Cryophotolysis of *ortho*-Nitrobenzyl Derivatives of Enzyme Ligands for the Potential Kinetic Crystallography of Macromolecules

Alexandre Specht,[b] Thomas Ursby,[a] Martin Weik,[c] Ling Peng,[b] Jan Kroon,[c] Dominique Bourgeois,*[a] and Maurice Goeldner*[b]

KEYWORDS:

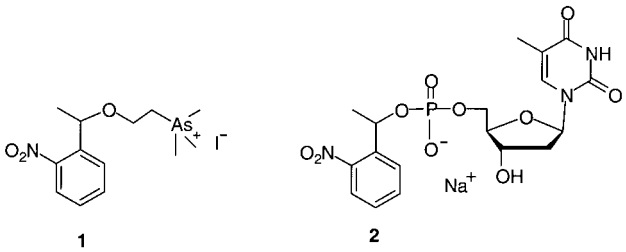
acetylcholinesterase · caged compounds · cryophotolysis · enzymes · hydrolases

Photolabile precursors of biologically active molecules (caged compounds) have been developed to allow temporally and spatially controlled release of bioactive compounds by rapid photolysis.[1] Caged compounds can be used to photochemically trigger an enzymatic reaction within an enzyme crystal and are thus valuable tools for studying protein dynamics at the atomic level by time-resolved X-ray crystallography.[2] Real-time-resolved X-ray crystallographic studies of fast biological processes have been applied mainly to a series of naturally light-sensitive proteins by using rapid data collection techniques (Laue diffraction) combined with high-intensity synchrotron X-ray beams.[3] However, caged ligands can also be used as photochemical triggers to capture transient structural species within enzyme crystals, provided photofragmentation reactions are fast, synchronous, and homogeneous throughout the crystals. The few successful examples which have been described so far concern enzymes whose reaction rates in the crystal were slow enough to allow both completion of the photolytic reaction and generally of the Laue data collection.[4] Alternatively, enzyme mutants may be produced to further increase the reaction intermediates' lifetime.[5] Unfortunately, a series of technical pitfalls are associated with this method, including difficulties with the Laue method (essentially sensitivity to crystal disorder)[6] as well as photochemical[7] and radiochemical damages[8] to the studied protein induced by intense UV light pulses and/or X-ray beams.

The study of fast enzymatic reactions by crystallography using caged compounds will require alternative methods such as kinetic crystallography,[3] where cryotechniques[9] are used to slow down and eventually completely stop the enzymatic reaction. Recently, the use of caged GTP was combined successfully with low-temperature crystallography on the Ras protein ($p21^{ras}$),[10] the photochemical fragmentation reaction being achieved at room temperature before rapid cooling of the crystal. However, achievable cooling rates of macromolecular crystals do not allow to apply this procedure to rapid enzymatic reactions,[11] such as those catalyzed by cholinesterases. Accordingly, we developed several caged cholinergic ligands to allow the photoregulation of the cholinergic activity.[12]

Here, we demonstrate the photofragmentation of caged compounds even at cryogenic temperatures as low as 100 K both in flash-cooled amorphous solution and in complex with a crystalline enzyme. This method allows to overcome the previously mentioned pitfalls. While the selected temperature does preclude any enzyme catalysis to proceed, there is no need for a synchronized photofragmentation on a rapid time scale, and low levels of photolyzing light can be used to minimize the radiation damages. Most importantly, this technique, in combination with appropriate temperature profiles, opens the possibility to trap reaction intermediates in protein crystals that can be studied by monochromatic X-ray crystallography.[13]

As a first example, we investigated the photolysis of a caged cholinergic ligand, *ortho*-nitro-α-methylbenzyl arsenocholine (**1**; Scheme 1), which possesses a potential advantage in future dynamic X-ray crystallographic studies on cholinesterases by



Scheme 1. ortho-*Nitrobenzyl derivatives* ***1*** *and* ***2*** *representing caged arsenocholine and caged deoxythymidine monophosphate, respectively.*

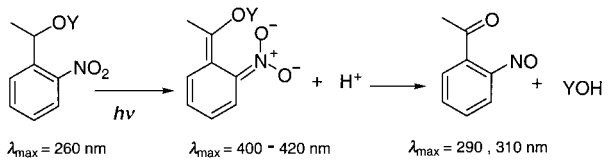
allowing the photolytic release of a cholinergic ligand containing a heavy atom. At room temperature, the photochemical fragmentation of **1** was demonstrated to be a fast and efficient photolytic reaction.[14] The formation of both arsenocholine and *ortho*-nitrosoacetophenone was shown to be concomitant with the consumption of the caged compound **1** during photolysis (data not shown), in agreement with the presumed fragmentation process (Scheme 2).[15]

Cryophotolysis of compound **1** in flash-cooled solution at 100 K was performed by applying successive Xe flashes (see

[a] *Dr. D. Bourgeois, Dr. T. Ursby*
Institut de Biologie Structurale
41 Rue Jules Horowitz
38043 Grenoble Cedex (France)
and
European Synchrotron Radiation Facility
B.P. 220, 38043 Grenoble Cedex (France)
Fax: (+33) 476-882-542
E-mail: bourgeoi@lccp.ibs.fr

[b] *Prof. Dr. M. Goeldner, A. Specht, Dr. L. Peng*
Laboratoire de Chimie Bioorganique, UMR 7514 CNRS
Faculté de Pharmacie, Université Louis Pasteur Strasbourg
BP 24, 67401 Illkirch Cedex (France)
Fax: (+33) 390-244-306
E-mail: goeldner@bioorga.u-strasbg.fr

[c] *Dr. M. Weik, Prof. Dr. J. Kroon*
Bijvoet Center for Biomolecular Research
Utrecht University
3584 CH Utrecht (The Netherlands)



1 Y= arsenocholine, **2** Y = dTMP

Scheme 2. *Proposed mechanism for the photofragmentation of* ortho*-nitrobenzyl derivatives.*

Experimental Section). Figure 1 shows the difference UV spectra recorded during cryophotolysis by reference to the nonphotolyzed sample. The increase in the negative peak around 260 nm, which is indicative of a fragmentation of the starting compound **1**, is proportional to the formation of a positive peak around

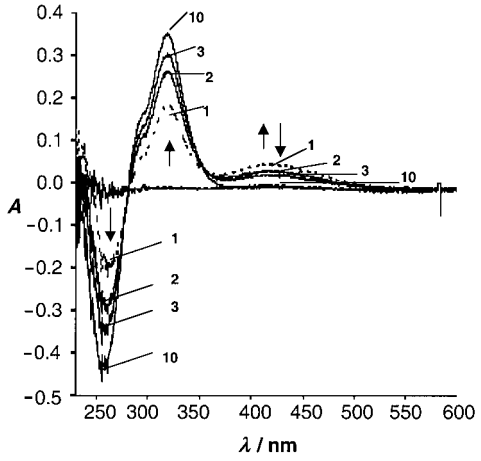


Figure 1. *Difference UV spectra showing progressive photolysis of caged arsenocholine* ***1*** *at 100 K after 0, 1, 2, 3, and 10 flashes, respectively (see labels). The spectrum recorded after one flash is shown as a dashed line.*

310 nm. This latter peak is characteristic of the nitroso derivative, which allows a quantification of the photolytic reaction. The peak formed initially around 400 nm decreases after subsequent photolysis and might be tentatively attributed to an *aci*-nitro intermediate (Scheme 2), suggesting also a photosensitivity of this intermediate.[16] Most importantly, exposure to one flash led to a ca. 50% conversion of **1**, and a maximum of ten successive flashes were sufficient for a complete photofragmentation as assessed by a stationary difference spectrum and the absence of residual absorbance at 260 nm in the non-difference spectrum. Independently, we checked that the photochemical experiment can be achieved under cryogenic conditions by using gentle laser photolysis at 355 nm (data not shown). A key advantage of laser photolysis relative to flash lamp photolysis is that, at 355 nm, the laser source produces only a negligible temperature elevation of the sample. This is particularly true in the presence of proteins, which strongly absorb light at 280 nm emitted by Xe sources. Even though the sample was kept at 100 K during photolysis, we cannot exclude that local heating occured as a consequence of UV photon absorption that may serve to carry forward the fragmentation reaction.

In a second example, we performed a cryophotolytic experiment at 100 K of the caged deoxythymidine monophosphate (caged dTMP) **2** (Scheme 1). Similar results were obtained by UV spectroscopic analysis indicating a complete fragmentation of compound **2** to dTMP and *ortho*-nitrosoacetophenone (data not shown).

To compare the identity of the products from the photolytic reaction under cryogenic conditions with those from photolysis at room temperature, we performed HPLC analyses of the products of the cryophotolysis experiments. We used a high concentration of caged ligands (up to 15 mM) to mimic the situation occurring in protein crystals and to obtain sufficient material for HPLC analyses (see Experimental Section). These analyses confirmed the conversion of **1** to arsenocholine and of **2** to dTMP, as well as the formation of the nitroso derivative in both cases (Figure 2). The conversion of either **1** or **2** was not

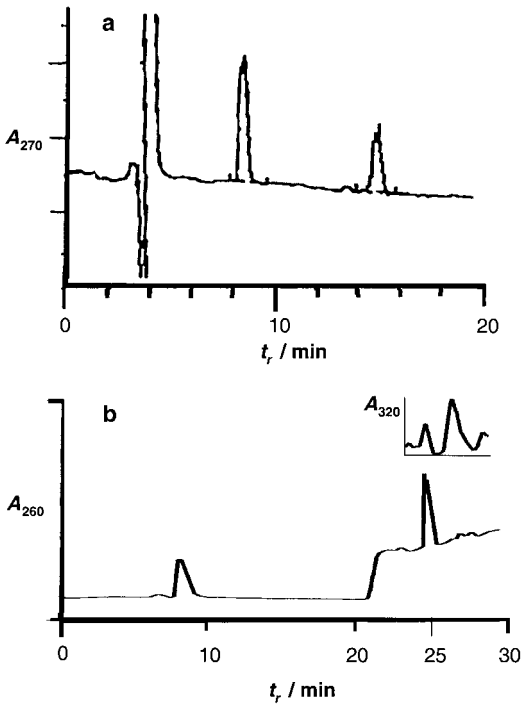


Figure 2. *HPLC analysis of the cryophotolysis reaction of* ***1*** *(a) and* ***2*** *(b) at 100 K. a) A sample of caged arsenocholine* ***1*** *was exposed to excess (50) Xe flashes. The retention times for* ***1*** *and* ortho*-nitrosoacetophenone are 9.2 and 15.0 min, respectively. b) A sample of caged dTMP (****2****) was exposed to 40 successive Xe flashes. The retention times for dTMP,* ***2****, and* ortho*-nitrosoacetophenone are 9.0, 25.3, and 27.1 min, respectively. The nitroso derivative is detectable at 320 nm (see insert).*

complete (about 40% and 58% for **1** and **2**, respectively), although UV spectroscopic analysis indicated a total photolytic conversion (Figure 1). We then performed photolysis of **1** either at lower concentration (7.5 mM) or at higher temperature (160 K) and the observed profiles showed increased conversion in both

  1439-4227/01/02/11 $ 17.50+.50/0 CHEMBIOCHEM **2001**, No. 11

cases: 65% and 82%, respectively (data not shown). The occurrence of a precipitation phenomenon that could explain this discrepancy and would be in agreement with the observed HPLC results, however, seems unlikely under the flash-freezing conditions used.

To extend this method to the study of functional proteins, we applied the cryophotolysis experiment (100 K) on trigonal crystals of *Torpedo californica* acetylcholinesterase (*Tc* AChE)[17] containing the caged arsenocholine derivative **1**. The formation of the enzyme–inhibitor complex was demonstrated by X-ray crystallographic analysis.[18] The UV difference spectrum obtained after 100 successive Xe flashes at 100 K shows the appearence of a peak around 310 nm (Figure 3), suggesting the

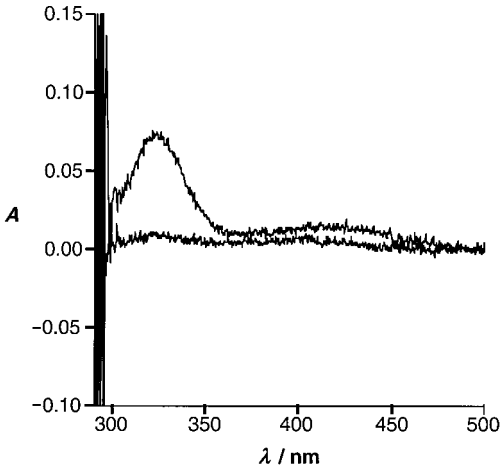


Figure 3. *Difference UV spectra recorded before and after photolysis of a crystal of* T. californica *AChE–caged arsenocholine complex at cryogenic temperature (100 K).*

likely formation of the nitroso derivative within the enzyme crystal, corresponding to the desired uncaging of the enzyme inhibitor. In an attempt to assess the formation of the nitroso compound in the enzyme crystal, we performed successive cryophotolysis experiments on twenty different crystal complexes. After solubilization of the crystals, we performed HPLC analyses on the combined material. Unfortunately, the obtained profiles did not allow the detection of any interpretable signal from the background noise.

The present study demonstrates for the first time an efficient photofragmentation reaction of caged ligands at 100 K in a glass formed from a buffered solution and within a protein crystal. The latter photofragmentation reaction will be applicable to any crystal of a protein–caged ligand complex and is therefore of general interest. For instance, the cryophotolysis at 100 K of NPE-caged ATP has been demonstrated to occur within crystals of the enzyme thymidylate kinase after x-ray analysis.[19] This method opens up new possibilities for studying reaction intermediates of functional proteins, provided that the intermediates build up in the crystal when a suitable temperature profile is subsequently applied.

Experimental Section

General: A Zorbax C_{18} column (4.6 × 250 mm) was used for HPLC analyses of **1** and a Hypersil C_{18} column (3.9 × 300 mm) was used for HPLC analyses of **2**. Xe flashes (1 ms) were generated at a frequency of 1 Hz from a xenon lamp (Rapp OptoElectronic, Hamburg, Germany) with a 265–380 nm bandpass filter and focused to 4 mm^2 (energy 20 $mJ\,mm^{-2}$, unless otherwise indicated). Laser light of 355 nm wavelength was obtained from the third harmonic of a passively q-switched YAG laser (Nanolase, Meylan, France), delivering gentle light pulses at a 10 kHz frequency, with an average output power of 0.75 mW per 0.01 mm^2. Difference UV spectra were recorded after each flash with a CCD spectrometer (Ocean Optics, Dunedin, USA), using a Deuterium monitoring light (Oriel, Stratford, USA). The output power of the lamp is in the lower microwatt range. A shutter was used to minimize the exposure of the sample to the lamp (a few seconds per recorded spectrum), so that residual photolysis by the monitoring light was negligible.

Cryophotolysis experiments: Caged arsenocholine **1**: A 15 mM solution of **1** in 50 mM MES buffer, pH 6, containing 25% glycerol, in a 0.1–0.2 mm cryoloop (Hampton Research, Laguna Miguel CA) was flash-cooled at 100 K (Oxford Cryosystems, Oxford, UK) and then exposed to successive Xe flashes.

Caged dTMP **2**: The same procedure as for **1** was applied using a solution of 13 mM **2** in 50 mM MES buffer, pH 6, 25% glycerol.

Preparation of HPLC samples: Samples from the cryophotolysis experiments were dissolved at 25 °C in 100 μL water. To obtain sufficient material for HPLC analysis, the above-mentioned experiments were repeated ten times for caged arsenocholine **1** (15 mM) and twenty times for caged dTMP **2**.

HPLC analyses of cryophotolysis products: Caged arsenocholine **1**: Samples (100 μL) were injected into a C_{18} column equilibrated with 32% (v/v) acetonitrile in a 0.1% aqueous trifluoroacetic acid (TFA) solution, at a flow rate of 1 $mL\,min^{-1}$. The compounds were detected by their UV absorption at 270 nm.

Caged dTMP **2**: Samples (100 μL) were injected into a C_{18} column. Elution was performed at a flow rate of 1 $mL\,min^{-1}$ with a 0.1% aqueous TFA solution for 10 min before using a linear gradient to 100% (v/v) acetonitrile over 30 min. The compounds were detected by their UV absorptions at 260 and 320 nm.

Photochemical conversion: The yields of the photochemical conversion were deduced from a HPLC calibration by using caged arsenocholine **1**, dTMP, caged dTMP **2**, and *ortho*-nitrosoacetophenone as references.

Cryophotolysis of caged arsenocholine 1 in AChE crystals: A crystal of *Tc* AChE (0.2 × 0.2 × 0.05 mm^3) was soaked in a solution of 7 mM **1** in 0.1 M MES buffer, pH 5.8, 36% (w/v) PEG 200, for 2–3 days and then rinsed (30 s) in 0.1 M MES buffer, pH 5.8, 36% PEG 200 (w/v), 20% glycerol. This crystal was flash-cooled to 100 K and exposed to 100 successive Xe flashes (5 mJ).

We thank Dr. Serge van Calenbergh for the synthesis of caged dTMP, and Lilly Toker and Dr. Israel Silman for providing purified Tc *AChE. This work was supported by the Centre National de la Recherche Scientifique, the EU Biotechnology Programs no. 96081 and BIO4-CT98-0354, the COST program D11/0014/99, the Association Française contre les Myopathies, and the Région Alsace. M.W. was supported by an EMBO long-term fellowship.*

[1] S. R. Adams, R. Y. Tsien, *Annu. Rev. Physiol.* **1993**, *35*, 755 – 784.
[2] D. W. J. Cruickshank, J. R. Helliwell, L. N. Johnson, *Time-Resolved Macromolecular Crystallography*, Oxford University Press, Oxford, **1992**.
[3] I. Schlichting, *Acc. Chem. Res.* **2000**, *33*, 532 – 538; M. H. B. Stowell, T. M. McPhillips, D. C. Rees, S. M. Soltis, E. Abresch, G. Feher, *Science* **1997**, *276*, 812 – 816; B. Perman, V. Srajer, Z. Ren, T. Y. Teng, C. Pradervand, T. Ursby, D. Bourgeois, F. Schotte, M. Wulff, R. Kort, K. Hellingwerf, K. Moffat, *Science* **1998**, *279*, 1946 – 1950.
[4] I. Schlichting, S. C. Almo, G. Rapp, K. Wilson, K. Petratos, A. Lentfer, A. Wittinghofer, W. Kabsch, E. F. Pai, G. Petsko, R. S. Goody, *Nature* **1990**, *345*, 309 – 315; B. L. Stoddard, P. Koenigs, N. Porter, K. Petratos, G. A. Petsko, D. Ringe, *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 5503 – 5507; B. L. Stoddard, B. E. Cohen, M. Brubaker, A. D. Mesecar, D. E. Koshland, *Nat. Struct. Biol.* **1998**, *5*, 891 – 897.
[5] J. M. Bolduc, D. H. Dyer, W. G. Scott, P. Singer, R. M. Sweet, D. E. Koshland, B. L. Stoddard, *Science* **1995**, *268*, 1312 – 1318.
[6] J. Hajdu, I. Andersson, *Annu. Rev. Biophys. Biomol. Struct.* **1993**, *22*, 467 – 498.
[7] G. Rapp, *Methods Enzymol.* **1998**, *291*, 202 – 222.
[8] I. Schlichting, R. S. Goody, *Methods Enzymol.* **1997**, *277*, 467 – 490; M. Weik, R. B. G. Ravelli, G. Kryger, S. McSweeney, M. L. Raves, M. Harel, P. Gros, I. Silman, J. Kroon, J. L. Sussman, *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 623 – 628.
[9] K. Moffat, R. Henderson, *Curr. Opin. Struct. Biol.* **1995**, *5*, 656 – 663.
[10] A. J. Scheidig, C. Burmester, R. S. Goody, *Structure* **1999**, *7*, 1311 – 1324.
[11] T. Y. Teng, K. Moffat, *J. Appl. Crystallogr.* **1998**, *31*, 252 – 257.
[12] L. Peng, I. Silman, J. L. Sussman, M. Goeldner, *Biochemistry* **1996**, *35*, 10854 – 10861; L. Peng, M. Goeldner, *Methods Enzymol.* **1998**, *291*, 265 – 278.
[13] A. Ostermann, R. Waschipky, F. G. Parak, G. U. Nienhaus, *Nature* **2000**, *404*, 205 – 208.
[14] L. Peng, F. Nachon, J. Wirz, M. Goeldner, *Angew. Chem.* **1998**, *110*, 2838 – 2840; *Angew. Chem. Int. Ed.* **1998**, *37*, 2691 – 2693.
[15] J. M. Walker, G. P. Reid, J. A. McCray, D. R. Trentham, *J. Am. Chem. Soc.* **1988**, *110*, 7170 – 7177.
[16] I. R. Dunkin, J. Gebicki, M. Kiszka, D. Sanin-Leira, *Spectrochim. Acta Part A* **1997**, *53*, 2553 – 2557.
[17] J. L. Sussman, M. Harel, F. Frolow, C. Oefner, A. Goldman, L. Toker, I. Silman, *Science* **1991**, *253*, 872 – 879.
[18] G. Koellner, M. Weik, A. Specht, L. Peng, M. Harel, D. Bourgeois, I. Silman, J. Kroon, M. Goeldner, J. Sussman, unpublished results.
[19] T. Ursby, M. Weik, E. Fioravanti, M. Delarue, M. Goeldner, D. Bourgeois, unpublished results.

Received: March 8, 2001
Revised version: July 9, 2001 [Z 240]

The Experimentally Elusive Oxidant of Cytochrome P450: A Theoretical "Trapping" Defining More Closely the "Real" Species

François Ogliaro, Samuël P. de Visser, Shimrit Cohen, Jose Kaneti, and Sason Shaik*[a]

KEYWORDS:

cytochromes · density functional calculations · hydrogen bonds · porphyrins · solvent effects

Cytochrome P450 is a vital enzyme in oxidative biotransformations, responsible for the detoxification of biological systems and for the synthesis of sex hormones.[1] Recent experimental results[2] demonstrate that, despite previous reports,[3, 4] the active species of the enzyme, compound I (Cpd I, **1**; Scheme 1), might have never been detected since it does not seem to accumulate during the catalytic cycle even at low temperature. To date, the only characterized Cpd I of a cysteinate enzyme belongs to chloroperoxidase (CPO).[5] However, here too the geometry of the species is unknown, and the precise identity of the ground state is still debated.[5, 6] Thus, a key species of one of the most important enzymes of biological systems is known to exist, but eludes detection.

We present here theoretical calculations of the so far most extensive and most realistic Cpd I model,[7, 8] with an account of the interaction types exerted by the apoprotein environment. We assign the ground state of Cpd I (**1**) as $^2A_{2u}$, thereby settling previous theoretical disagreements and hopefully contributing toward an eventual resolution of the experimental controversy. The calculations project the unusual nature of this Cpd I that behaves as a chameleon species by adopting its electronic and geometric features to the protein environment to which it has to accomodate.

Our benchmark system **2** (Scheme 1) involves octamethyl porphyrin and an axial cysteinato ligand. From an electronic point of view, methyl substituents are good representations of the side chains in **1**, while avoiding complications due to internal rotations of the long side chains. Noncovalent interactions revealed by mimetic systems,[9] mutation studies,[10] and X-ray crystal structures of P450 enzymes[3, 11] were taken into account as follows: a) Embedding of **2** in a polarizing medium of a low dielectric constant ($\varepsilon = 5.7$) serves to mimic the effect of polarization by the dipoles of the protein pocket near Cys 357 (using the numbering system in $P450_{cam}$).[11] b) An internal NH ··· S

[a] *Prof. Dr. S. Shaik, Dr. F. Ogliaro, Dr. S. P. de Visser, S. Cohen, Dr. J. Kaneti*
Department of Organic Chemistry
and
Lise Meitner-Minerva Center for Computational Quantum Chemistry The Hebrew University
Givat Ram Campus, 91904 Jerusalem (Israel)
Fax: (+ 972) 2-6584680
E-mail: sason@yfaat.ch.huji.ac.il