values of 4.55 and 4.75 for the pK of hydrazoic acid, determined under different experimental conditions (Cotton & Wilkinson, 1980; Quintin, 1940).

Mechanism III is consistent with the absence of any pH effect on k_{off}, since at all pH values from 3.0 to 6.6 the enzyme–azide complex contains all of the protons originating from hydrazoic acid and transfers all of them back to the azide ligand during its departure.

Hydrazoic acid contains one labile N–H bond which can be broken and replaced by another, such as Fe–N. If the acid does not dissociate, then formation of the new bond is precluded. We also know that the hydrazoic acid proton is not transferred to solvent during the binding reaction. Hence, we conclude that the proton is transferred exclusively to the enzyme upon reaction of hydrazoic acid with chloroperoxidase. The simple reaction, shown in eq 6, can be expanded as shown in Figure 5. The proton from hydrazoic acid is transferred to a distal basic group B^-, which facilitates azide binding to iron(III). The newly formed B–H acid group, in turn, facilitates the dissociation process. It converts azide anion into the much better leaving group hydrazoic acid. From the principle of microscopic reversibility, we know that a common pathway must be shared by the forward and reverse reactions.

The pK value of the native enzyme distal basic group must be below 3 since we see no indication of it in our k_{on} results. Similarly, the pK value of the same group in the enzyme–azide complex must be above 6.6. Thus, it is greatly increased upon hydrazoic acid binding. Such pK shifts are the essence of acid–base catalysis in enzymology (Jencks & Sayer, 1975; Jencks, 1976). Resonance structures for the azide ion show formal charges of −1 on both terminal N atoms. Such a charge could help stabilize a B–H hydrogen bond to the azide ligand as shown in Figure 5. The deuterium isotope effects indicate that proton transfer is more rate limiting for the dissociation process.

A potential rival to eq 6 is mechanism IV:

$$HE + N_3^- \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} HE{\cdot}N_3 \qquad (8)$$

The total number of protons is the same in mechanisms III and IV, and a distinction between such mechanisms is an old and well-documented kinetic enigma (Frost & Pearson, 1961). The fashion in which mechanisms III and IV predict the same pH–rate profile is shown schematically in Figure 6. The only possible way of distinguishing the two may be by use of the diffusion-controlled limit rule, and this appears to be valid here. Mechanism IV appears to predict a k_4 value in excess of the diffusion-controlled limit (Appendix). In the earlier study of the kinetics of azide binding to horseradish peroxidase, it was concluded from the pH dependence of k_{on} that a proton played an important role (Morishima et al., 1978). We agree with that conclusion and infer from our chloroperoxidase results that the proton must originate from hydrazoic acid.

Our results provide supporting evidence for the proposed mechanism for peroxidase compound I formation (Dunford & Stillman, 1976; Dunford, 1982). It has been proposed that proton transfer from hydrogen peroxide to a distal basic group

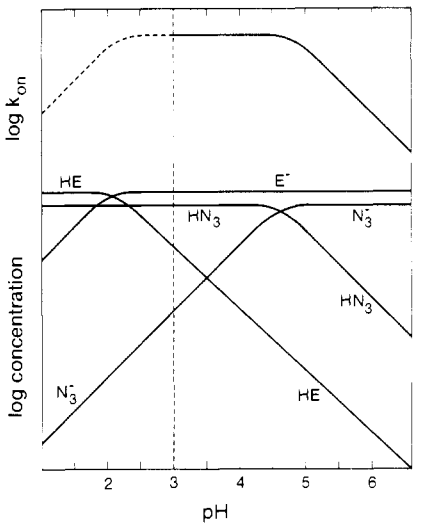

FIGURE 6: Schematic representation of two mechanisms which in principle can be fit to the experimental data. Ionization of the enzyme distal basic group must occur at a pH <3 since its influence is not observed in the accessible pH range. The shape of the observed pH–rate constant profile for k_{on} can be reproduced by the sum of the HN_3 and E^- relative concentration curves (mechanism III) and by the sum of the N_3^- and HE relative concentration curves (mechanism IV). However, the latter mechanism is excluded because it predicts a rate constant which exceeds the diffusion-controlled limit.

is an important part of the mechanism. However proof was lacking, because once compound I is formed the proton is lost from the enzyme. Since the overall process is concerted, no detectable intermediates occur along the reaction coordinate unless nonphysiological conditions are employed (Balny et al., 1987; Job & Dunford, 1978). In our study of azide binding, we have proof that proton transfer to the enzyme does occur. Our results are also in complete accord with the conclusions reached in a recent equilibrium study of ligand binding to chloroperoxidase (Sono et al., 1986).

APPENDIX

In well-buffered solutions proton equilibria are maintained. Thus hydrazoic acid and azide anion are in equilibrium throughout the course of the binding reaction.

Mechanism I:

$$\begin{array}{ccc} & HN_3 & \\ & K_A \uparrow\downarrow H^+ & \\ E + N_3^- & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & E \cdot N_3^- \end{array} \qquad (9)$$

The velocity of the forward reaction is given by

$$V = k_{on}[E]_{tot}[azide]_{tot} = k_1[E][N_3^-] \qquad (10)$$

For this mechanism it is assumed that no important ionizations in the enzyme active site occur for azide binding. Therefore, $[E]_{tot} = [E]$.

$$k_{on} = \frac{k_1[N_3^-]}{[azide]_{tot}} = \frac{k_1[N_3^-]}{[N_3^-] + [HN_3]}$$

$$k_{on} = \frac{k_1}{1 + [H^+]/K_A} \qquad (11)$$

Equation 11 predicts a pH–rate profile for k_{on} which is the mirror image of that in Figure 4. Therefore, mechanism I is invalid.

Mechanism II:

$$\begin{array}{ccc} E + HN_3 & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & E \cdot N_3^- + H^+ \\ K_A \uparrow\downarrow H^+ & & \\ N_3^- & & \end{array} \qquad (12)$$

Since a proton is lost to solution in the forward reaction, it must be regained in the reverse reaction (principle of microscopic reversibility). However, we can state with certainty that there is no pH dependence to the k_{off} data (Figure 4); therefore, mechanism II is invalid.

Mechanism III:

$$\begin{array}{ccc} E + HN_3 & \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} & E \cdot HN_3 \\ K_A \uparrow\downarrow H^+ & & \\ N_3^- & & \end{array} \qquad (13)$$

$$k_{on} = \frac{k_3}{1 + K_A/[H^+]} \qquad (7)$$

Equation 7 can be fit to the k_{on} data with great precision; it also predicts the pH independence of k_{off}. Mechanism III does not give any information about the location of the proton in the enzyme–ligand complex (see Discussion), but it is phenomenologically of the correct form.

Mechanism IV:

$$\begin{array}{ccc} HE + N_3^- & \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} & HE \cdot N \\ K_E \uparrow\downarrow H^+ \quad H^+ \downarrow\uparrow K_A & & \\ E^- \qquad HN_3 & & \end{array} \qquad (14)$$

Finally, we discuss a mechanism in which the kinetics are governed by an enzyme active site ionization which occurs outside the experimentally accessible pH range. This leads to

$$k_{on} = \frac{k_4}{(1 + K_E/[H^+])(1 + [H^+]/K_A)} \qquad (15)$$

Since k_{on} is unaffected by K_E at low pH (Figure 6), we estimate $K_E \geq 10^{-2}$ M. Using $K_A = 2.5 \times 10^{-5}$ and pH 3 as an example where $k_{on} = 7.5 \times 10^7\ M^{-1}\ s^{-1}$, one can calculate from eq 15 that $k_4 \geq 3.4 \times 10^{10}\ M^{-1}\ s^{-1}$. This exceeds the diffusion-controlled limit (Caldin, 1964; Dunford & Hasinoff, 1986) and is therefore physically impossible. Electrostatic attraction cannot be invoked to rationalize a larger diffusion-controlled limit. At an ionic strength of 0.4 M the screening effect of the inert electrolyte overwhelms electrostatic attraction between ionic reactants (Holzwarth, 1979b).

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Active Site Selective Labeling of Serine Proteases with Spectroscopic Probes Using Thioester Peptide Chloromethyl Ketones: Demonstration of Thrombin Labeling Using N^{α}-[(Acetylthio)acetyl]-D-Phe-Pro-Arg-CH_2Cl†

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ABSTRACT: The feasibility of a new approach to incorporation of spectroscopic probes into the active sites of certain serine proteases has been demonstrated. The method is based on inactivation of a serine protease with a thioester derivative of a peptide chloromethyl ketone. The thiol group generated by reaction of the covalent enzyme–inhibitor complex with NH_2OH provides a unique site for subsequent labeling with thiol-reactive probes. To evaluate the method, N^{α}-[(acetylthio)acetyl]-D-Phe-Pro-Arg-CH_2Cl was synthesized by reaction of the thrombin-specific tripeptide chloromethyl ketone with succinimidyl (acetylthio)acetate and purified by sulfopropyl-Sephadex and Sephadex G-10 chromatography. Reverse-phase high-performance liquid chromatography indicated that the product was 90 ± 3% pure. The compound was quantitated by using 5,5′-dithiobis(2-nitrobenzoic acid) to measure the concentration of thiol produced in the presence of NH_2OH. On this basis, titrations of the irreversible loss of human α-thrombin activity had end points of 1.1 ± 0.1 mol of inhibitor/mol of active sites, indicating a 1:1 stoichiometry for inactivation. Incubation of N^{α}-[(acetylthio)acetyl]-D-Phe-Pro-Arg–thrombin with 5-(iodoacetamido)fluorescein in the presence of NH_2OH resulted in incorporation of 0.96 mol of the fluorescence probe/mol of active sites and the appearance of fluorescein fluorescence associated with the active site containing B-chain on sodium dodecyl sulfate–polyacrylamide gels. Fluorescence labeling of thrombin required reaction of the inhibitor at the active site as well as subsequent generation of the thiol group with NH_2OH. It is concluded that active site selective labeling can be achieved by using this approach, which is likely to be applicable to other proteases, peptide chloromethyl ketones, and a wide variety of probes.

Use of covalent spectroscopic probes for studying the functions of serine proteases, such as the enzymes of the blood coagulation system, is well established. Several reagents derived from synthetic inhibitors (Powers & Harper, 1986) have been described for active site affinity labeling of serine proteases with spectroscopic probes. These include sulfonyl fluoride (Berliner & Wong, 1974; Vaz & Schoellmann, 1976) and organophosphorous (Epstein et al., 1979; Hsia et al., 1972) derivatives of fluorophores and spin labels or acylating agents (Haugland & Stryer, 1967; Moorman & Abeles, 1982) based on these probes which react with the active center serine residue of the enzymes. Irreversible inactivation of proteases by alkylation of the active site histidine residue with derivatives of amino acid or peptide chloromethyl ketones has also been

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