Investigation of the Active Center of Trypsin Using Photochromic Substrates*

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ABSTRACT: Two photochromic compounds, *p*-phenylazobenzoyl-L-arginine methyl ester hydrochloride (PABE) and its hydroxamide (PABH), were examined as substrates of trypsin. Both compounds can exist as planar trans or nonplanar cis isomers which are interconvertible under the influence of light of selected wavelengths. *trans*-PABE is hydrolyzed faster than *cis*-PABE: k_{cat} (trans), 30 sec^{-1}; k_{cat} (cis), 18 sec^{-1}. The kinetic constants for the PABH isomers are: trans: K_m, 1.6 mM; k_{cat}, 1.3 sec^{-1}; cis: K_m, 9.1 mM; k_{cat}, 4.0 sec^{-1}. The data for PABH, in which K_m represents a true binding constant and k_{cat} the rate constant for acylation of the enzyme, indicate that the binding of the planar trans isomer includes more nonproductive modes than does the binding of the cis. This may be taken to support the suggestion of Mares-Guia *et al.* (*J. Biol. Chem. 242*, 5777, 1967) that there is a hydrophobic slit at the active center of trypsin which acts as a primary binding site for the methylene groups of the basic side chains of specific substrates.

Studies on the mechanism of action of trypsin have, generally speaking, focussed on two separate but interrelated functional aspects of the active center of the enzyme: (a) the binding of specific substrates, and (b) the subsequent hydrolytic process. Trypsin, as a "serine esterase," has an active serine residue which, in cooperation with a nearby histidine, participates directly in the catalytic mechanism (Hartley, 1960). Substrate binding can apparently take place at more than one site at the active center of the enzyme. At low concentrations, arginine-containing substrates, such as TAME,[1] bind mainly to a "primary" binding site; at higher concentrations, binding can occur at a secondary site as well, resulting in activation (acceleration) of the hydrolytic process (Trowbridge, *et al.*, 1963). Neutral substrates such as benzoyl citrulline methyl ester do not bind to the primary site, but bind elsewhere, possibly to the secondary site, although this has by no means been established (Sanborn and Hein, 1968).

There is evidence that at least one of the binding sites has hydrophobic character (Erlanger, 1958; Cohen *et al.*, 1962; Erlanger and Cohen, 1963; Mares-Guia and Shaw, 1965; Mares-Guia *et al.*, 1967; Seydoux *et al.*, 1969). Mares-Guia and his coworkers have concluded, mainly from thermodynamic evidence, that the primary binding site is a hydrophobic slit into which the methylene carbons of the arginine side chain are inserted, the slit being located between an anionic region and the catalytic site of the active center.

Recent investigations in our laboratory have been concerned with photochromic, enzyme-specific derivatives of azobenzene, which can exist as cis or trans isomers that are interconvertible under the influence of light of selected wavelengths (Kaufman *et al.*, 1968; Bieth *et al.*, 1969, 1970; Deal *et al.*, 1969). In the trans configuration, the two benzene rings and the azo bond are in the same plane. Exposure to near-uv light causes conversion to the cis configuration in which the two benzene rings are no longer in the same plane, one of them occupying a plane that places it about 56° from the plane of the azo nitrogens and the other ring (Hampson and Robertson, 1941). It seemed possible that this type of compound could be useful in providing evidence for or against the existence of a hydrophobic binding slit at the active site of trypsin, since a slit would accommodate the planar, trans configuration more easily than it would the bulky, cis configuration. With this in mind, two photochromic derivatives were synthesized and tested as substrates for trypsin: *p*-phenylazobenzoyl-L-arginine methyl ester hydrochloride (PABE) and *p*-phenylazobenzoyl-L-arginine hydroxamide hydrochloride (PABH). The kinetics of their hydrolysis by trypsin was examined.

Materials and Methods

Worthington trypsin (twice crystallized, dialyzed salt free, lyophilized) was used in all experiments. Concentrations were determined according to Schwert and Takenaka (1955).

Substrates. PABE. To 5 g (0.02 mole) of arginine methyl ester dihydrochloride (Fisher and Suzuki, 1905) in 20 ml of water was added 20 ml of chloroform. The mixture was stirred and cooled in an ice bath while a total 0.8 g (0.02 mole) of MgO and 4.9 g (0.02 mole) of *p*-phenylazobenzoyl chloride (in a total of 18 ml of chloroform) were added in three portions over a period of 30 min. Then 25 ml of chloroform and 20 ml of water were added and the reaction was stirred for an additional 30 min. The reaction mixture was acidified to about pH 2 using concentrated hydrochloric acid. The water layer was removed and put aside; the chloroform layer was washed once with water and then discarded. To the combined water layers was added enough sodium chloride to produce 90% saturation, resulting in the precipitation of an oil, which began to crystallize in a short time. After remaining overnight in the refrigerator, the crystals were collected by filtration and dried over P_2O_5 in a desiccator for several days. Recrystallization was from hot water: yield, 4.6 g (53%); mp 113°. *Anal.* Calcd for $C_{20}H_{26}ClN_6O_3 \cdot H_2O$ (451.95): C, 53.15; H, 6.24; N, 18.60. Found: C, 53.66; H, 5.86; N, 18.76. The water of hydration could be removed by heating at 100°

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[1] Abbreviations used are: PABE, *p*-phenylazobenzoyl-L-arginine methyl ester hydrochloride; PABH, *p*-phenylazobenzoyl-L-arginine hydroxamide hydrochloride; TAME, *p*-toluenesulfonyl-L-arginine methyl ester hydrochloride; BAA, benzoyl-L-argininamide hydrochloride.

in vacuo over P_2O_5 for 1 hr. *Anal.* Calcd for $C_{20}H_{26}ClN_6O_3$ (433.93): C, 55.36; H, 6.04; N, 19.37. Found: C, 55.27; H, 5.75; N, 19.23.

PABH. PABE (500 mg, 1.1 mmoles) was dissolved in a mixture of 5 ml of water and 3 ml of ethanol. To this solution was added a basic solution of hydroxylamine, which was prepared by mixing 7.7 ml of 30% NaOH with 1.6 g of hydroxylamine hydrochloride in 5 ml of water. An oil began to be deposited but the addition of 5 ml of methanol yielded a clear solution. After standing overnight the solution was acidified with hydrochloric acid to pH 1; crystallization commenced. The crystals were finally collected by suction, washed with a small amount of ice-cold water, and air-dried: yield, 205 mg (40%); mp 170.5° (dec). *Anal.* Calcd for $C_{19}H_{24}N_7ClO_3 \cdot H_2O$ (452.95): C, 50.36; H, 5.56; N, 21.64. Found: C, 50.73; H, 5.25; N, 21.11.

Titrimetric Method. Kinetic data were obtained according to the method of Inagami and Sturtevant (1960), using a Radiometer pH-Stat titrator. Steady-state rates were obtained at 26.5° at pH 8.0 in 150 ml of a solution containing 0.2 M KCl and 0.01 M $CaCl_2$, under nitrogen, using 0.05 N NaOH as titrant. An enzyme concentration of 2.24×10^{-9} M was used in each experiment.

Hydroxamide Hydrolysis. Rates of hydrolysis were obtained by measuring residual hydroxamide at 500 nm according to the method of Hogness and Niemann (1953). Experiments were performed at 26.5°, using an enzyme concentration of 9.01×10^{-7} M in 5 ml of 0.1 M Tris-chloride buffer (pH 8.0) containing 15% dimethyl sulfoxide and 0.01 M $CaCl_2$.

Irradiation of the substrates by either uv light or photoflood was essentially as described previously (Bieth *et al.*, 1969). PABE was dissolved in 2.5 ml of 0.02 M Tris-chloride buffer (pH 8.0) and irradiated in a quartz cuvet either with uv light (Spectroline B-100) for 20 min or with a photoflood for 3–4 min. The spectra were always monitored. The solution (100 μl) was then added to 200 ml of 0.02 M KCl in each experiment in which the titrimetric method was used. PABH was dissolved in 0.1 M Tris-chloride buffer (pH 8.0) containing 15% dimethyl sulfoxide and 0.01 M $CaCl_2$ and irradiated with uv light for about 25 min, *i.e.*, until the spectrum indicated at least 90% conversion into cis isomer.

All experiments employing uv-irradiated substrates were carried out in a darkened room with the aid of red safety lamps, except in those cases where a photoflood was used to convert a cis substrate into the trans configuration in the midst of an experiment. Spectra were checked both before and after each experiment.

As described previously (Kaufman *et al.*, 1968), trans forms of these azo compounds, which are the more stable isomers, exhibit a peak at 330 nm. The cis isomer, which forms upon irradiation of the trans isomer with uv light, displays no peak at 330 nm but acquires a smaller one at 255 nm. No conversion of the cis into the trans isomer occurred in the dark but rapid conversion occurred upon exposure to a photoflood or to light of 420 nm.

Results

Hydrolysis of PABE. Preliminary experiments showed that PABE concentrations as low as 3×10^{-5} M resulted in enzyme saturation. Because of limitations in the precision of the titrimetric equipment, we, therefore, could determine V_{max} and k_{cat} but not K_m of the hydrolytic reaction. The results are given in Table I. The trans isomer is hydrolyzed about twice as fast as the cis isomer. It must be stressed that this represents a significant difference since the experimental procedure was designed so that the cis and trans constants were obtained in a single run. The hydrolysis of the cis isomer was followed first, for a period of time sufficient to abstract a velocity constant, after which it was converted to the trans isomer *in situ* (using a photoflood light) for the determination of the velocity constant of the trans isomer. In Figure 1 is shown an actual tracing of a run; a change in slope after exposure to light is readily apparent.

TABLE I: Kinetic Constants for Tryptic Hydrolysis of PABE and PABH.[a]

Constant	PABE		PABH	
	cis	trans	cis	trans
k_{cat} (sec^{-1})	18	30	4.0	3.3
K_m (mM)			9.1	1.6
k_{cat}/K_m			440	813

[a] 26.5°, pH 8.0, 0.01 M Ca^{2+}. For additional conditions, see Experimental Section.

Hydrolysis of PABH. It was possible to determine K_m and k_{cat} of the hydrolysis of PABH, since saturation of the enzyme did not occur even at concentrations of the order of 1 mM of either substrate. Figure 2 represents reciprocal plots for both isomers. The calculated K_m and k_{cat} values are given in Table I. Also given are the calculated values for the ratio k_{cat}/K_m. The cis isomer is hydrolyzed more rapidly but is bound less efficiently. The important ratio k_{cat}/K_m, which is more meaningful with respect to "kinetic specificity" (Bender and Kezdy, 1965), is higher for the trans isomer. In this respect, the relationship is similar to that which was found for the two isomers of PABE.

Discussion

The Michaelis–Menten mechanism for enzyme catalysis is expressed as

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E \cdot S \xrightarrow{k_2} E + P \qquad K_m = \frac{k_2 + k_{-1}}{k_1} \qquad (1)$$

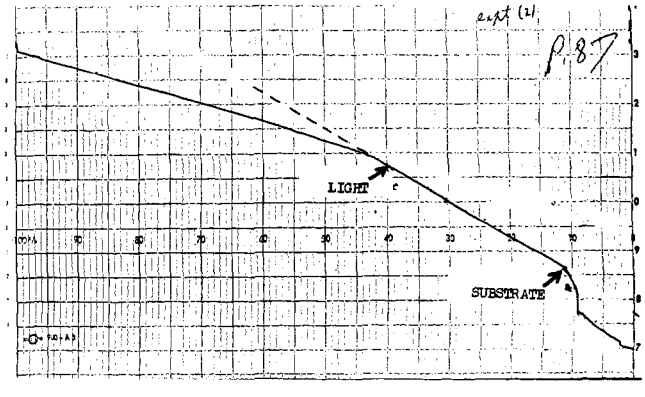

FIGURE 1: pH-Stat trace of hydrolysis of *cis*-PABE followed by conversion to trans isomer by photoflood. Vertical axis represents time; horizontal axis represents quantity of base added to keep pH constant.

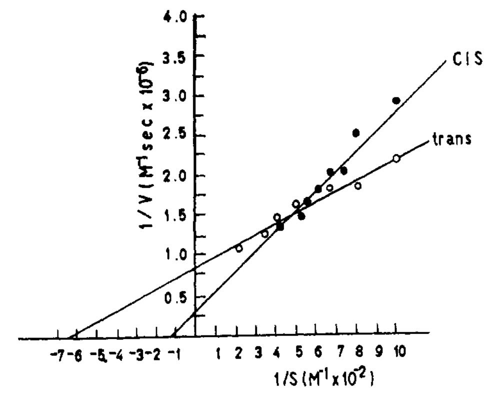

FIGURE 2: Double-reciprocal plot of kinetics of hydrolysis of *cis*- and *trans*-PABH. Conditions in Experimental Section.

For all "serine esterases," however, the hydrolytic mechanism can be represented as follows (Sturtevant, 1960).

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}S \xrightarrow{k'_2} \underset{P_1}{\overset{}{E{-}S + }} \xrightarrow{k_3} E + P_2 \qquad K_s = \frac{k'_2 + k_{-1}}{k_1} \quad (2)$$

where E·S is the Michaelis–Menten complex and E–S represents enzyme in which acylation of the serine hydroxyl by the acyl portion of the substrate has occurred. Therefore, k'_2 is the rate constant for acylation and k_3 for deacylation of the enzyme. The consequences of this mechanism with respect to the problems investigated will be elaborated later in the discussion.

Before examination of the experimental data, let us attempt to predict the behavior of the cis and trans derivatives, on the assumption that a hydrophobic slit is present at the active center of trypsin. The suggestion of Mares-Guia and his coworkers is that the hydrophobic slit represents part of the primary binding site, *i.e.*, the site involved in binding the side chain of the arginine (or lysine) residue of a specific substrate. On that basis alone, we could not predict whether the cis or trans isomer would be hydrolyzed more efficiently since in the productive mode of binding the *p*-phenylazobenzoyl moiety will not be in the slit. However, substrate to enzyme binding also includes nonproductive modes (Hein and Niemann, 1962). One of these modes is likely to involve binding of the hydrophobic *p*-phenylazobenzoyl group at the hydrophobic site. If this site is a slit, it will be able to accommodate the planar trans isomer but not the bulky, nonplanar cis form (Hampson and Robertson, 1941). We might predict, therefore, a more favorable binding constant for the trans isomer than for the cis because kinetically determined binding constants include *all* modes of binding. However, nonproductive modes will not lead to acylation of the enzyme. In fact, they would act to lower the effective concentration of active enzyme, which would be reflected kinetically in a lower rate constant for acylation of the enzyme by the trans isomer. One would not be able to predict *a priori* the effect of planarity (or nonplanarity) on the rate of deacylation because only productively bound substrate will get as far as this step.

Let us now go back to eq 2. Both ester and amide substrates are believed to be hydrolyzed by the two-step mechanism shown in this equation. With esters, however, the rate-determining step is believed to be the deacylation step, *i.e.*, $k'_2 \gg k_3$. Sturtevant (1960) has pointed out that the following relationships hold

$$\frac{1}{k_{cat}} = \frac{1}{k'_2} + \frac{1}{k_3}$$

$$K_m = K_s \frac{k_3}{k'_2 + k_3}$$

Thus, for esters, k_{cat}, which represents the rate constant at saturation, should be very nearly the same as k_3, the deacylation rate constant. Furthermore, K_m should be very much lower than the true E·S binding constant K_s. In fact, the finding that PABE saturates the enzyme at low concentration (*ca.* 10^{-5} M) is a clear indication that in the tryptic hydrolysis of this substrate $k'_2 \gg k_3$ and, hence, that k_{cat} is equivalent to k_3.

In the hydrolysis of amides and hydroxamides, $k'_2 < k_3$. Hence, k_{cat} more closely resembles k'_2, and K_m derived from the Lineweaver–Burk plot is not very different from K_s, the true binding constant.[2]

If we examine the kinetic constants for the tryptic hydrolysis of *cis*- and *trans*-PABE, we find that the trans isomer is the better substrate. Since k_{cat} for PABE represents the deacylation rate constant, we can say (although we never could have predicted it *a priori*) that the bulky cis configuration may sterically interfere with concerted processes which contribute to the deacylation step.

With respect to PABH, we find that the trans isomer is bound more favorably (since for this type of substrate $K_m \doteq K_s$). On the other hand, k_{cat} ($\doteq k'_2$) of the cis isomer is greater than k_{cat} of the trans isomer. This is as predicted above for the case in which there exists a hydrophobic slit which can accommodate the trans isomer in a nonproductive mode. As noted earlier, however, a better measure of "kinetic specificity" lies in the value of the ratio of k_{cat}/K_m. In this case, the trans isomer, as with PABE, is the more specific substrate.

The value of K_m for *trans*-PABH is similar to the K_m reported for BAA (Harmon and Niemann, 1949). This is also consistent with the presence of a hydrophobic slit since it would be expected that the planar benzoyl moiety of BAA, like *trans-p*-phenylazobenzoyl, could also be accommodated by the slit.

In summary, the data are consistent with the presence of a hydrophobic binding slit at the active center of trypsin. However, other appropriate substrates must be examined before we can accept its existence with some certainty. The data indicate also that the area occupied by the acylamido moiety (in the productive mode of binding) might be of limited breadth, though not likely a slit, because the trans isomers of both PABE and PABH are better substrates than the bulky cis isomers.

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[2] We have calculated the binding constants (K_s) and the k'_2 for the cis and trans hydroxamates (Sturtevant, 1960): K_s (cis), 11.6 × 10^{-3} M; K_s (trans), 1.65 × 10^{-3} M; k'_2 (cis), 5.3 sec^{-1}; k'_2 (trans), 1.4 sec^{-1}.

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Properties of Synthetic Polydeoxyribonucleotide Complexes Containing Adenine and Bromouracil*

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ABSTRACT: The homopolymer polydeoxyribobromouridylic acid (dBrU) has been prepared using *Escherichia coli* DNA polymerase I. Two- and three-stranded base-paired homopolymer complexes with polydeoxyriboadenylic acid (dA) have been made, and interactions occurring between the complexes and their component strands have been studied at 0.1 M sodium ion. The thermal stabilities of the homopolymer pair dA·dBrU and the alternating copolymer d(A-BrU)·d(A-BrU) have been determined at pH 7 as a function of ionic strength, and at 0.1 M sodium ion as a function of pH. In addition, the thermal stabilities of dA·dT and d(A-T)·d(A-T) have been determined at 0.1 M sodium ion as a function of pH. For both d(A-BrU) polymers and d(A-T) polymers, stability is affected by base sequence. The homopolymer pair is more stable than the copolymer pair at all pH values and ionic strengths studied. Other polymer systems show no base sequence effect. Possible sources of the base sequence dependence are discussed.

Synthetic polynucleotides serve as model compounds for naturally occuring nucleic acids and afford the opportunity to study the reactivity of molecules having a relatively simple, repeating composition. Polymers can be studied which differ from one another in chemically simple ways. In this study, we have prepared the homopolymer dBrU[1] and the base-paired two- and three-stranded complexes dA·dBrU and dA·dBrU$_2$, and we have compared certain properties of these polymer complexes to those of closely related polymer complexes which differ only in the 5-carbon pyrimidine substituent or the 2′-hydroxy sugar substituent or in base sequence alone.

The properties of deoxy polymers containing BrU are of interest since BrU has been used extensively in biological experiments in several different connections. When BrU is incorporated into DNA, it acts as a mutagen, it increases the density of the DNA, and it sensitizes the DNA to uv inactivation *in vivo*. The effects on the properties and reactivities of DNA containing BrU should be understood in order to allow most effective use of BrU as a tool in biological experiments. BrU increases the thermal stability of DNA and synthetic polynucleotides. Certain aspects of this stabilization have been studied in the work reported here, with emphasis on the effect of base sequence on the increase in thermal stability.

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[1] Abbreviations used are: A, BrU, U, T: adenine, bromouracil, uracil, and thymine, respectively. Deoxynucleoside triphosphates, dXTP. Abbreviations for the synthetic polynucleotides and polynucleotide complexes are those of the IUPAC–IUB Commission (*Biochemistry 9*, 4025 (1970)), for example, dA·dBrU$_2$ denotes the three-stranded homopolymer complex composed of one polydeoxyadenylate strand and two polydeoxybromouridylate strands, while d(A-BrU) denotes the deoxy copolymer containing adenine and bromouracil in strictly alternating sequence. Classes of polymer complexes are also referred to. The "deoxy A-BrU polymers" refers to both copolymer and homopolymer complexes. T_m = the temperature at the midpoint of an absorbancy transition, pH_m = the pH at the midpoint of an absorbancy transition. ϵ_P is the molar extinction coefficient relative to phosphorus. The symbol 2 → 3 represents a transition from a two- to a three-stranded complex, with similar connotation for other types of transitions.