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The Development and Application of Photosensitive Caged Compounds to Aid Time-Resolved Structure Determination of Macromolecules [and Discussion]

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The development and application of photosensitive caged compounds to aid time-resolved structure determination of macromolecules

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Rapid photochemical release of biologically active molecules, typically enzyme substrates or effectors of proteins, within crystals is likely to play an important role in time-resolved macromolecular crystallography. Photosensitive or 'caged' compounds in which functional groups are protected by the 1-(2-nitrophenyl)ethyl group are potentially useful because many such compounds are efficiently and fairly rapidly photolysed (product quantum yield *ca.* 0.5 and photolysis rate *ca.* 100 s^{-1} for esters of weakly acidic phosphates) and have proved effective probes of physiological mechanisms. However, their availability and successful application are unlikely to be universal, and in some cases limitations may arise because of low quantum yield, a photolysis rate that is slow compared with the mechanism being studied or the toxicity of the by-product, 2-nitrosoacetophenone. 3,5-Dinitrophenyl and 3′,5′-dimethoxybenzoin esters are two other potentially useful photosensitive classes of compound (Kirby & Varvoglis, *J. chem. Soc. chem. Commun.* 406 (1967); Sheehan *et al.*, *J. Am. chem. Soc.* **93**, 7222–7228 (1971); Baldwin *et al.*, *Tetrahedron* **46**, 6879–6884 (1990)). 3,5-Dinitrophenyl phosphate has a product quantum yield of 0.67 and releases P_i at greater than 10^4 s^{-1}. However the dinitrophenyl group is not generally photosensitive: for example the P^3-3,5-dinitrophenyl ester of ATP photolyses very inefficiently at pH 7. The 3′,5′-dimethoxybenzoin group is a promising photosensitive group for phosphate esters and the P^3-3′,5′-dimethoxybenzoin ester of ATP photolyses at greater than 10^5 s^{-1} at neutral pH and 20 °C though with only about 4% photolysis on 347 nm pulse irradiation.

1. Introduction

Dynamic structural analysis of enzyme catalysed reactions and of protein–ligand interactions is a principal target of time resolved macromolecular crystallography. Initiation of such processes by photochemical release of substrates has been achieved in several laboratories. We are addressing the synthetic organic chemistry and photochemistry that are essential components of this approach. Two proteins in which time-resolved structure analysis has been aided by such photosensitive or 'caged' compounds are Ha-ras p21 (Schlichting *et al.* 1990) and glycogen phosphorylase (Duke *et al.* 1991). The relevant photochemical reactions are shown in equations (1) and (2) respectively.

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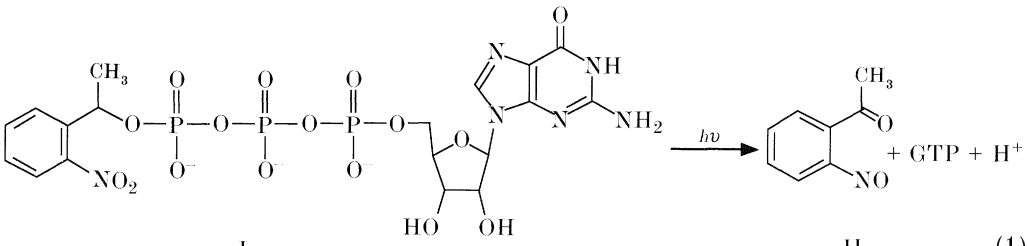

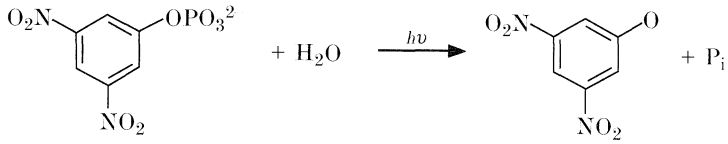

In this article we discuss some of the practical and theoretical considerations in the use of various caged compounds. Generally the two most important properties that a caged compound requires are rapid and extensive conversion to photo-products on pulse illumination. In addition it is important to know whether a caged compound binds to the macromolecule in the crystal and whether by-products of the photolysis react with the macromolecule. Another consideration is the ease of synthesis of the caged compound: in particular it may be useful to have the caged compound radiolabelled so that the extent of photolysis may be readily assessed. Finally we need to be alert to problems in the use of caged compounds that might occur because of intense X-ray sources: caged GTP has been reported to be sensitive to X-ray irradiation (Marx *et al.* 1990). So that the topics discussed herein are not too diffuse, the examples we use to illustrate the approach are generally relevant to studies of Ha-ras p21 (Scheidig *et al.* 1992) and glycogen phosphorylase (Duke *et al.* 1992).

2. Synthesis and characterization of caged compounds

By far the most frequently used caged compounds are substituted 2-nitrobenzyl derivatives. There is an extensive literature on syntheses of unsubstituted 2-nitrobenzyl derivatives because of their importance as protecting groups that may be removed photochemically under otherwise mild conditions. However, substituted rather than unsubstituted 2-nitrobenzyl derivatives are normally used as caged compounds because the photolyses proceed more smoothly with substituted compounds and by-products are nitrosoketones rather than the highly reactive 2-nitrosobenzaldehyde (Kaplan *et al.* 1978). Syntheses of 2-nitrobenzyl derivatives have been facilitated by the availability of 1-(2-nitrophenyl)diazoethane and related compounds. These diazo compounds react with weak acids in mixed organic/aqueous solvents to form in high yield caged compounds that are readily isolated (Walker *et al.* 1989). Stronger acids are also esterified by the diazo compounds but require a homogeneous solvent such as dimethylformamide or dimethylsulphoxide (Wootton & Trentham 1989). A particular advantage of this synthetic route is that radiolabelled caged compounds may readily by synthesised.

While 1-(2-nitrophenyl)diazoethane has been applied widely for synthesis of caged nucleotides it does raise some problems because multiple isomeric forms may arise as a chiral centre is introduced at the methine carbon atom leading to diastereoisomeric products. Furthermore the reagent does not discriminate between oxygen and sulphur atoms in thiophosphates. Thus caged GTP(γS) exists as four diastereoisomers when an oxygen atom links the thiophosphate group to the 1-(2-nitrophenyl)ethyl group (O-caged GTP(γS)) and two more diastereoisomers when linked through the sulphur atom (S-caged GTP(γS)). While these compounds can be resolved at least partially by chromatography, there may be advantage in forming an ester specifically through the sulphur atom. This can be achieved by use of 1-(2-nitrophenyl)ethyl bromide.

1-(2-Nitrophenyl)ethyl bromide is obtained quantitatively (as determined by ^{1}H NMR) by treatment of 2-ethylnitrobenzene with equimolar N-bromosuccinimide (recrystallised from water) and 0.008 molar ratio of benzoyl peroxide under reflux for 18 h in carbon tetrachloride. Succinimide is filtered off from the cooled solvent (4 °C). The alkyl bromide is then used as a concentrated solution in dimethylformamide. In experiments done in collaboration with Dr J. F. Wootton, S-alkylation of GTP(γS) was achieved by stirring 28 µmol GTP(γS) in 1.8 ml of 20 mM K_2HPO_4, 5 mM dithiothreitol at pH 7.9 with an equal volume of dimethylformamide containing 180 µmol of 1-(2-nitrophenyl)ethyl bromide at room temperature for 17 h, by which time no starting material remained. S-Caged GTP(γS) was recovered in 46 % overall yield following standard purification protocols (Walker *et al.* 1989).

As noted above, derivatization with α-substituted 2-nitrophenyldiazomethanes introduces a chiral centre at the α-carbon atom. If the caged compound interacts with proteins, then two distinct protein-ligand complexes may arise, as occurs for example when caged GTP, I, binds to Ha-ras p21 (Schlichting *et al.* 1989). The two diastereoisomers of caged GTP can be resolved by chromatography as have the isomers of caged ATP (Walker *et al.* 1988) but a better approach to the separate isomers is based on their synthesis from resolved (*R*)- and (*S*)-1-(2-nitrophenyl)ethanol (Corrie *et al.* 1992). Use of resolved caged GTP isomers derived from the two enantiomeric alcohols leads to improved high resolution structures of the caged GTP–Ha-ras p21 complex (Scheidig *et al.* 1992).

Characterization of 1-(2-nitrophenyl)ethyl caged compounds uses conventional analytical techniques (Walker *et al.* 1988). Enantiomers and diastereoisomers may be distinguished optically (Corrie *et al.* 1992). (*R*)- and (*S*)-1-(2-nitrophenyl)ethyl phosphates have intense circular dichroic (CD) spectra with a high dissymmetry ($\Delta\epsilon/\epsilon$) ratio. For the (*S*)-isomer $\Delta\epsilon/\epsilon = 0.0045$ and $\Delta\epsilon = 3.4\ \mathrm{M^{-1}\ cm^{-1}}$ at 342 nm, the wavelength of one of the peaks in the CD spectrum. CD spectra thus provide a convenient means to distinguish isomers of caged nucleotides (figure 1).

The use of 1-(2-nitrophenyl)ethyl phosphate, a photosensitive precursor of P_i, has proved problematic in crystallographic studies of glycogen phosphorylase (Hajdu & Johnson 1990). This is because thiols used to remove the photolysis by-product 2-nitrosoacetophenone, II, tended to crack the crystals. 3,5-Dinitrophenyl phosphate, III, has been used as an alternative photosensitive precursor of P_i (equation (2)).

3,5-Dinitrophenyl phosphate was synthesized and characterized as described by Duke *et al.* (1991). Its favourable photochemical properties are described below. Kirby & Varvoglis (1967*a*, *b*) who drew attention to the photosensitivity of dinitrophenyl phosphate also reported that the P-3,5-dinitrophenyl ester of AMP is photosensitive in aqueous pyridine. The photochemistry of other 3-nitrophenyl or

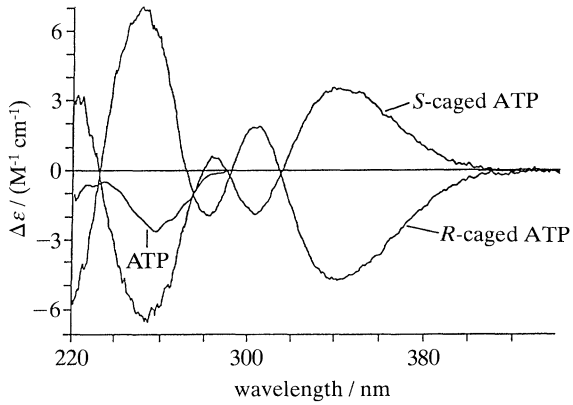

Figure 1. CD spectra of the (*R*)- and (*S*)-diastereoisomers of P^3-1-(2-nitrophenyl)ethyl esters of ATP (caged ATP) and of ATP. (Reproduced from Corrie *et al.* 1992. © 1992 The Royal Society of Chemistry.)

3,5-dinitrophenyl derivatives in alkali is also well established (Havinga *et al.* 1956; Bonilha *et al.* 1979). Based on these observations the P^3-3,5-dinitrophenyl ester of ATP, DNP-caged ATP, was synthesized as a possible photosensitive precursor to ATP that might be generated more rapidly than nucleotides generated from 1-(2-nitrophenyl)ethyl caged nucleotides as in equation (1).

DNP-caged ATP was synthesized by condensation of 3,5-dinitrophenyl phosphate with ADP which had been activated by reaction with carbonyldiimidazole (Hoard & Ott 1965). The reaction product was allowed to stand for 30 min in aqueous triethylamine to allow any 2′,3′-cyclic carbonate to hydrolyse (Maeda *et al.* 1977). The product was purified by chromatography on DEAE-cellulose in 26% overall yield based on ϵ 27,770 M^{-1} cm^{-1} at its λ_{max} of 251 nm in aqueous solution at pH 7; ϵ was calculated from the sum of extinction coefficients at 251 nm for ATP and 3,5-dinitrophenol (neutral form). DNP-caged ATP was characterized and shown to be pure by ^{31}P NMR spectroscopy and HPLC using anion exchange and reverse phase columns.

3. Extent of photolysis of caged compounds

From the photochemical viewpoint probably the most difficult technical problem is to achieve sufficient conversion of caged compound in a single pulse of light so that all the protein sites are occupied by the photoreleased ligand. The problem is accentuated when the stoicheiometry of the protein active site to photoreactive species is one to one as occurs, for example, in the cases of the caged GTP–Ha-ras p21 complex (Schlichting *et al.* 1990) the photolabile covalent inhibitor–chymotrypsin complex (Stoddard *et al.* 1991) and photoactive yellow protein (Moffat *et al.* 1992). The above three examples have, of course, the important advantage that the photoreleased ligand does not have to diffuse to the protein active site within the crystal lattice.

So that the photochemical reaction proceeds fairly uniformly throughout the crystal, it is desirable that a significant fraction, say 20%, of the incident light be transmitted. This corresponds to an absorption of 0.7. Hence the extinction

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Table 1. *Extinction coefficients at 347* nm *of photosensitive groups*

photosensitive group	$\epsilon/(M^{-1}\ cm^{-1})$ at 347 nm
1-(2-nitrophenyl)ethyl[a]	660
3,5-dinitrophenyl[b]	3000
3′,5′-dimethoxybenzoin[c]	170
1-(3,4-dimethoxy-6-nitrophenyl)ethyl[d]	5000
2-hydroxy-4-(N,N-diethylamino)cinnamate[e]	*ca.* 5000

[a] McCray *et al.* (1980); [b] Parke (1961); [c] Corrie & Trentham (1992); [d] Wootton & Trentham (1989); [e] Stoddard *et al.* (1990).

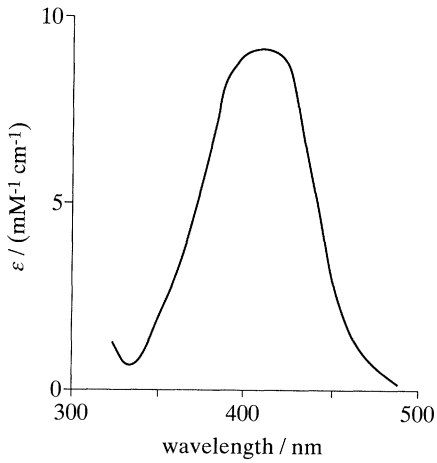

Figure 2. Absorption spectra of the *aci*-nitro anion of caged ATP (IV, scheme 1). The spectrum of the *aci*-nitro anion was derived from measurements at 5 nm intervals between 325 nm and 490 nm of the absorption change following 1 ms pulse irradiation from a xenon-arc flash lamp (Rapp & Güth 1988) containing a Hoya U-350 band-pass filter. The solution at 22 °C contained initially 0.50 mM caged ATP, 2 mM $MgCl_2$, 2 mM dithiothreitol, 100 mM KCl, 100 mM sodium carbonate buffer at pH 10.0. Under these conditions the decay rate of the aci-nitro anion is 1.5 s^{-1} (Walker *et al.* 1988). Measurements were made across the wavelength range with frequent assays at 400 nm to monitor the gradual photolysis of caged ATP: the fraction of solution exposed to the photolysis beam was less than 1%. The spectrometer was calibrated using as a standard a Cary 118 double beam spectrometer and a solution of 4-nitrophenol at pH 10. The 4-nitrophenolate ion has an absorption spectrum similar in shape to that of the aci-nitro anion. Each has a 70 nm bandwidth with λ_{max} at 400 nm and 406 nm respectively. The extinction coefficient of the aci-nitro anion at 406 nm is 9.1×10^3 M^{-1} cm^{-1} (Walker *et al.* 1988).

coefficient, ϵ, of a caged compound would need to be 700 M^{-1} cm^{-1} if it were present at 20 mM in an otherwise transparent crystal of 0.5 mm thickness. Extinction coefficients at 347 nm, the emission wavelength of a frequency-doubled ruby laser, of several photosensitive groups are listed in table 1. For the geometry and concentration used in the above example, the 1-(2-nitrophenyl)ethyl group as used in caged GTP (equation (1)) is ideal.

The absorption properties of intermediates and photoproducts could also have a bearing on the effectiveness of the photolysis. For example, in the photolysis of the 1-(2-nitrophenyl)ethyl group, the first characterized intermediate is an aci-nitro compound (IV, scheme 1). Its spectrum has been measured during the photolysis of caged ATP (figure 2) and caged carbamoyl choline (Walker *et al.* 1986) and is

relatively transparent at 347 mm. If that had not been the case and assuming very rapid aci-nitro anion formation, the surface of the crystal closest to the incident beam would have become opaque during the 50 ns laser pulse and blocked irradiation deeper into the crystal. The same considerations apply to rapidly formed photoproducts.

When multiple irradiation pulses are needed (Schlichting *et al.* 1990; Duke *et al.* 1991), consideration of the absorption of photoproducts is important even when they are formed relatively slowly, as can be seen from consideration of the by-products in equations (1) and (2). 2-Nitrosoacetophenone, II, interacts with proteins and hence is routinely removed *in situ* with thiols. For the adduct formed between 2-nitrosoacetophenone and dithiothreitol, $\epsilon = 850$ M^{-1} cm^{-1} at 380 nm (Walker *et al.* 1988) and approximately equals that of the 1-(2-nitrophenyl)ethyl group at 347 nm (i.e. 660 M^{-1} cm^{-1}). The absorption of 3,5-dinitrophenol (pK_a 6.7) extends to visible wavelengths at neutral pH ($\epsilon = 2800$ M^{-1} cm^{-1} for the anion at 400 nm (Parke 1961)) and may well interfere with photolysis of 3,5-dinitrophenyl phosphate when a xenon-arc flash lamp is used.

It is useful to relate the energy available in a laser or arc lamp pulse to that needed to photolyse a caged compound in a crystal. Extending the above example: let the crystal have dimensions 0.5 mm × 1 mm × 1 mm, then a 20 mM solution of caged compound within the crystal comprises 6×10^{15} molecules. The number of photons needed to impinge on the sample so that 80% of the molecules are excited is 7.5×10^{15}, and the quantity of energy required at 347 nm is 4.3 mJ (corresponding to 7.5×10^{15} $h\nu$). Both a ruby laser (347 nm) and a xenon-arc lamp (between 300–360 nm) can provide greater than 100 mJ per pulse. Limitations from the point of view of energy input are more likely to arise because higher energies may physically move the crystal and possibly cause heating. These factors and other potential artefacts have been carefully considered in muscle fibre experiments with caged ATP (Ferenczi *et al.* 1984; Goldman *et al.* 1984).

A critical parameter for a caged compound is its quantum yield, Q_p, defined as the fraction of molecules of product formed per photon absorbed. Q_p may be measured by various standard photochemical methods (reviewed by McCray & Trentham 1989). Values in excess of 0.5 are considered high and are a useful goal when synthesising new caged compounds.

An equally important though empirical parameter is the fraction of caged compound photolysed to product in a single laser or xenon-arc pulse, which we term F_p. F_p is directly related to Q_p but may be larger than Q_p especially for values of $Q_p < 0.1$. The reason is probably that a single pulse of light may induce several excitation events provided the duration of the pulse is long compared with excited state lifetime of the caged compound. The longer period of a xenon-arc pulse compared with that of a laser may, in certain cases, make it a more favourable light source. High extents of photolysis of carbon monoxide from carbonmonoxy-haemoglobin have been ascribed to multiple excitations (Greene *et al.* 1978), and are probably responsible for the many-fold greater value of F_p compared with Q_p found for the photosensitive calcium releasing agent, nitr 5 (Adams *et al.* 1988). Even when Q_p is relatively large, F_p may exceed Q_p (Ferenczi *et al.* 1984). The higher the energy of the light pulse the greater the possible value of F_p.

Other things being equal, it has been found that the higher the value of Q_p, the more effective the caged compound. Q_p for caged GTP (equation (1)) is 0.63 (assuming it to be the same as for caged ATP). The extent of photolysis of 3,5-

dinitrophenyl phosphate was measured relative to that of caged ATP, by pulse-irradiating (50 ns) a mixture of the two compounds, at concentrations such that each had the same absorption at 347 nm, the wavelength of the frequency-doubled ruby laser. The extent of photolysis was monitored by measuring the decrease in quantities of samples of the caged compounds from peak-area absorptions following resolution by anion exchange HPLC. 3,5-Dinitrophenyl phosphate photolysed 6% more than caged ATP, suggesting $Q_p = 0.67$ for the former.

DNP-caged ATP was at least 100-fold less photosensitive than 3,5-dinitrophenyl phosphate in aqueous solvents at pH 7. This rules out DNP–caged-ATP as a useful photochemical source of ATP in biological studies, and supports the argument of Kirby & Varvoglis (1967*b*) that the special photolability of 3,5-dinitrophenyl phosphate derives from its capacity to cleave via a mechanism not accessible to phosphate diesters.

4. Rates of photolysis of caged compounds

Historically photosensitive groups have been used as protecting groups that may be removed by light under otherwise mild conditions. Illumination has been continuous and little insight was gained about the rate of photolysis. Application of caged compounds to study physiological mechanisms has required knowledge about the kinetics of their photolysis. This knowledge is equally important in time-resolved macromolecular structure studies as exemplified in Ha-ras p21 and glycogen phosphorylase. Kinetic mechanisms and rates can be investigated by flash photolysis techniques. In general dark reactions following initial photochemical excitation are likely to control the rate of release of the biologically active compound. Kinetics of elementary steps have usually been measured by optical spectroscopy and a major difficulty is that frequently there is no convenient spectrophotometric probe of the product of biological interest.

A detailed analysis has been undertaken of the photolysis kinetics and mechanism of 1-(2-nitrophenyl)ethyl esters of nucleotides (McCray *et al.* 1980; Walker *et al.* 1988; Wootton & Trentham 1989). From these studies we know that nucleotides are released on flash photolysis in exponential processes whose rate constants vary from 7 to 300 s^{-1} at pH 7 and 21 °C in aqueous solvents. The rate constants can be estimated over a range of pH, temperature, Mg^{2+} concentration and ionic strength by extrapolating data obtained with caged ATP, caged ATP analogues and caged cyclic nucleotides (McCray & Trentham 1989). Spectrophotometric probes used to analyse these photolyses include the chromophore of the aci-nitro anion (figure 2), fluorescent nucleotide analogues, proton indicators, the nitroso absorption band, the absorption of adducts between thiols and the by-product 2-nitrosoacetophenone, and biological assays of ATP released from caged ATP.

The relatively slow release kinetics of nucleotides places limits on structural studies of muscular contraction. Structural changes can be observed from low angle X-ray diffraction data collection in 200 μs time bins following length step changes in muscle fibres that are complete in 120 μs (Irving *et al.* 1992; Piazzesi *et al.* 1992). Similar time resolution of protein structure changes from Laue diffraction analysis may be expected in due course. To try and overcome the potential time resolution constraints of caged nucleotides, we have synthesised the P^3-3′,5′-dimethoxybenzoin ester of ATP, V, DMB–caged-ATP, and are investigating its photochemical properties (Trentham *et al.* 1992). Its fractional extent of photolysis, F_p, was 0.04

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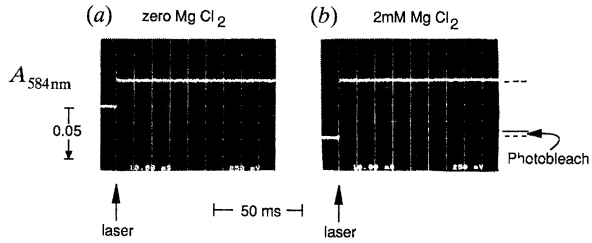
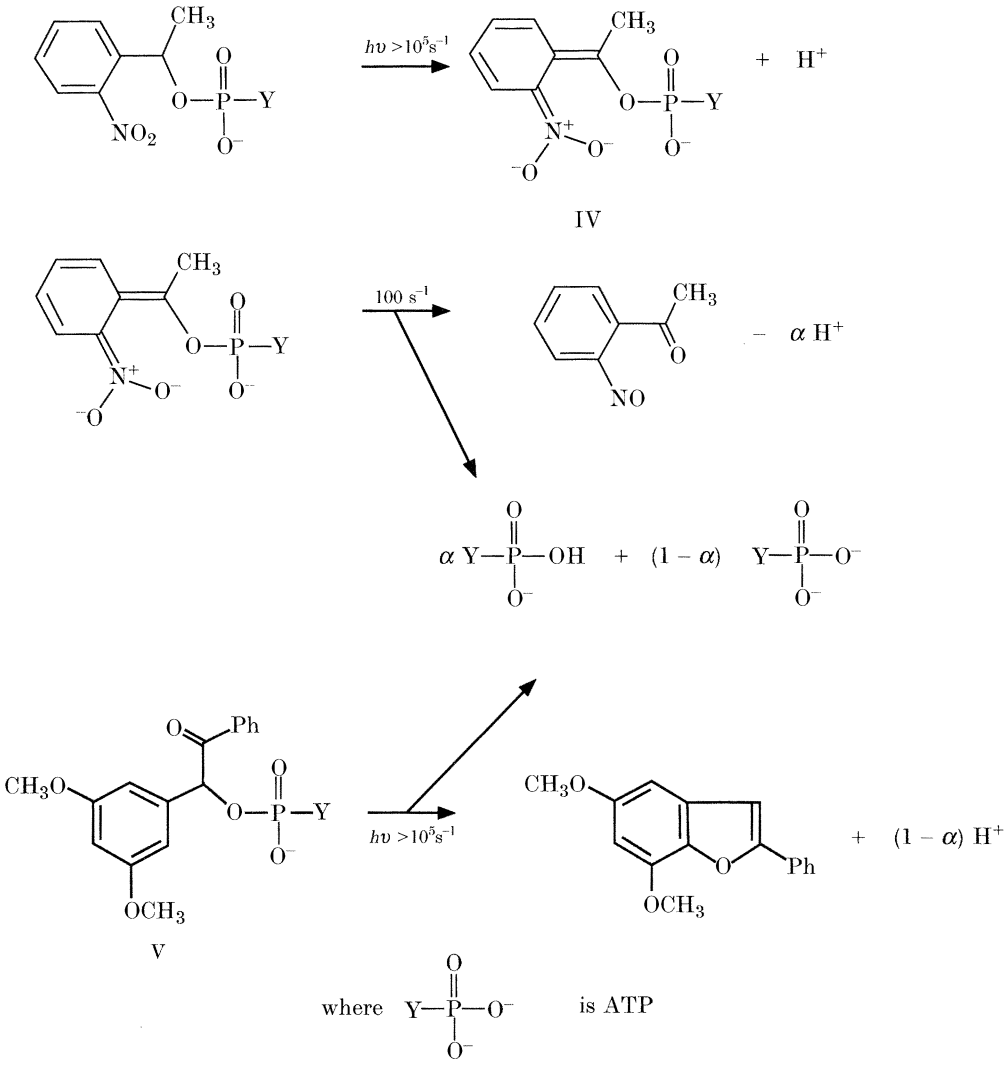


Figure 3. Kinetic records of proton release on photolysis of the P^3-3′,5′-dimethoxybenzoin ester of ATP (DMB–caged-ATP) at pH 6 and 22 °C. In record (*a*) the solution contained 1 mM DMB–caged-ATP, 100 mM KCl, 1 mM 2-(*N*-morpholino)ethanesulphonic acid and chlorophenol red (absorption of the solution was 0.9 at 584 nm in 4 mm path length cell). In record (*b*) the solution also contained 2 mM $MgCl_2$. The vertical arrows mark the timing of a 50 ns frequency doubled ruby laser pulse at 347 nm. The horizontal arrow indicates the absorbance change due to photobleaching.

Scheme 1



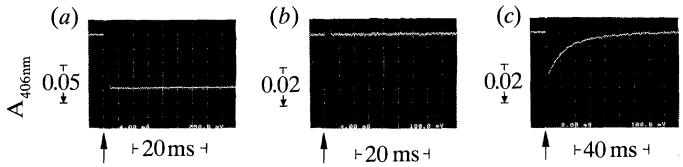


Figure 4. Comparison of the photolysis kinetics of 3,5-dinitrophenyl phosphate and the P-1-(2-nitrophenyl)ethyl ester of AMP (caged AMP) at pH 7.0 and 21 °C recorded at 406 nm in a 4 mm cell. Aqueous solutions buffered with potassium phosphate contained in (*a*) 0.5 mM 3,5-dinitrophenyl phosphate and in (*c*) 0.1 mM caged AMP. Arrows mark the time of a 1 ms irradiation from a xenon-arc flash lamp (Rapp & Güth 1988) containing a Hoya U-350 band-pass filter. Record (*a*) shows formation of 3,5-dinitrophenol (as the anion, pK_a 6.7). Record (*b*) is a blank and shows the absence of optical artefacts other than the light pulse. Record (*c*) shows the rapid formation of the aci-nitro intermediate (as in scheme 1) followed by its decay with concomitant AMP release.

when a 30 μl 5 mM sample of DMB–caged-ATP was irradiated with a 130 mJ frequency-doubled ruby laser pulse at 347 nm in a 1 mm path-length cell at pH 7.

The rate of ATP release from DMB–caged-ATP may be measured using proton indicators based on the approach developed for caged ATP. Proton release studies near pH 7 with caged ATP showed that on flash photolysis in the absence of Mg^{2+} a proton was released rapidly and then a fraction of a proton per caged ATP photolysed was taken up at the rate of ATP release (Walker *et al.* 1988). This is because ATP (pK_a 6.5 of the γ-phosphate group (Martell & Schwarzenbach 1956)) takes up protons in the final step concomitant with ATP release and the decay of the aci-nitro intermediate (scheme 1). A similar experiment was carried out with DMB–caged-ATP; again there was net proton release but in this case all proton changes occurred within the time resolution of the measurement showing that DMB–caged-ATP photolysed much more rapidly at greater than 10^5 s^{-1} (figure 3, scheme 1). As expected, when Mg^{2+} was added a larger proton release occurred (figure 3), because MgATP is a stronger acid than ATP^{3-} with a pK_a 4.5 (Martell & Schwarzenbach 1956).

The kinetics of photolysis of 3,5-dinitrophenyl phosphate are straightforward to measure because of the chromophore of 3,5-dinitrophenolate anion at 400 nm. The data in figure 4 permit comparison of the photolysis kinetics of P-1-(2-nitrophenyl)ethyl ester of AMP (100 s^{-1}) and of 3,5-dinitrophenyl phosphate (greater than 10^4 s^{-1}) at pH 7.

5. Conclusion

The general strategy of rapid photochemical release of biologically active compounds from photosensitive but biologically inert precursors is a useful way to probe physiological mechanisms. A similar approach is already showing its value for analysis of enzyme mechanisms and other biological processes through time-resolved macromolecular crystallography. Because free diffusion of ligands and effector molecules within the crystal lattice of macromolecules may well be restricted, it is possible that success will sometimes depend (unlike the general case in physiology) on the caged compound being bound to the site of action of the photoreleased product. In these situations a major challenge will be to achieve rapid photochemical

conversions that approach 100 %. More photochemistry specifically oriented towards the problem is needed. In addition developments in the use of pulsed lasers to obtain high photon flux for photochemical reactions while at the same time minimizing heating and movement artefacts are desirable.

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Discussion

R. M. Sweet (*Department of Biology, Brookhaven National Laboratory, Upton, U.S.A.*). Can you prepare nitrobenzyl derivatives of primary amines?

D. R. Trentham. Yes (Wilcox *et al.* 1990). In addition 2-nitrobenzylcarbonyl related

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES

derivatives of amines were introduced by Patchornik *et al.* (1970) and these promise to be of value in view of their high quantum yield with respect to photolysis (*ca.* 0.7) (Cameron & Fréchet, 1991; Fodor *et al.* 1991; Khan *et al.* 1992).

K. Moffat (*Department of Biochemistry and Molecular Biology, University of Chicago, U.S.A.*). The kinetics of product release from 2-nitrobenzyl phosphate esters and 2-nitrobenzyloxycarbonyl derivatives of amines (Khan *et al.* 1992) are relatively slow, in the millisecond range. Do you see prospects of photogeneration of biochemically useful compounds on a much faster timescale, say microsecond or even nanosecond?

D. R. Trentham. Yes. If the rate of photolysis is solely limited by the lifetime of the excited state whether it be singlet or triplet, we can expect product release in the microsecond to picosecond range (reviewed by McCray & Trentham 1989).

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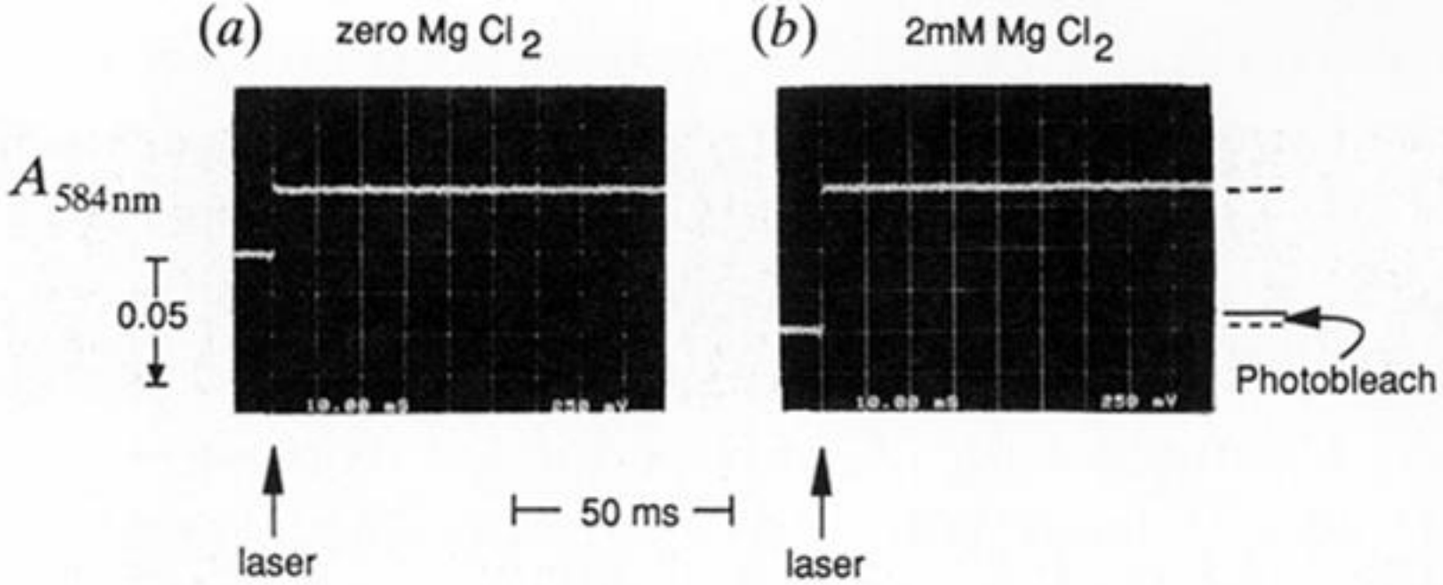
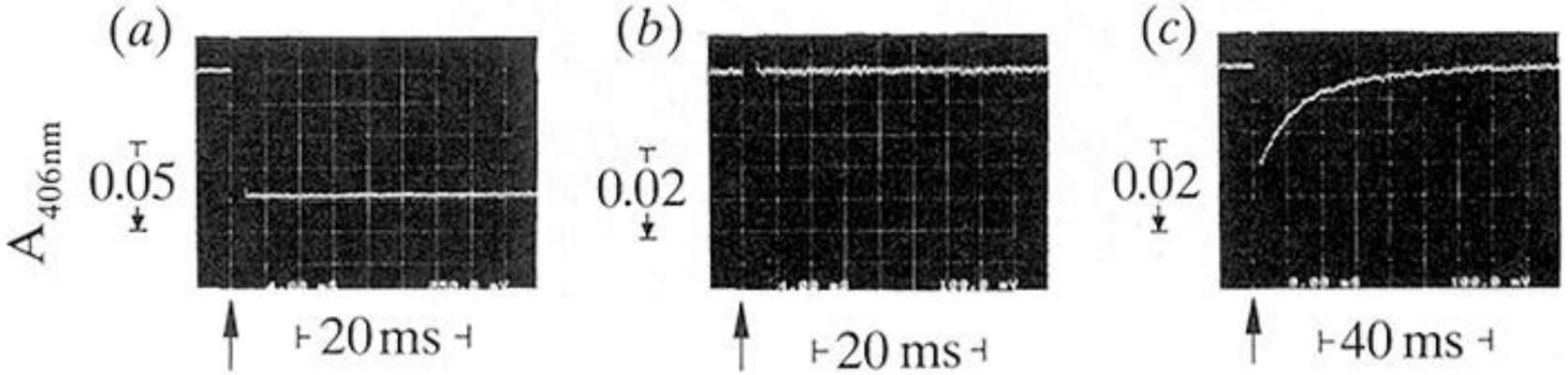
PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY A MATHEMATICAL, PHYSICAL & ENGINEERING SCIENCES



igure 3. Kinetic records of proton release on photolysis of the P³-3′,5′-dimethoxybenzoin ester of TP (DMB–caged-ATP) at pH 6 and 22 °C. In record (*a*) the solution contained 1 mM DMB–caged-TP, 100 mM KCl, 1 mM 2-(*N*-morpholino)ethanesulphonic acid and chlorophenol red (absorption f the solution was 0.9 at 584 nm in 4 mm path length cell). In record (*b*) the solution also contained mM $MgCl_2$. The vertical arrows mark the timing of a 50 ns frequency doubled ruby laser pulse at 47 nm. The horizontal arrow indicates the absorbance change due to photobleaching.



igure 4. Comparison of the photolysis kinetics of 3,5-dinitrophenyl phosphate and the P-1-(2-itrophenyl)ethyl ester of AMP (caged AMP) at pH 7.0 and 21 °C recorded at 406 nm in a 4 mm ell. Aqueous solutions buffered with potassium phosphate contained in (*a*) 0.5 mM 3,5-dinitrophenyl hosphate and in (*c*) 0.1 mM caged AMP. Arrows mark the time of a 1 ms irradiation from a xenon-c flash lamp (Rapp & Güth 1988) containing a Hoya U-350 band-pass filter. Record (*a*) shows rmation of 3,5-dinitrophenol (as the anion, pK_a 6.7). Record (*b*) is a blank and shows the absence optical artefacts other than the light pulse. Record (*c*) shows the rapid formation of the aci-nitro termediate (as in scheme 1) followed by its decay with concomitant AMP release.