EPR Spectroscopy of Triplet Aryl Nitrenes Covalently Bound to α-Chymotrypsin. Application of Low-Temperature Methods to Photoaffinity Labeling

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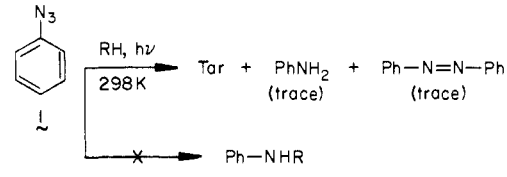
Abstract: Reaction of α-chymotrypsin with various aryl azido acyl imidazoles leads to covalent attachment of the aryl azide to the enzyme via an ester linkage. Photolysis of frozen solutions of the modified enzymes at 77 K leads to intense EPR signals of the related triplet nitrenes. The position of the EPR resonance fields and the decay kinetics of the nitrenes can be interpreted in terms of the label being within or outside the binding pocket of the enzyme. Photolysis of methyl *m*-azidobenzoate in toluene at 298 K gives very little nitrene-derived product. At this temperature, ring expansion to a ketenimine that ultimately polymerizes is the major process. Photolysis of methyl *m*-azidobenzoate in frozen toluene at 77 K gives the triplet nitrene, which undergoes secondary photolysis leading to formal CH insertion into the solvent matrix. It is demonstrated that photolysis of α-chymotrypsin covalently modified with a *m*-azidobenzoyl group at serine-195 leads to more efficient labeling at 77 K than at 298 K.

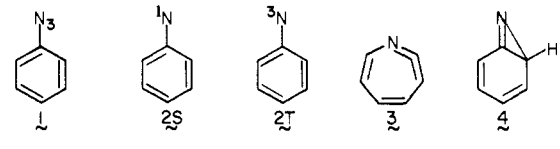
It has been more than 20 years since photoaffinity labeling was invented by Westheimer.[2] In the photoaffinity labeling experiment, a substrate of a biological receptor is chemically appended with a photosensitive functional group.[3] Ideally, the new light-sensitive substrate will still bind to the macromolecular receptor. Upon photolysis, the appendage is converted into a reactive intermediate that it is hoped will react with the biological target to ultimately form a new covalent bond between substrate and receptor. It is crucial to the success of the photoaffinity labeling experiment that the reactive intermediate produced be so highly reactive that it will react in the original binding site of the biological receptor. The experiment will fail if the photolytically produced intermediate is chemically discriminating and reacts sluggishly thereby allowing it to depart from the binding site prior to reaction with the biological target. The reaction will also fail if the light-sensitive substrate is not in the binding site of the natural ligand during photolysis. It is extremely unlikely that an intermediate generated outside the binding site will react inside the binding site.[3] To our knowledge, no one, as yet, has used direct spectroscopic methods to monitor a reactive intermediate bound to a biological macromolecule and to ascertain whether it is formed and reacts in the binding site. In this paper, we will report the direct spectroscopic detection of triplet aryl nitrenes covalently bound to α-chymotrypsin using EPR spectroscopy. We will show that this methodology is quite similar in philosophy to the spectroscopy of other reporter groups bound to enzymes. It has been demonstrated that spectroscopic analysis of nitroxide spin labels (EPR),[4] fluorinated materials (NMR),[5] and aromatics (fluorescence spectroscopy)[6] bound to enzymes gives an insight into the local environment around the reporter molecule. We will show that similar conclusions can be drawn from low-temperature EPR spectroscopy of triplet nitrenes bound to α-chymotrypsin. We will also demonstrate that low-temperature photoaffinity labeling improves the yield of permanent covalent attachment between the nitrene and the biological target.

Aromatic azides have been the most frequently utilized photoaffinity labels. The popularity of azides in these experiments is usually based on practicality; aryl azides are relatively easy to synthesize and handle.[7] Unlike aryl diazo compounds, azides will generally tolerate the pH range necessary in an experiment involving biomolecules. It is unfortunately true, however, that the photochemistry of simple aryl azides seems to be less than desirable for photoaffinity labeling purposes. Photolysis of phenyl azide in a hydrocarbon solution leads mainly to intractable, tarry material and only trace amounts of aniline and azobenzene.



Conspicuous by its absence is the complete lack of adducts between phenylnitrene and solvent. No formal CH insertion products (e.g., the desired products in a PAL experiment) are formed on photolysis of phenyl azide in hydrocarbon solutions.[7,8] Obviously, this does not bode well for a successful PAL experiment based on these reagents.

The photochemistry of aryl azides is highly complex.[9] At least four distinct reactive intermediates have been invoked at one time or another to explain the photochemistry of phenyl azide **1**: singlet phenylnitrene **2S**, triplet phenyl nitrene **2T**, 1,2-azacycloheptatetraene **3**, and benzazirine **4**. It is perhaps a prerequisite to



a successful PAL experiment to know the identity(ies) and yield(s) of the reactive intermediates formed on photolysis of a given azide. It will be nearly impossible to understand the photochemistry of an azide in the binding site of an enzyme when one does not understand the photochemistry of that azide in simple solution. Ideally, one should be able to choose experimental conditions that will produce only one reactive intermediate and that will also induce high yields of insertion into unactivated CH bonds.

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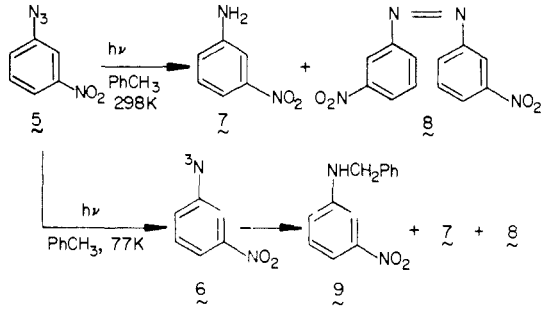
Table I. Resonance Fields (Gauss) Observed for Triplet Nitrenes upon Photolysis (5 min, λ 313 nm) of Various Aryl Azides in Different Matrices

nitrene methyl ester	$\|D/hc\|$[d]	cyclohexane	toluene	propanol	CH_3OH	GW[a]	nitrene	enzyme-bound GW[a]	GW(I)[a,c]
14a	1.038	6815	6815	6835	3825	6830	**14b**	6807	6835
15a	0.847	6325	6325	6335	6335	6325	**15b**	6317	6350
16a	0.949	6600	6650	6683	6660	6660	**16b**	6685	6700
17a	1.080	6927	6918	6942	6937	6938	**17b**	6930	6930

[a] Glycerol–water. [b] Modified enzyme. [c] Saturated indole solution. [d] $|D/hc|$ values for methyl esters in glycerol–water, all values ±0.05 cm^{-1}.

We have recently discovered that the photochemistry of aryl azides is highly sensitive to temperature.[10] Photolysis of phenyl azide and its simple derivatives at 77 K gives only the triplet phenylnitrene reactive intermediates. The 3-nitrophenyl azide (**5**) example motivated the present study.[11] Photolysis of **5** in



toluene at 298 K gives a 4.7% yield of 3-nitroaniline (**7**), a 24.1% yield of azo compound **8** and indetectably small amounts of the desired adduct **9**. However, when the photolysis was repeated at 77 K, the yield of adduct **9** rose from 0 to 29.5%, and the material balance of triplet nitrene **6** derived product rose from 28.9% at 298 K to 53.2% at 77 K. Thus, it occurred to us that performing photoaffinity labeling experiments using aryl azides at 77 K might have several advantages over traditional ambient-temperature photoaffinity labeling work. Low-temperature experiments also have the advantage of allowing direct EPR spectroscopic detection of triplet nitrenes. In this paper, we are pleased to report the application of low-temperature conditions to the photoaffinity labeling of α-chymotrypsin.

Results

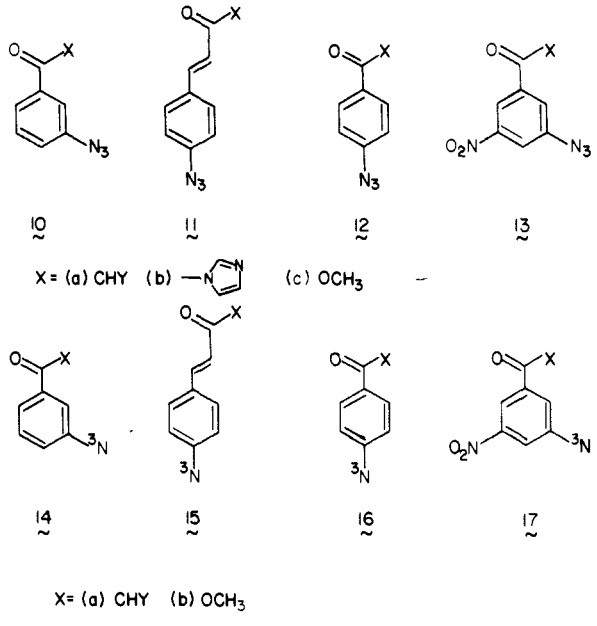
EPR Spectroscopy of Triplet Nitrenes Bound to α-Chymotrypsin. The structure and mechanism of action of α-chymotrypsin (CHY) is perhaps as well-known as that of any enzyme.[12] It is not the purpose of this work to further elucidate the structure and activity of α-chymotrypsin. This enzyme was chosen to test the utility of low-temperature methodology precisely because it is a well-understood protein. CHY is a serine-based protease specific for the hydrolysis of aromatic esters and amides. The extensive literature concerned with this protein provides a check on the validity of the conclusions we will draw upon analyzing the EPR spectra of nitrenes bound to CHY.[13] It is well appreciated from X-ray crystallography of α-chymotrypsin complexes with aromatic ligands such as indole that CHY contains an aromatic binding pocket, known as the "tosyl hole".[13] Aryl esters and amides in the noncovalent aromatic binding site react with a particularly nucleophilic hydroxyl moiety of serine-195 to give an inactive ester adduct, which can be isolated at low pH (pH ~3–4.5). At higher pH, the serine-195 ester linkage of the modified enzyme is hydrolyzed to regenerate native α-chymotrypsin, which has recovered its catalytic activity.

Treatment of α-chymotrypsin with the appropriate azidobenzoyl or cinnamoylimidazole **10b–13b** led to modified enzymes **10a–13a**,

Table II. Pseudo-First-Order Rate Constants for Triplet Nitrene Decay in Glycerol–Water at 173–174 K

azide	nitrene	rate, s^{-1}	azide	nitrene	rate, s^{-1}
10a	**14a**	$3.29 \pm 0.17 \times 10^{-4}$	**10c**	**14b**	$>1 \times 10^{-2}$
11a	**15a**	$<1 \times 10^{-4}$	**11c**	**15b**	$>1 \times 10^{-2}$
12a	**16a**	$3.54 \pm 0.12 \times 10^{-3}$	**12c**	**16b**	9×10^{-3}
13a	**17a**	$6.47 \pm 0.10 \times 10^{-3}$	**13c**	**17b**	$>1 \times 10^{-2}$

which were purified by gel permeation chromatography and concentrated by lyophilization. The modified enzymes **10a–13a**



could be obtained anywhere from ~50 to 100% inactivated by standard assays based on cinnamoylimidazole[14] and proflavine.[15] More than 80% of the original enzymatic activity of the modified proteins could be recovered by hydrolysis of the acyl enzymes at pH 7.[16]

The catalytic specificity of α-chymotrypsin originates in the tosyl hole binding pocket.[12,13] The steric requirements that a substate must meet to fit into the tosyl hole are extremely well-known from X-ray crystallography of modified enzymes.[13] These studies have been complemented by solution-phase EPR analysis of nitroxide[4] spin labels and fluorine NMR[5] analysis of the appropriately modified enzymes. On the basis of this work, we expected that the aryl azide moieties of **10a** and **11a** bound to serine-195 would fit into the tosyl hole, whereas those of **12a** and **13a** would not. In fact, **11a** has been synthesized previously[17] and was used in one of the earliest applications of photoaffinity labeling. The chemical labeling pattern observed on photolysis of **11a** was in accord with expectations that it was in the aromatic binding pocket.

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Methyl esters **10c–13c** and modified enzymes **10a–13a** were dissolved in the appropriate degassed medium, frozen to 77 K, and photolyzed ($\lambda > 310$ nm, 30 s) in the cavity of an EPR spectrometer. Triplet nitrene EPR spectra were observed in all cases. The resonance fields of the nitrene methyl esters were solvent dependent, with higher field positions generally observed in polar hydroxylic matrices. As expected for nitrenes inside the hydrophobic binding pocket, the resonance fields obtained by photolysis of **10a** and **11a** were at much lower field than those obtained from methyl esters **10c** and **11c** in the same solvent matrix (Table I). Significantly, the resonance fields observed on photolysis of azides **12a** and **13a** (which do not fit into the binding pocket) were consistent with a polar environment and were very similar to the resonance fields observed on photolysis of methyl esters **12c** and **13c** in glycerol–water. Indole is known to bind to the active site of α-chymotrypsin.[13] Berliner has shown that upon addition of indole to a solution of CHY covalently modified with an aryl sulfonyl nitroxide spin label the indole will ultimately occupy the aromatic binding pocket and effectively displace the spin label from the tosyl hole.[4] Berliner has observed similar indole displacements of fluorinated aromatic probes of chymotrypsin using ^{19}F NMR.[5] Accordingly, indole was added to solutions of **10a–13a** prior to cooling and photolysis. The resonance fields obtained by low-temperature photolysis of modified enzymes **10a** and **11a** in the presence of indole were much higher than in its absence and indicated that the nitrenes **14a** and **15a** had indeed been displaced from the hydrophobic binding pocket. Only small or zero effects of indole were observed with nitrenes **16a** and **17a**, which were not expected to fit in the tosyl hole binding pocket.

Decay Kinetics of Triplet Nitrenes Covalently Bound to α-Chymotrypsin. We next turned our attention to the kinetics of decay of the matrix-isolated nitrene photoaffinity labels. The lifetimes of the triplet nitrenes **14a,b–17a,b** were determined by EPR following brief irradiation of glycerol–water samples at 173–174 K (the nitrenes do not react appreciably in any matrices in the dark at 77 K). The nitrene methyl esters **14b–17b** decayed more rapidly than the matrix-isolated nitrenes **14a–17a**, which are derived from photolysis of modified enzymes. A reasonable mechanism for the decay of a triplet nitrene is hydrogen atom abstraction. Glycerol is an excellent hydrogen atom donor, hence the relatively rapid decay of nitrenes **14b–17b** derived from the azido methyl esters. The diminished reactivity of nitrenes **14a** and **15a** (derived from the modified enzymes **10a** and **11a**) relative to methyl esters **14b** and **15b** was consistent with the enzyme-bound nitrenes being in the binding pocket of α-chymotrypsin and shielded from the excellent hydrogen atom donating glass. Meta-substituted methyl ester nitrene **14b** is more reactive than its para-substituted nitrene isomer **16b** in glycerol–water at 173–174 K. However, under the same conditions the nitrene derived from the para-substituted modified enzyme **16a** (which is outside the tosyl hole) is more than 10 times as reactive as the meta-labeled species **14a**, which is believed to be in the binding pocket. In fact, the para-substituted nitrene **16a** bound to the enzyme reacts within a factor of 3 as rapidly as does the simple methyl *p*-nitrenobenzoate (**16b**) in glycerol–water. The nitrenocinnamate **15a** (derived from **11a**), which resides within the binding pocket of the enzyme, reacted at least 100 times more slowly than nitrene **15b** (derived from the methyl cinnamyl ester **11c**) in glycerol–water. The kinetic evidence for disubstituted phenylnitrene **17a** indicated that, as expected, it was outside the binding pocket. In every case, the analysis of the triplet EPR spectroscopic data, the kinetic analyses, and previous structural work[4,5,13] are in agreement.

Matrix Chemistry of Methyl *m*-Azidobenzoate. EPR spectroscopy of triplet nitrenes is a very sensitive tool for detecting a triplet nitrene, but it is not readily calibrated to determine the amount of triplet present in a sample. It is conceivable that only a small fraction of the azides form triplet nitrenes on photolysis at 77 K. We have measured the size of the nitrene signals derived from azides **10a–13a** and their corresponding methyl esters **10c–13c** under conditions identical in azide concentration (10^{-3} M), temperature (77 K), glass (glycerol–water), and photolysis time $\lambda > 320$ nm (25 s). In all cases, the nitrene EPR signals obtained from the modified enzymes and the corresponding methyl esters were of similar intensity, demonstrating that the basic azide photochemistry and quantum yields of triplet nitrene formation were similar. However, this did not reveal what percentage of the azides was converted into triplet nitrenes under the conditions employed in the EPR experiments.

Scheme I

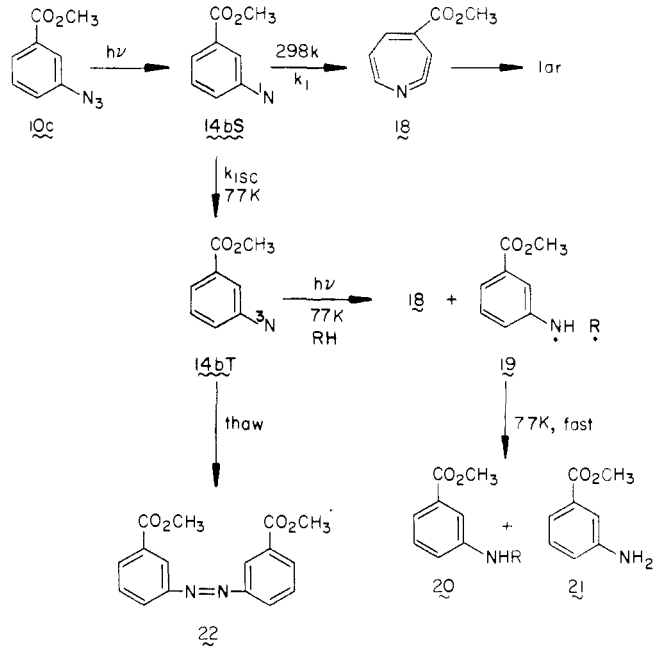


Table III. Temperature Dependence of Products Formed on Photolysis[a] of Methyl 3-Azidobenzoate in Toluene

	absolute yields, %			
T (±3°), K	**21**	**20**	**22**	total, %
298	0.2	*h*	*h*	0.2
203	*h*	*h*	*h*	*h*
123[c]	0.4	30.3	33.0	63.7
77[c]	0.4	21.5	55.6	77.5
77[d]	0.2	39.0	33.4	72.6
4[c,e,f]	0.7	26.4	42.9	70.0
4[c,e,g]	1.1	31.7	33.0	65.8

[a] Rayonet Photochemical Reactor, all products analyzed by GC. [b] 4.85×10^{-2} M **10c** and naphthalene (internal standard) in toluene. [c] The frozen solution was thawed directly after photolysis. [d] The frozen solution was warmed to and kept at −150 °C after photolysis before it was thawed to room temperature. [e] Photolysis was carried out using a Hg arc lamp equipped with a $CuSO_4$ filter. [f] Time of photolysis was 0.2 h. [g] Time of photolysis was 0.5 h. [h] Yield much less than 0.2%.

We therefore undertook a model study of the products formed on photolysis of methyl *m*-azidobenzoate (**10c**) in toluene. Photolysis of **10c** in toluene solutions at 298 and 223 K gave considerable tar and much less than 1% yield of identifiable products. Thus, this reagent would hardly seem to be a suitable reagent for a solution-phase photoaffinity labeling study as there is no evidence for a covalent adduct between a reactive intermediate derived from **10c** and toluene at 223 and 298 K. An entirely different pattern is revealed on photolysis of **10c** in a frozen toluene polycrystal. At 77 K it was possible to realize 63.7–77.5% material balances of products **20–22**, products that can easily be attributed to triplet nitrene **14b** (Scheme I). Significantly, very little tar was produced in the matrix photolyses and it was possible to achieve a 39% yield of adduct **20**, the desired type of product in photoaffinity labeling.

The pattern of observations that emerges from Table III is completely consistent with our previous mechanistic analysis of phenyl azide.[10] The pertinent details are summarized in Scheme I. Photolysis of phenyl azide (**1**) or methyl *m*-azidobenzoate (**10c**)

Table IV. Number of Catalytically Active Sites (Percent) of Native and Modified α-Chymotrypsin before and after Photolysis[a]

A (enzyme)	B (before $h\nu$, $-NH_2NH_2$)[b]	C ($-NH_2NH_2$)[c]	D	E (298 K)[d]	F	G	H (after $h\nu$, 77 K)[e]	I	J
				Trial 1					
CHY	100	89		71	(80)[f]		81	(91)[f]	
10a	18	68	(76)[f]	57	(64)[f]	80[g]	40	(45)[f]	(49)[g]
A (enzyme)	B (before $h\nu$, $-NH_2NH_2$)[b]	C ($-NH_2NH_2$)[c]	D	E (after $h\nu$, 298 K)[d]	F	G	H (77 K)[e]	I	J
				Trial 2					
CHY	100	71		77	(108)[f]		75	(106)[f]	
10a	46	70	(99)[f]	64	(90)[f]	(83)[f]	36	(50)[f]	(48)[g]

[a] Assays were measured by the Shonbaum method.[14] [b] Activities measured before treatment with hydrazine. [c] Activities measured after treatment with hydrazine, gel permeation chromatography, and lyophilization. [d] Photolysis at 298 K followed by treatment as per c. [e] Photolysis at 77 K followed by treatment as per c. [f] This value obtained by comparison with unphotolyzed native enzyme treated with hydrazine as per c. [g] This value obtained by comparison with photolyzed native enzyme (*d*) treated with hydrazine as per c.

leads to nitrogen extrusion and the corresponding singlet nitrenes (**2S** and **14bS**, respectively). The singlet nitrenes can either ring expand (k_1) to a ketenimine (**3** or **18**) or can intersystem cross (k_{isc}) to the ground-state triplet nitrenes (**2T** or **14bT**). A combination of product studies, laser flash photolysis studies, and matrix isolation spectroscopy convinced us that the ratio of k_1 to k_{isc} was strongly temperature dependent.[10] At 298 K $k_1 \gg k_{isc}$ and little or zero triplet nitrene is formed. The product formed at 298 K is the ketenimine (**3** or **18**), which polymerizes in the absence of a strong nucleophile. The parent ketenimine can only be intercepted with a potent nucleophile such as an alkylamine. Singlet phenylnitrene (**2S**) has never been intercepted with an external trap.[7,8,10] At 77 K, $k_1 \ll k_{isc}$ and triplet nitrenes **2T** and **14bT** are formed cleanly. At this temperature, the triplet nitrenes are held rigidly in a frozen matrix. They can be detected by EPR and by UV–vis[18] and fluorescence spectroscopy.[10] The triplet nitrenes are not reactive intermediates under conditions of matrix isolation. They are persistent, and they can accumulate and ultimately compete with azide precursor for the absorption of light. Photolysis of parent triplet phenylnitrene at 77 K leads cleanly to its isomerization to ketenimine **3**.[10,19] Matrix photolysis of 2,6-difluorophenylnitrene or pentafluorophenylnitrene leads exclusively to hydrogen atom abstraction to give a radical pair that collapses to form formal CH insertion adducts in greater than 90% yield.[20] Both processes occur upon photolysis of triplet nitrene **14bT** in frozen toluene. The nitrene abstracts a benzylic hydrogen to form radical pair **19**, which can collapse (even in the matrix) to form adduct **20**. Thawing a matrix containing incompletely photolyzed **14bT** results in its dimerization to azo compound **22**.

Evidence for the matrix photochemical ring expansion of **14bT** to **18** was deduced from UV–vis spectroscopy. Brief photolysis (313 nm) of methyl *m*-azidobenzoate (**10c**) produces a new absorption spectrum shown in Figure 1. This spectrum is very similar to that of parent triplet phenylnitrene (**2T**) reported by Reiser,[18] Waddell,[21] and ourselves.[10] Continued photolysis of the initially formed photoproduct rapidly destroys it and leads to a new species whose absorption spectrum is shown in Figure 2. The spectrum of the secondary photoproduct observed at 77 K is virtually identical to the transient spectrum of ketenimine **3** produced at 298 K by flash spectroscopy of phenyl azide.[10,22] The missing 20–30% of the material balance in the matrix photolysis of **10c** at 77 K can be easily explained by secondary photolysis to give **19** and **18**, the latter species ultimately polymerizing upon melting the matrix.

Photochemistry of Modified Enzyme 10a. Model studies with methyl *m*-azidobenzoate (**10c**) convinced us that the efficiency of PAL would significantly improve at 77 K relative to 298 K.

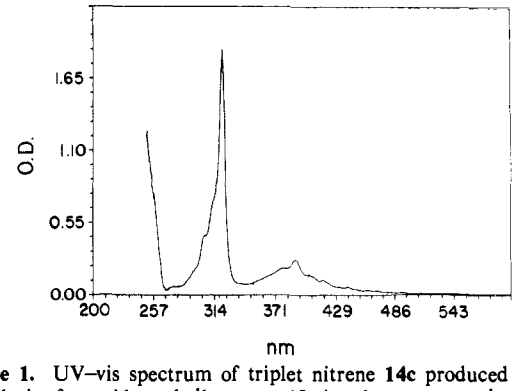


Figure 1. UV–vis spectrum of triplet nitrene **14c** produced by brief photolysis of *m*-azidomethylbenzoate **19c** in ether–pentane alcohol glass at 77 K.

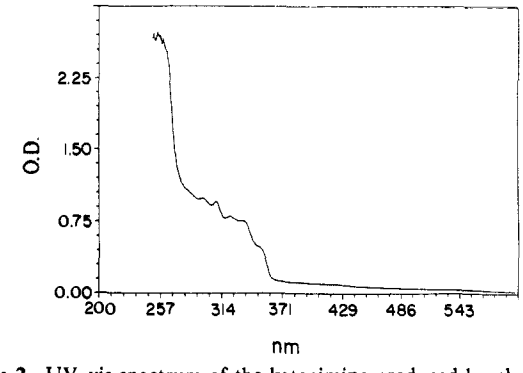
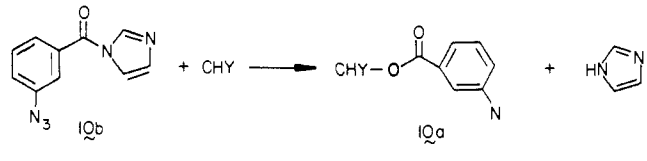
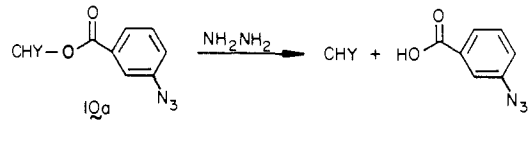


Figure 2. UV–vis spectrum of the ketenimine produced by photorearrangement of triplet nitrene **14c** at 77 K.

Thus, the corresponding modified enzyme **10a** was photolyzed at both of these temperatures. The activities of the enzymes were measured by the Schonbaum assay, which in effect counts the number of catalytically active sites present in the sample.[14] The activities are reported in percentages of active sites of a sample relative to the untreated native enzyme (Table IV, columns B, C, E, and H). Modified enzyme **10a** was prepared by treatment of chymotrypsin with (*m*-azidobenzoyl)imidazole (**10b**) as previously described. The modified enzyme can be deacylated with



hydrazine to regenerate the catalytic activity.[23]



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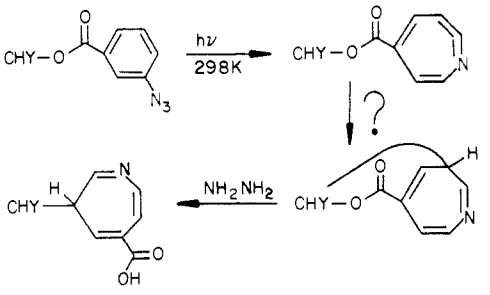
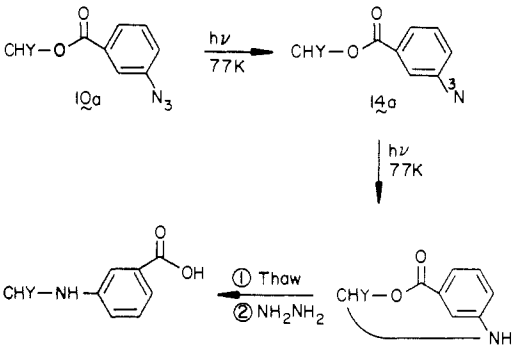
Treatment of native chymotrypsin with hydrazine followed by gel permeation chromatography and concentration by lyophilization gave protein that was 71–89% as active as the untreated native enzyme (column C). Presumably some denaturation or contamination of the enzyme with salt occurred during this process. Photolysis of native enzyme at 298 or 77 K followed by treatment with hydrazine and reisolation produced a further slight decrease in activity in trial 1 and no further decrease in activity in trial 2 (columns E and H). It can be concluded that freezing chymotrypsin to 77 K and then irradiating and thawing does not destroy its catalytic activity. The two trials were performed on modified enzymes synthesized on different days with differing initial incorporation of the PAL reagent. Treatment of modified enzyme **10a** (no photolysis) with hydrazine followed by reisolation returned 68–70% (column C) of the catalytic activity when compared to the untreated native enzyme and 76–99% of the catalytic activity of similarly hydrazine-treated native enzyme (column D).

Assuming that the photochemistry of enzyme **10a** is similar to that of model azide **10c**, then we expect that photolysis of **10a** at 298 K will generate a ketenimine. The ketenimine may react



with a neighboring nucleophilic amino acid residue to form a new covalent link to the binding site and thereby irreversibly destroy the catalytic activity of the protein. The ketenimine being relatively long-lived[10] in the absence of an amino acid derived nucleophile may eventually react with water. Photolysis of **10a** at 298 K followed by hydrazine deacylation and reisolation returns protein that is 57–64% as active as native enzyme (column E), 64–90% as active as native enzyme treated with hydrazine and reisolated (column F), and 80–83% as active as native enzyme that had been photolyzed, treated with hydrazine, and reisolated (column G). If photolabeling had been 100% efficient, the catalytic activity of the recovered enzyme would equal the percent of incorporation of the original label (18–46%, column B). If one assumes that the loss of activity in the recovered photolyzed enzyme results from irreversible photolabeling of the binding site, then the efficiency of photoaffinity labeling at 298 K is at best small. This analysis assumes that ketenimine–amino acid adducts are stable to treatment with hydrazine. This is probably a good assumption for amine–ketenimine adducts because the adduct of diethylamine and parent ketenimine **3** is stable to large excesses of diethylamine.[7-10] It is possible that adducts of the ketenimine derived from modified enzyme **10a** with sulfur nucleophiles are decomposed by hydrazine.

Modified enzyme **10a** was then photolyzed at 77 K, thawed, treated with hydrazine to effect deacylation, purified by gel permeation chromatography, and concentrated by lyophilization. The activity of the recovered protein **10a** that was photolyzed at 77 K was only 48–49% that of native enzyme photolyzed at 77 K and reisolated (column J). Thus, low-temperature photolysis of **10a** was much more effective at destroying the catalytic activity of α-chymotrypsin than room-temperature photolysis. The loss in active sites is presumed to result from covalent attachment of the label to the binding site. In trial 2, the loss of active sites was almost equal to the stoichiometric amount of azide present in the unphotolyzed sample, which implies nearly complete conversion of azide in trial 2 to covalently bound product. In the more highly



modified enzyme (trial 1), the efficiency of labeling is somewhat less than in trial 2. This may be due to the fact that it is more difficult to effect complete photochemical conversion of azide to nitrene, and complete secondary photolysis of triplet nitrene to a radical pair, than in the experiment using less azide label (trial 2). The increased loss of active sites in **10a** may also reflect the increased stability of nitrene-derived adducts to hydrazine relative to ketenimine-derived adducts.

Conclusions

It is our contention that the basic photophysics and photochemistry of aryl azides immobilized in organic polycrystals and covalently bound to α-chymotrypsin cooled in a rigid glass will be quite similar. Thus, we feel confident that the EPR signals of nitrenes bound to α-chymotrypsin do not correspond to a trivial photoprocess. On the basis of product studies of **10c** in toluene, we suspect there is at least a 60–70% yield of triplet nitrene held rigidly inside the binding site of α-chymotrypsin. In the ambient-temperature solution-phase photochemistry of modified enzyme **10a**, a ketenimine such as **18** is produced that can only react with nucleophiles or solvent. In the photolysis of **10a** frozen in a glass, one can demonstrate that (1) a triplet nitrene is formed in high yield in a hydrophobic environment (presumably the tosyl hole), (2) the rigid glass prohibits nitrene diffusion out of the binding site, hence, it must react in the binding site, (3) on the basis of model studies, there is reason to expect formal insertion into nearby unactivated CH bonds, and (4) that consequently that the efficiency of PAL of α-chymotrypsin is much greater at 77 K than at 298 K.

Experimental Section

General Methods. Melting points were obtained by using an electrothermal melting point apparatus and are uncorrected. NMR spectra were recorded on a Varian EM-390 (90-MHz) spectrometer. All ^{1}H chemical shifts are reported relative to TMS as an internal standard. Infrared (IR) spectra were recorded on a Perkin-Elmer 457 grating spectrophotometer. Ultraviolet (UV) spectra were recorded either on a Cary 14 or on a Perkin-Elmer Lambda 3 UV–vis spectrophotometer.

Materials. 3-Aminobenzoic acid, 4-aminobenzoic acid, and 4-aminocinnamic acid hydrochloride were purchased from Aldrich. 3-Amino-4-nitrobenzoic acid was prepared by a literature method.[24] α-Chymotrypsin was purchased from ICN Pharmaceutical Co. 4-Azidocinnamic acid, *m*-azidobenzoic acid, *p*-azidobenzoic acid, and 3-azido-5-nitrobenzoic acid were prepared by literature methods.[24,25]

General Procedure for Preparation of Acyl Imidazoles 10b–13b. (a) (4-Azidocinnamoyl)imidazole (11b). 4-Azidocinnamic acid (378 mg, 0.02 mol) was mixed with freshly distilled thionyl chloride and heated to reflux over a steam bath for 2 h. Excess $SOCl_2$ was removed under vacuum. Dry benzene (5 mL) was added to the residue, and the remaining $SOCl_2$ was removed as an azeotrope. The acid chloride was dissolved in 5 mL of dry benzene, and the solution was added dropwise to a suspension of imidazole (272 mg, 0.04 mol) in 25 mL of benzene at 0 °C. The temperature was allowed to rise to room temperature, and after 1 h of stirring, the imidazolium chloride formed was filtered. The filtration was concentrated, and the solid residue was recrystallized in

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benzene to yield 288 mg (60%) of **13b**: mp 165–166 °C dec; IR (KBr) 2100, 1690 cm^{-1}; ^1H NMR ($CDCl_3$) δ 6.9–83 (m, 9 H).

(b) (3-Azidobenzoyl)imidazole (10b): 56%; mp 81–83 °C (benzene–hexane); IR ($CHCl_3$) 2100, 1690 cm^{-1}; ^1H NMR ($CDCl_3$–acetone-d_6) δ 7.12–8.0 (m, 7 H).

(c) (4-Azidobenzoyl)imidazole (12b): 70%; mp 66–68 °C (benzene–hexane); IR ($CHCl_3$) 2100, 1685 cm^{-1}; ^1H NMR ($CDCl_3$) δ 7.0–8.1 (m, 7 H).

(d) (3-Azido-5-nitrobenzoyl)imidazole (13b): 18%; mp 100–103 °C (benzene–hexane); IR (KBr) 2120, 1705, 1530, 1520, 1380, 1350, 1265, 960 cm^{-1}; ^1H NMR ($CDCl_3$) δ 8.5 (s, 1 (H), 8.0 (s, 1 H), 7.2 (s, 2 H), 6.9 (s, 1 H).

EPR Experiments. Solutions of methyl esters **10c–13c** (1×10^{-3} M) in cyclohexane, toluene, 2-propanol, methanol, and glycerol–water (1:1, pH 3.0) were prepared. The solutions (0.5 mL) were placed in quartz EPR tubes, degassed by 3 freeze–jump–thaw cycles, and then sealed under vacuum. Samples of modified enzymes **10a–13a** were prepared by putting 12.5 mg of **10a–13a** in quartz EPR tubes and adding 0.5 mL of glycerol–water (1:1, pH 3.0), which was previously deoxygenated by bubbling with nitrogen gas. The solutions obtained were frozen in liquid nitrogen and sealed under vacuum.

A Varian E-line Series 112 spectrometer was used to obtain EPR spectra. To obtain spectra at 77 K, the samples were immersed in a quartz Dewar (Wilmad) containing liquid nitrogen, which fit into the cavity of the EPR spectrometer. EPR spectra were obtained at 173–174 K by using the Varian variable-temperature controller.

The output of a 1000-W mercury–xenon arc lamp passing through a $CuSO_4$ solution filter ($\lambda > 320$ nm) was focused into the cavity for photolysis. The samples were photolyzed for 30 s, and the field positions of the triplet nitrene signals were recorded. The kinetics of the decay reaction of the triplet nitrene at 173–174 K were measured by observing the decrease in the intensity of the signals at regular intervals. Pseudo-first-order rate constants were obtained by fitting the initial 20% of the signal decay.[23]

Product Studies of Methyl *m*-Azidobenzoate (10c) in Toluene. Solutions of **10c** in toluene for product studies (0.5 mL) were sealed in 5-mm Pyrex tubes, which were prewashed with ammonium hydroxide solution and oven-dried. The samples were degassed by using 3 freeze–jump–thaw cycles, sealed under vacuum, and photolyzed for 4 h with two southern New England ultraviolet RPR 3500-Å lamps. The product mixtures were analyzed with a Hewlett-Packard 8530 A gas chromatograph using a 6 ft × $^1/_8$ in. 5% SE-30 column. The yields and identities of the products were determined by coinjection of authentic samples and by mass spectroscopy.

Photochemistry of Modified Enzyme 10a. Samples were prepared by dissolving 93–94 mg each of native chymotrypsin and modified enzyme **10a** in 5 mL of aqueous HCl solution of pH 3.0. Solutions (1.0 mL) were placed in 6-mm Pyrex tubes previously cleaned and dried and sealed with rubber septa. The solutions were photolyzed for 2 h with four Southern New England ultraviolet RPR 3500-Å lamps.

The photolyzed enzyme solutions were treated with sufficient 25% aqueous hydrazine solution[24] to raise the pH of the solutions between pH 7 and 8. The samples were kept at that pH for 1 h to effect complete hydrolysis of the ester linkage in modified enzyme **10a**. The solutions were then passed through a column of Sephadex G-25 (10 cm × 1 cm) and eluted with aqueous HCl solution of pH 3.0. The eluent fractions with high absorbances at 280 nm were collected, and the enzyme was obtained by lyophilization using a Virtis Freeze Mobile 6 lyophilizer. In the control experiments, samples of native α-chymotrypsin and modified enzyme **10a** were photolyzed and treated with hydrazine and reisolated by lyophilization after being passed through a Sephadex G-25 column.

The enzymes, both photolyzed and unphotolyzed, were assayed using cinnamoyl imidazole at pH 5 as described in literature.[14]

General Procedure for the Preparation of Modified Enzymes 10a–13a. α-chymotrypsin (100 mg) was dissolved in 3.0 mL of 0.1 M acetate buffer (pH 5) in a centrifuge tube. To this was carefully added a solution of 10 mg of the acyl imidazole in 200 mL acetonitrile. After careful mixing for 15 minutes, the mixture was centrifuged to separate the solid material. The clear supernatant solution was immediately transferred onto a Sephadex G-25 column (40 cm × 2.5 cm) which was equilibrated with aqueous HCl solution (pH 3) and eluted with the same solution. Five-mL fractions were collected and monitored for the protein absorption at 280 nm. The fractions containing the protein were combined and lyophilized to obtain the modified enzyme.

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Observation of Secondary Bonding in Solution: Synthesis, NMR Studies, and the Crystal Structure of $Te^{VI}[OTe^{IV}(C_8H_8)(S_2P(OEt)_2)]_6$†

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Abstract: The compound $Te[OTe(C_8H_8)(S_2P(OEt)_2)]_6$ (**1**), an unexpected hydrolysis product of $C_8H_8Te[S_2P(OEt)_2]_2$, was subsequently synthesized under very mild conditions and appears to be the first organotellurium compound containing both tellurium(IV) and tellurium(VI). Crystals of **1** are triclinic, space group $P\bar{1}$: a = 14.287 (2), b = 14.579 (2), c = 14.652 (2) Å; α = 85.30 (1), β = 84.36 (1), γ = 60.20 (1)°; Z = 1. The central Te(VI) atom is surrounded octahedrally by six oxygen atoms, which bridge to Te(IV) centers. Each Te(IV) center is coordinated asymmetrically by a dithiophosphate ligand. The sulfur atom of the longer Te–S bond also forms a secondary interaction with an adjacent Te(IV) atom within the same molecule. Each Te(IV) atom has a stereochemically active lone electron pair occupying one of the coordination positions and results in a seven-coordinate 1:2:2:2 geometry. Variable-temperature ^{13}C, ^{31}P, and ^{125}Te NMR indicate that intramolecular monodentate–bidentate exchange of the dithiophosphate ligand is slow on the NMR time scale. NMR studies on $Te[OTe(C_8H_8)(S_2P(O^iPr)_2)]_6$ indicate that, as a result of extensive secondary bonding, this molecule is remarkably rigid in solution at room temperature, with the structure in solution being similar to that determined for $Te[OTe(C_8H_8)(S_2P(OEt)_2)]_6$ in the solid state. This represents the first occasion for which the secondary bonds observed in the solid state persist in solution.

Synthesis of known mixed-valent tellurium(IV)–tellurium(VI) complexes has until now involved use of the highly electron withdrawing ligand, $OTeF_5$.[3-6] The complex $Te(OTeF_5)_4$, characterized by ^{125}Te and ^{19}F NMR spectroscopies,[4] contains a central tellurium(IV) atom bonded via oxygen to four tellurium(VI) atoms. Complexes such as $F_4Te(OTeF_5)_2$, $F_2Te(OTeF_5)_4$, $FTe(OTeF_5)_5$, and $Te(OTeF_5)_6$, which contain Te(VI)–O–Te(VI) bonds, have also been studied.[3] The X-ray structure determi-

† Hexakis[[2-[(diethoxythiophosphoryl)thio]-1,3-dihydro-2λ⁴-benzotellurol-2-yl]oxy]tellurium(VI).

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