Photoregulation of Enzyme Activity. Photochromic, Transition-State-Analogue Inhibitors of Cysteine and Serine Proteases

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Abstract: Two azobenzene derivatives, the aldehyde **1** and the boronic acid **2**, were synthesized and shown to be reversible, photoregulatable inhibitors of the cysteine protease, papain, and the serine proteases, chymotrypsin and subtilisin. *trans*-**1** was found to be a potent, reversible inhibitor of papain (K_i = 2.1 μM) while its cis isomer was about 40 times weaker. Irradiation of a solution containing papain and *trans*-**1** with 330 > λ > 370 nm light resulted in a photostationary state mixture of 83% *cis*-**1** and a corresponding 500% increase in enzyme activity. The original activity level was subsequently restored by irradiation with λ > 400 nm light which converted the inhibitor back to its trans configuration. The cycle was repeated to give an identical change in enzyme activity. Similar results were obtained with **2** as a photoswitchable inhibitor of chymotrypsin although the change in enzyme activity was much smaller (13%). In the case of subtilisin, the relative inhibitory abilities of *trans*-**2** and *cis*-**2** were found to be dependent upon the conditions of the experiment, particularly the concentration of KCl. In the absence of KCl, *cis*-**2** was a better inhibitor of subtilisin than *trans*-**2**, but in the presence of 0.5 M KCl the reverse trend was observed.

Introduction

General methods that simplify the technique of protein affinity chromatography would be of practical value, especially in the area of enzyme purification.[1] Work on the purification of cobalamin-binding proteins has demonstrated that affinity adsorption ligands that can be photoconverted from a configuration with strong affinity for a desired enzyme to a configuration with a weak affinity provide a novel approach to enzyme purification under mild conditions.[2,3] Since transition-state-analogue enzyme inhibitors can be utilized as enzyme affinity adsorption ligands,[4] it is possible that appropriately immobilized photoisomerizable transition-state-analogue enzyme inhibitors may act as photoswitchable affinity ligands. An initial requirement is the development of such a series of photochromic enzyme inhibitors. Here we report on the novel photochromic, transition-state-analogue protease inhibitors, **1** and **2**, and their ability to reversibly bind and inhibit the activity of certain cysteine and serine proteases in a photoregulatable manner.[5]

The general design of these inhibitors can be considered as the structural sum of two components, a head and a tail. The head consists of a primary recognition moiety known to have a propensity for reversible binding in the enzyme active site. The tail is a photochromic moiety whose molecular shape can be controlled in a photoswitchable manner. Photomodulation of the shape of the tail results in regulation of the inhibitory ability of the inhibitor and therefore enzyme activity. Such a strategy should be applicable to other enzyme/reversible-inhibitor systems.

In the case of **1**, the head is an aldehyde moiety, known to bind reversibly in the active site of cysteine proteases by forming a labile, covalent thiohemiacetal linkage with the active site cysteine residue;[6] for **2** the head is an aryl boronic acid which has an affinity for the active site of serine proteases via reversible formation of a tetrahedral boronate adduct with the active site serine.[7] In both cases the photochromic tail is an azobenzene derivative which can be reversibly photoisomerized between trans and cis configurations. Isomerization of the more stable trans form to the cis isomer is accompanied by a large change in molecular dipole moment (ca. 0–3 D) and molecular geometry (planar to nonplanar).[8]

Results and Discussion

At low substrate concentrations *trans*-**1** acts as a competitive inhibitor of the cysteine protease, papain; at higher concentrations mixed kinetics are observed ($K_i > K_i'$) and K_i = 2.1 μM was determined (Figure 1).[9] Irradiation of a solution of *trans*-**1** with UV light (filtered to allow passage of 330 > λ > 370 nm)[10] results in clean photoisomerization to produce a photostationary state of 83% *cis*-**1** (Figure 2) as determined by HPLC. For this photostationary mixture, K_i = 8.8 μM. *cis*-**1** can therefore be estimated to be about 40 times weaker an inhibitor of papain than its trans isomer. Irradiation of *cis*-**1** with visible light (filtered

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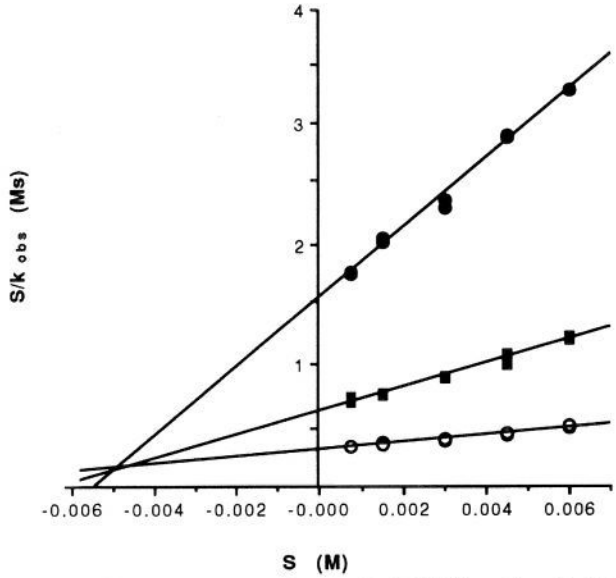


Figure 1. Half-reciprocal plots for the mixed inhibition of papain (12 μM), in 100 mM potassium phosphate buffer, pH 7.4: (○) no inhibitor, (●) *trans*-**1** (9 μM), (■) cis/trans photostationary mixture of **1** (9 μM) obtained by UV irradiation.

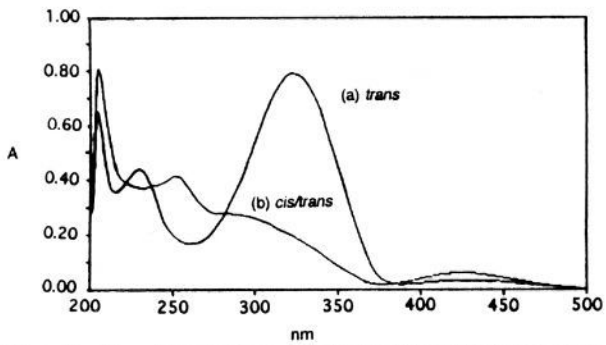
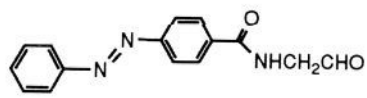
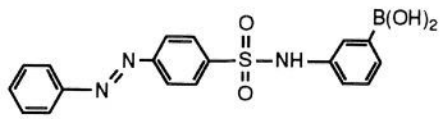


Figure 2. Absorption spectrum of (a) *trans*-**1** in 100 mM potassium phosphate buffer, pH 7.4, and (b) the photostationary, 83:17, cis/trans mixture of **1** achieved after UV ($330 > \lambda > 370$ nm) irradiation.



trans-**1**



trans-**2**

to allow $\lambda > 400$ nm) was observed to result in complete restoration of the trans configuration. In situ photoregulation of papain activity was accomplished in the following manner.[10] Addition of *trans*-**1** (18 μM) to a solution of papain (12 μM) was found to lower its ability to hydrolyze *N*-benzoyl-DL-arginine-*p*-nitroanilide[11] from a rate of 480 to 18 nM min^{-1}. UV irradiation of this sample raised the hydrolysis rate to 114 nM min^{-1}. Subsequent visible irradiation returned the hydrolysis activity to 17 nM min^{-1}. The cycle was repeated again and an identical

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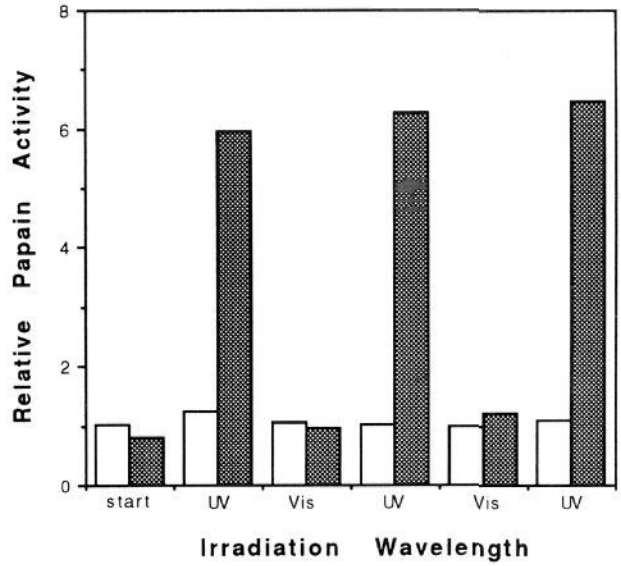

Figure 3. Relative papain activity as a function of light regulated inhibition by **1**. (▨) Relative rate of *N*-benzoyl-DL-arginine-4-nitroanilide hydrolysis for a solution of papain (12 μM), **1** (18 μM), β-mercaptoethanol (6.7 mM), EDTA (1 mM), and KCl (300 mM) in 3% methanol/phosphate buffer (100 mM, pH 7.35), after successive irradiations by UV ($330 > \lambda > 370$ nm) and visible ($\lambda > 400$ nm) light; (□) hydrolysis activity for a control solution kept in the dark.

change in activity levels was observed (Figure 3). Thus, the hydrolytic activity of papain in the presence of inhibitor **1** could be reversibly switched from a relative activity of 1.0 after irradiation with visible light to an activity of 6.3 after UV irradiation.

A similar study was conducted with compound **2** as a photoregulatable inhibitor of the serine proteases, chymotrypsin[12] and subtilisin. *trans*-**2** was a competitive inhibitor of chymotrypsin, $K_i = 11$ μM. Photoisomerization with UV light produced a photostationary state of 80% *cis*-**2** and an apparent $K_i = 41$ μM. With this difference in K_i's, a 2- to 3-fold change in enzyme activity can be predicted upon photoswitching.[9] Attempts to produce an in situ photoswitchable inhibitor/chymotrypsin system, however, were hindered by problems maintaining enzyme activity and inhibitor solubility over the time period of the experiment.[13] These problems were solved by including potassium chloride (KCl) and bovine serum albumin (BSA) as solution additives. Unfortunately, this resulted in a 10-fold increase in K_i which attenuated the range over which enzyme activity could be modulated. Addition of *trans*-**2** (42.3 μM) to a pH 8.4 solution of chymotrypsin (50 nm), BSA (4 μM), and KCl (0.5 M) lowered the rate of MeO-Suc-Ala-Ala-Pro-Phe-*p*-nitroanilide hydrolysis[14] from 716 to 590 nM min^{-1}. UV irradiation of this sample raised the hydrolysis rate to 670 nM min^{-1}. Subsequent visible irradiation returned the hydrolysis activity to 600 nM min^{-1}. Further cycling of the irradiation wavelength produced virtually identical changes in activity levels (Figure 4). Thus, the hydrolytic activity of chymotrypsin in the presence of inhibitor **2** could be reversibly switched from a relative activity of 1.00 after irradiation with visible light to an activity of 1.13 after irradiation with UV light.

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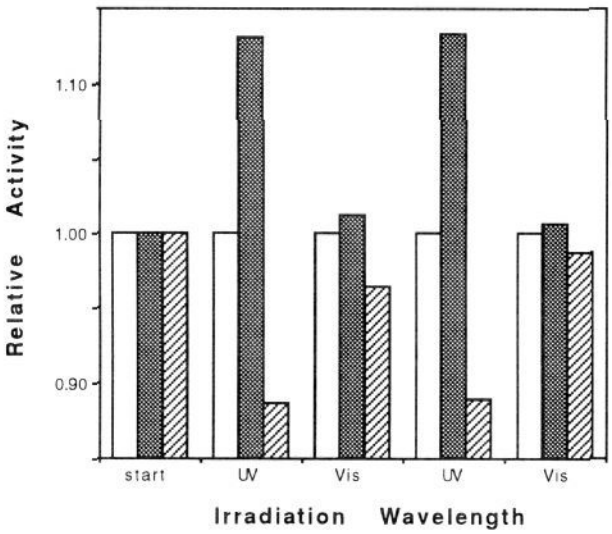

Figure 4. Relative chymotrypsin and subtilisin activities as a function of light modulated inhibition by **2**. (▨) Relative rate of MeO-Suc-Ala-Ala-Pro-Phe-4-nitroanilide hydrolysis for a solution of chymotrypsin (50 nM), **2** (42 μM), BSA (4 μM), and KCl (500 mM) in 3% acetonitrile/phosphate buffer (40 mM, pH 8.4), after successive irradiations by UV ($330 > \lambda > 370$ nm) and visible ($\lambda > 400$ nm) light; (▨) Relative rate of 4-nitrophenyl butyrate hydrolysis for a solution of subtilisin (250 nM), **2** (220 μM), and BSA (4 μM) in 7% acetonitrile/phosphate buffer (40 mM, pH 8.4), after successive irradiations by UV and visible light; (□) hydrolysis activity for chymotrypsin and subtilisin control solutions kept in the dark.

In the case of subtilisin, the relative inhibitory abilities of *trans*-**2** and *cis*-**2** were found to be dependent on the conditions of the experiment. When the sample included the additives described in the chymotrypsin experiments above, trans-**2** (visible irradiation, K_i = 0.9 mM) was observed to be a slightly better subtilisin inhibitor than *cis*-**2** (UV irradiation, K_i = 1.4 mM). However, in the absence of KCl, which was not necessary for the enzyme's stability in this case, *trans*-**2** (K_i = 0.5 mM) was a weaker subtilisin inhibitor than *cis*-**2** (after UV irradiation, K_i = 0.3 mM). Irradiation of the enzyme–inhibitor system with UV light resulted in a decrease in hydrolysis activity of 12% which was restored by irradiation with visible irradiation (Figure 4). The reason for this reversal in K_i upon KCl addition is not clear; speculative explanations include an enzyme conformational change or a relative change in inhibitor partition coefficients.

Since aldehydes are known to act as inhibitors of serine proteases,[7] **1** was briefly examined as a chymotrypsin inhibitor. The modest inhibition (K_i = 0.15 mM) observed, combined with poor inhibitor solubility prevented the in situ photoregulation experiment from being attempted. Current efforts are focussed on attaching structural analogues of these inhibitors to solid supports and determining their ability to act as photoswitchable enzyme affinity adsorption ligands.

Experimental Section

Materials. The following reagents were obtained commercially: 4-(phenylazo)benzoylchloride (Eastman Kodak); 4-(phenylazo)-benzenesulfonyl chloride (TCI America); *N*-benzoyl-DL-arginine 4-nitroanilide hydrochloride, MeO-succinyl-Ala-Ala-Pro-Phe 4-nitroanilide, and 4-nitrophenyl butyrate (Sigma); BSA (Serva). All enzymes were obtained commercially and were used without further purification: subtilisin BPN′ and α-chymotrypsin (Sigma); papain (2 × recrystallized, Worthington).

Synthesis. *N*-(4-(Phenylazo)benzoyl)aminoacetaldehyde (1). Aminoacetaldehyde diethyl acetal (0.59 mL, 4.08 mmol) was added dropwise to a stirring, room temperature solution of 4-(phenylazo)benzoylchloride (1.00 g, 4.08 mmol) and triethylamine (5.70 mL, 4.08 mmol) in ethyl acetate (20 mL). A precipitate formed immediately and after 2 h, TLC indicated the reaction was complete. The precipitate was collected, rinsed with water, and recrystallized twice from aqueous ethanol to give *N*-(4-(phenylazo)benzoyl)aminoacetaldehyde diethyl acetal as an orange solid (1.20 g, 73%): mp = 109–110 °C; TLC (silica, 1:1 ethyl acetate/hexanes), R_f 0.82; ^{1}H NMR (300 MHz, $CDCl_3$) 7.93 (m, 7 H), 7.55 (d, 2 H, J = 9 Hz), 6.43 (br t, 1 H, NH), 4.64 (t, 1 H, J = 5.5 Hz, CH), 3.77 (m, 2 H), 3.63 (m, 4 H), 1.25 (t, 6 H, J = 7Hz, CH_3); *m/e* (FAB) 342 (M + 1), 296 (M – OEt). Anal. Calcd for $C_{19}H_{23}N_3O_3$: C, 66.84; H, 6.79; N, 12.31. Found: C, 66.65; H, 6.74; N, 12.26.

Removal of the acetal was achieved by stirring in a 50% solution of tetrahydrofuran and 0.1 M HCl until TLC indicated consumption of the protected material (usually 24 h). Rotary evaporation of the tetrahydrofuran produced a precipitate which was collected, rinsed with water, and dried via desication. The product **1** was purified by preparative TLC (silica gel, 1:1 ethyl acetate/hexanes), R_f = 0.62, and shown to be pure by HPLC analysis (silica, 1:1 hexanes/ethyl acetate): (yield ~20%); mp = 124–126 °C; ^{1}H NMR (300 MHz, $CDCl_3$) 9.20 (s, 1 H, OCH), 8.00 (s, 5 H, Ph-N), 7.96 (d, 2 H, J = 9 Hz), 7.56 (d, 2 H, J = 9 Hz), 6.98 (br t, 1 H, NH), 4.50 (2 H, d, J = 4 Hz); *m/e* (FAB) 268 (M + 1), 209 (M – $NHCH_2COH$); UV λ_{max} = 324 nm (ϵ = 15 864 cm^{-1} M^{-1} in 100 mM potassium phosphate, pH 7.4).

***N*-((4-(Phenylazo)phenyl)sulfonyl)-3-aminobenzeneboronic Acid (2).** To an ice-cooled solution of 3-aminobenzeneboronic acid monohydrate (0.50 g, 3.23 mmol) in 50% acetone–water, adjusted to pH 8 with NaOH, was added dropwise, a solution of 4-(phenylazo)benzenesulfonyl chloride (0.90 g, 3.23 mmol) in acetone (10 mL). After stirring for 1 h, the acetone was removed by rotary evaporation. The residual alkaline layer was washed twice with methylene chloride, acidified to pH 5 with 20% HCl, and extracted with methylene chloride. After drying, the organic solvent was evaporated to leave **2** as an orange solid which was recrystallized twice from aqueous ethanol (1.12 g, 80%): mp = 147–149 °C; TLC (silica, 25:25:2, ethyl acetate/hexanes/ethanol) R_f trans = 0.66, cis = 0.54; ^{1}H NMR (300 MHz, $CDCl_3$) 9.07 (s, 1 H, NH), 7.95 (m, 7 H), 7.71 (s, 1 H), 7.58 (m, 5 H), 7.26 (2 H, m); *m/e* (positive ion FAB in glycerol matrix)[15] 438 (M + 57); molecular weight via NaOH titration = 381 (calcd 389);[16] UV λ_{max} = 323 nm (ϵ = 21 440 cm^{-1} M^{-1} in 100 mM potassium phosphate, pH 7.4). Anal. Calcd for $C_{18}H_{16}BN_3O_4S$: C, 56.71; H, 4.23; N, 11.02; S, 8.41. Found: C, 56.93; H, 4.33; N, 10.98; S, 8.61.

Instrumentation. UV spectra were obtained on a computer driven Perkin-Elmer Lambda 2 spectrophotometer, equipped with a constant temperature cell-holder. The kinetic data were collected using Perkin-Elmer Computerized Spectroscopy Software and analyzed graphically[9] using Cricket Graph Software. Irradiation experiments utilized a 150 W Oriel Xe arc lamp equipped with a housing condensor to collimate the beam. An Oriel 59810 filter was used for UV ($330 > \lambda > 370$ nm) light and a 59494 filter for visible ($\lambda > 400$ nm) light. Organic sample in glass containers and enzyme solutions in plastic containers were held two inches away from the lamp output lens; control experiments showed there was no sample heating due to the irradiation.

Determination of Inhibition Constants. The trans inhibitors were added to the enzyme solutions as concentrated organic solutions (**1** in methanol and **2** in acetonitrile) protected from the light. The cis inhibitors were obtained by irradiating the organic solutions until photostationary states had been achieved. In the dark, the cis isomers spontaneously converted to the trans isomers with half-lives of about 8 h at room temperature in organic or aqueous solutions. In the presence of excess β-mercaptoethanol or cysteine this process was accelerated up to 100-fold. The enzyme/inhibitor solutions were incubated for 5 min before the substrate was added.

Papain was freshly activated using β-mercaptoethanol and EDTA, at pH 6, and its ability to hydrolyze *N*-benzoyl-DL-arginine-4-nitroanilide was determined by the rate of 4-nitroaniline production observed at 410 nm.[11] Inhibition assays consisted of papain (12 μM) in 1 mL of potassium phosphate solution (100 mM, pH 7.35), EDTA (1 mM), β-mercaptoethanol (6 mM), KCl (300 mM), and 3% organic solvent v/v.

Inhibition of chymotrypsin activity was determined using MeO-Suc-Ala-Ala-Pro-Phe-4-nitroanilide as the substrate[14] in solutions containing chymotrypsin (100 nM), BSA (4 μM), potassium phosphate (40 mM, pH 8.4), KCl (500 mM), and 3% organic v/v. The same conditions, with and without added KCl, were used to determine subtilisin (250 nM) inhibition which was monitored via hydrolysis of 4-nitrophenyl butyrate.[17] Background hydrolysis of the substrate at this pH was negligible.

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Enzyme/Inhibitor Irradiation Experiments. A magnetically stirred solution (10 mL) of papain (12 μM), **1** (18 μM), β-mercaptoethanol (6.7 mM), EDTA (1 mM), and KCl (300 mM) in 3% methanol/potassium phosphate buffer (100 mM, pH 7.35) was alternately irradiated by UV (usually 20 min to reach photostationary state) and then visible light. After each irradiation, a 1-mL aliquot was withdrawn and its ability to hydrolyze *N*-benzoyl-DL-arginine-4-nitroanilide was determined. The results were compared to an identical control solution that remained in the dark. Similarly, a 10-mL solution of chymotrypsin (50 nM), **2** (42 μM), BSA (4 μM), and KCl (500 mM) in 3% acetonitrile/potassium phosphate buffer (40 mM, pH 8.4) was assayed for MeO-Suc-Ala-Ala-Pro-Phe-4-nitroanilide hydrolysis activity after successive irradiations by UV and visible light. In the case of subtilisin, aliquots from a 10-mL solution of subtilisin (250 nM), **2** (220 μM), and BSA (4 μM) in 7% acetonitrile/potassium phosphate buffer (40 mM, pH 8.4) were assayed for 4-nitrophenyl butyrate hydrolysis activity after successive irradiations by UV and visible light.

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