[12] Fourier Transform Infrared Photolysis Studies of Caged Compounds

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Introduction

Time-resolved Fourier transform infrared (FTIR) difference spectroscopy has been established as a new tool to study molecular reaction mechanisms of proteins at the atomic level with nanosecond time resolution.[1,2] In photobiological proteins the reaction can be started directly by a short laser flash using the intrinsic chromophores. This approach has been successfully applied to the membrane proteins bacteriorhodopsin and the photosynthetic reaction center.[3] A much broader applicability can be achieved by the use of caged compounds. In this case biologically active molecules are released from inactive photolabile precursors. The use of caged compounds has become widespread in the last 10 years.[4–6] They allow the initiation of a protein reaction with a nanosecond UV laser flash.

This article presents FTIR photolysis studies of caged phosphate, caged GTP, caged ATP, and caged calcium. This should provide a good basis for further FTIR studies on molecular reaction mechanisms of proteins using caged compounds. Finally, as an example, FTIR studies on the GTP-binding protein H-*ras* p21 are presented.

Experimental Setup

Fourier transform infrared measurements are performed on an IFS 66v spectrometer (Bruker, Karlsruhe, Germany). The experimental setup is shown in Fig. 1. The MIR beam source is a water-cooled globar. The beam splitter consists of a KBr crystal. A HgCdTe (MCT) detector that is sensitive in the spectral region between 900 and 5000 cm^{-1} is used. Single-beam

[1] K. Gerwert, *Curr. Opin. Struct. Biol.* **3,** 769 (1993).
[2] R. Rammelsberg, B. Hessling, H. Chorongiewski, and K. Gerwert, *Appl. Spectrosc.* **51,** 558 (1997).
[3] K. Gerwert, *Biochim. Biophys. Acta* **1101,** 147 (1992)
[4] J. A. McCray and D. R. Trentham, *Annu. Rev. Biophys. Biophys. Chem.* **18,** 239 (1989)
[5] J. E. T. Corrie and D. R. Trentham, *in* "Bioorganic Photochemistry: Biological Application of Photochemical Switches" (H. Morrison, ed.), Wiley, New York, 1993.
[6] S. R. Adams and R. Y. Tsien, *Annu. Rev. Physiol.* **55,** 755 (1993).

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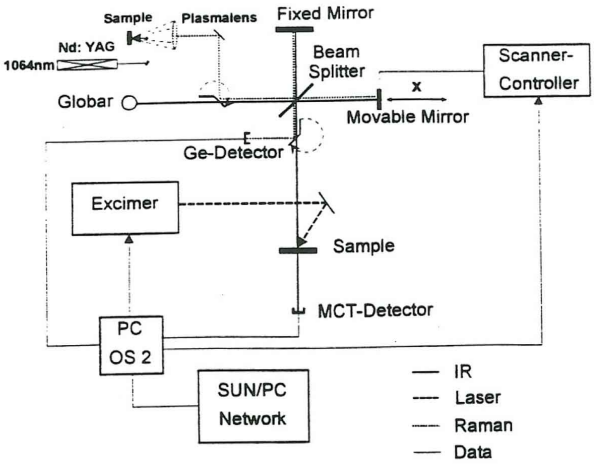


FIG. 1. Experimental setup for photolysis, FTIR, and Raman measurements of caged compounds.

spectra are measured. The spectrometer is controlled by a personal computer (PC) equipped with the program Opus (Bruker).

Photolysis of the caged compounds is performed by ultraviolet (UV) flashes at the wavelength λ of 308 nm [excimer laser, LPX 240 (Lambda Physics, Göttingen, Germany)]. The laser flashes are applied on the sample at an angle of 45° to the IR beam. The duration of a UV laser pulse is about 20 nsec, with the energy reaching up to 200 mJ per flash. Twenty to 60 flashes are used to achieve the complete photolysis of caged compounds, except in the case of DM-nitrophen, for which approximately 100 flashes are necessary. The home-built sample chamber of the FTIR spectrometer IFS 66v is purged with dried air. The metal sample holder is connected to a thermostat. The sample solution is prepared between two CaF_2 windows of 20 mm diameter. A spacer ring made of Mylar 2.5 μm thick is used in order to keep a defined distance between the windows. In the absence of other specification, spectral resolution of 4 cm^{-1} is used.

Fourier transform infrared measurements of H-*ras* p21 are performed with the truncated form of human H-*ras* p21c (1–166). For the nucleotide exchange of the protein-bound GDP for caged GTP, an excess of the desired caged GTP isotopomer in the presence of alkaline phosphatase is

used.[7] During the GTPase reaction of H-*ras* p21-GTP, spectra are collected up to 200 min after photolysis.

Raman spectra are recorded on a IFS 88 (Bruker) spectrometer with an FRA106 Raman module equipped with a germanium detector. For excitation, a Nd–YAG (neodymium/yttrium–aluminum–garnet) laser (1064 nm) is used. The sample, in a quartz capillary 0.8 mm in diameter, is placed in the thermostatted sample holder.

Synthesis

The synthesis of 1-(2-nitrophenyl)ethyl phosphate (caged phosphate) is performed, as described by Dantzig *et al.*,[8] via the reaction of 1-(2-nitrophenyl)diazoethane and orthophosphate.[9] $^{18}O_4$-Labeled orthophosphate is synthesized as described by Hackney *et al.*[10]

P^3-[1-(2-Nitrophenyl)ethyl]guanosine 5′-triphosphate (caged GTP, see Scheme 3) and P^3-[1-(2-nitrophenyl)ethyl]guanosine 5′-*O*-(γ-thio)triphosphate (caged GTPγS) are synthesized following the procedure of Walker *et al.*[9] by the esterification of guanosine 5′-*O*-triphosphate (GTP) and the γS analog guanosine 5′-*O*-(γ-thio)triphosphate (GTPγS, Merck, Darmstadt, Germany), respectively, with 1-(2-nitrophenyl)diazoethane. The ^{18}O-labeled isotopomers are prepared starting with the sulfur analogs guanosine 5′-*O*-(α-thio)triphosphate (GTPαS), guanosine 5′-*O*-(β-thio)triphosphate (GTPβS), and P^3-[1-(2-nitrophenyl)ethyl]guanosine 5′-*O*-(γ-thio)triphosphate (caged GTPγS) by oxidation with *N*-chlorosuccinimide and hydrolysis with $H_2{}^{18}O$.[11,12] Guanosine 5′-*O*-[α-^{18}O]triphosphate (GTPα^{18}O, Scheme 3), guanosine 5′-*O*-[β-^{18}O]triphosphate (GTPβ^{18}O, Scheme 3), and guanosine 5′-*O*-[γ-^{18}O]triphosphate (GTPγ^{18}O, Scheme 3) are then esterified with 1-(2-nitrophenyl)diazoethane as described earlier.

Caged ATP (P^3-[1-(2-nitrophenyl)ethyl]adenosine 5′-triphosphate) and DM-nitrophen [1-(4,5-dimethoxy-2-nitrophenyl)-1,2-diaminoethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] are available commercially (Calbiochem, La Jolla, CA).

[7] J. John, I. Schlichting, E. Schiltz, P. Rosch, and A. Wittinghofer, *J. Biol. Chem.* **264,** 13086 (1989).

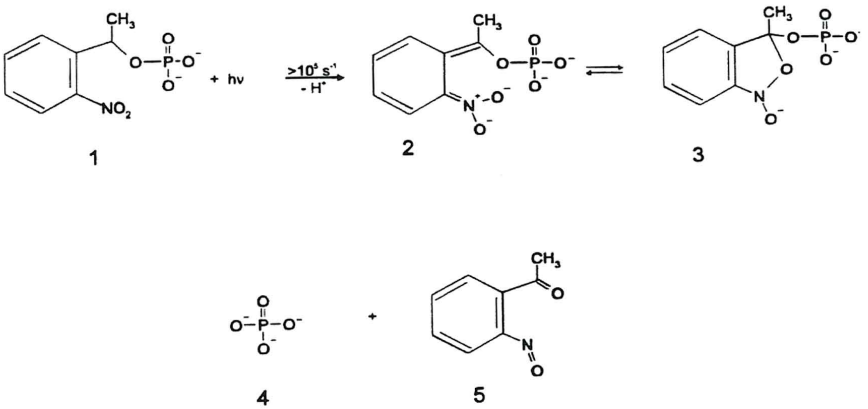
[8] J. A. Dantzig, Y. E. Goldman, N. C. Millar, J. Lacktis, and E. Homsher, *J. Physiol.* **451,** 247 (1992).

[9] J. W. Walker, G. P. Reid, J. A. McCray, and D. R. Trentham, *J. Am. Chem. Soc.* **110,** 7170 (1988).

[10] D. D. Hackney, K. E. Stempel, and P. D. Boyer, *Methods Enzymol.* **64,** 60 (1980).

[11] B. A. Connolly, F. Eckstein, and H. H. Fuldner, *J. Biol. Chem.* **257,** 3382 (1982).

[12] J. Feuerstein, Dissertation, Universität Hannover, Germany (1987).



SCHEME 1. Reaction scheme for the photolysis of caged phosphate.[9]

Caged Phosphate

The 1-(2-nitrophenyl)ethyl moiety is used to protect phosphate, nucleotides, and nucleotide analogs.[9,13] The application of UV flashes leads to the release of the desired phosphate compound. The mechanism of photolysis of compounds containing the 2-nitrobenzyl group was the topic of several investigations.[9,14,15]

Scheme 1 shows the generally accepted reaction pathway developed by Walker *et al.*[9] for the photorearrangement of caged phosphate compounds. After photolysis, a rapid formation of intermediate **2**, called the *aci*-nitro anion, occurs. It decays subsequently in a dark reaction to orthophosphate **4** and the byproduct 2-nitrosoacetophenone **5**. The intermediate may comprise a number of rapidly interconvertible forms such as **3**, but UV–VIS studies[9] and single-wavelength time-resolved IR measurements[15] are able to resolve only a single intermediate for caged ATP.

A typical FTIR difference spectrum of caged phosphate photolysis is shown in Fig. 2. A spectrum is measured before the photolysis and is taken as a reference. From this spectrum and the spectrum taken after the photolysis of the caged phosphate, the difference spectrum is calculated. The flat baseline shows the difference between two spectra measured before the flash as a control. Only those vibrational modes cause bands in the

[13] J. W. Walker, G. P. Reid, and D. R. Trentham, *Methods Enzymol.* **172,** 288 (1989).
[14] S. Schneider, *J. Photochem. Photobiol. A* **55,** 329 (1991).
[15] A. Barth, J. E. T. Corrie, M. J. Gradwell, Y. Maeda, W. Mantele, T. Meier, and D. R. Trentham, *J. Am. Chem. Soc.* **119,** 4149 (1997).

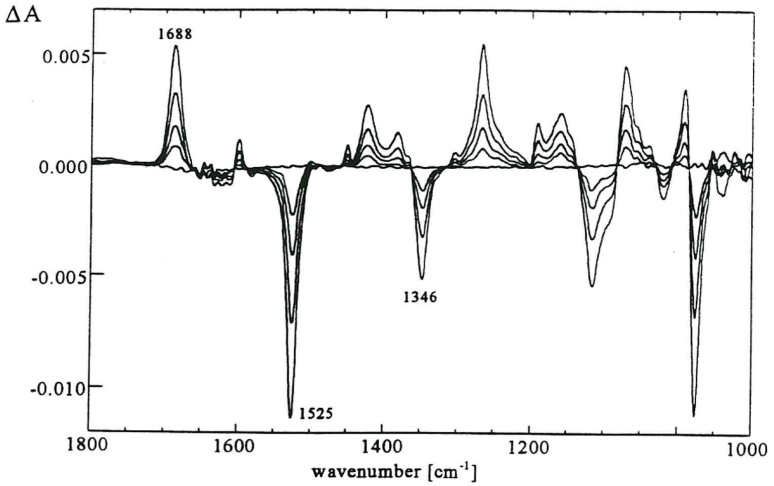


FIG. 2. Fourier transform infrared difference photolysis spectra of caged phosphate: 50 m*M* caged phosphate, 0.1 *M* MOPS, pH 7.0, baseline and a spectrum after the first, second, fourth, and ninth flash.

difference spectrum that undergo reaction-induced absorbance changes. Negative bands in the difference spectrum are due to the caged phosphate **1** in Scheme 1, whereas positive bands are due to the photolysis products, orthophosphate **4** and 2-nitrosoacetophenone **5**. Characteristic bands of 1-(2-nitrophenyl)ethyl derivatives are the disappearing asymmetric and the symmetric (NO_2) stretching vibrations [$\nu_{as}(NO_2)$ = 1525 cm^{-1}; $\nu_{sy}(NO_2)$ = 1346 cm^{-1}]; and the positive band is due to the carbonyl group of 2-nitrosoacetophenone **5** [ν(C=O) = 1688 cm^{-1}].[5,16-18]

Band Assignment by Isotopic Labeling

Unequivocal assignment of IR bands is made possible by isotopic labeling. For identification of phosphate bands we used $^{18}O_4$-labeled caged phos-

[16] N. B. Colthup J. W. Daly, and S. E. Wiberly, "Introduction to Infrared Raman Spectroscopy," Academic Press, London, 1990.

[17] D. Lien-Vien, N. B. Colthup, and S. E. Wiberley, "The Handbook of Infrared and Raman Characteristic Frequencies of Organic Molecules," Academic Press, San Diego, 1991.

[18] H. Georg, A. Barth, W. Kreutz, F. Siebert, and W. Mantele, *Biochim. Biophys. Acta* **1188,** 139 (1994).

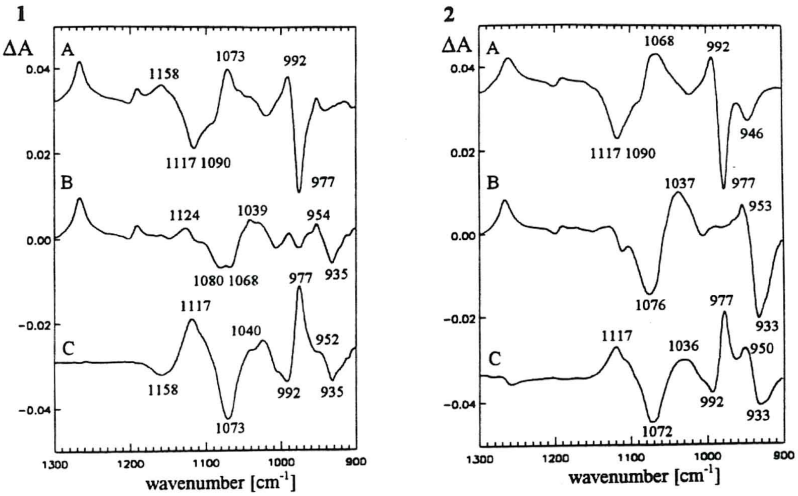


FIG. 3. Photolysis of 50 m*M* caged phosphate: **(1)** pH 7.0, 0.2 *M* MOPS: **(2)** pH 8.0, 0.2 *M* HEPES: (A) caged phosphate [$^{16}O_4$]; (B) caged phosphate [$^{18}O_4$]; (C) difference B − A.

phate. The higher mass of the oxygen isotope leads to a shift of all phosphate bands to lower wavenumbers.

Figure 3 **(1)** shows photolysis difference spectra of the unlabeled caged phosphate (A) and the ^{18}O-labeled caged phosphate (B) at pH 7.0. The double difference B minus A (C) represents the spectral differences between the unlabeled and the ^{18}O-labeled phosphate moiety of the caged compound. Figure 3 **(2)** shows the equivalent spectra measured at pH 8.0. The isotopical shifts are obvious. At pH 7.0, two species of orthophosphate, mono- and dihydrogen phosphate (pK_{a1} = 2, pK_{a2} = 6.72, pK_{a3} = 11.96, I = 0.1 *M*, 25°),[19] are present [Fig. 3 **(1)**]. To correctly assign the bands present at pH 7.0 to one of these two species, one has to measure the difference photolysis spectra at different pH values. Phosphate bands appear below 1300 cm^{-1}, and therefore only this spectral region is shown in Fig. 3.

The positive bands in spectra A and B correspond to the orthophosphate that is liberated by the photolysis of caged phosphate. The band at 1158 cm^{-1} [Fig. 3 **(1)**] is shifted to 1124 cm^{-1} and can be assigned to the (PO_2^-)

[19] "Critical Stability Constants. Vol. 4. Inorganic Complexes" (R. M. Smith and A. E. Marell, eds.), Plenum Press, New York, 1976.

TABLE I
ASSIGNMENT OF PHOSPHATE BANDS DUE TO ISOTOPIC LABELING

Caged phosphate	Wavenumber (cm^{-1})	Phosphate	Wavenumber (cm^{-1})
$(PO_3^{2-})_{deg}$	1117	$H_2PO_4^-$ $(PO_2^-)_{as}$	1158
$(PO_3^{2-})_{deg}$	1090	$H_2PO_4^-$ $(PO_2^-)_{ss}$	1073
$(PO_3^{2-})_{sy}$	977	HPO_4^{2-} $(PO_3^{2-})_{deg}$	1068
		HPO_4^{2-} $(PO_3^{2-})_{ss}$	992

asymmetric stretching vibration of $H_2PO_4^-$.[20] Indeed, this vibration is nearly completely absent at pH 8.0 [Fig. 3 **(2)**] where HPO_4^{2-} is the prevalent species. The broad band at 1073 cm^{-1}, which is shifted to 1039 cm^{-1}, is caused by the (PO_2^-) symmetric stretching vibration of $H_2PO_4^-$ and by (PO_3^{2-}) degenerate stretching vibration of HPO_4^{2-} [20,21] that appears at 1068 cm^{-1} at pH 8.0. The monohydrogen phosphate has an additional characteristic vibration at 992 cm^{-1} caused by the symmetric stretching vibration of the (PO_3^{2-}) moiety[20,21] that is shifted to 954 and 953 cm^{-1}, respectively.

The negative bands at 1117, 1090, and 977 cm^{-1} (Table I) belong to the unlabeled caged phosphate and are shifted to 1080, 1068, and 935 cm^{-1}, respectively, by isotopic labeling. The deprotonated monomethyl phosphate has two degenerate (PO_3^{2-}) stretching vibrations at 1115 and 1090 cm^{-1} and a (PO_3^{2-}) symmetric stretching vibration at 983 cm^{-1}.[22] Monoesters of phosphoric acid are reported to have two pK_a values, $pK_1 \approx 1.5$ and $pK_2 \approx 6.3$[23]; thus the FTIR spectrum of caged phosphate at pH 8.0 should be dominated by the completely deprotonated caged phosphate dianion. Because a band at 1117 cm^{-1} with a shoulder at 1090 cm^{-1} and a band at 977 cm^{-1} are observed in this spectrum, the same assignment could be given for caged phosphate.

Influence of Magnesium on Caged Phosphate

It is important to know the influence of magnesium on the FTIR spectrum of the phosphate moiety for the band assignments in protein measurements because magnesium is an essential cofactor for several phosphate and nucleotide binding enzymes, e.g., ATPases, kinases, and ion pumps.

[20] A. C. Chapman and L. E. Thirlwell, *Spectrochim. Acta* **20**, 937 (1964).
[21] E. Steger and K. Herzog, *Z. Anorg. Allg. Chem.* **331**, 169 (1964).
[22] T. Shimanouchi, M. Tsuboi, and Y. Kyogoku, *Adv. Chem. Phys.* **7**, 435 (1964).
[23] W. D. Kumler and J. J. Eiler, *J. Am. Chem. Soc.* **65**, 2355 (1943).

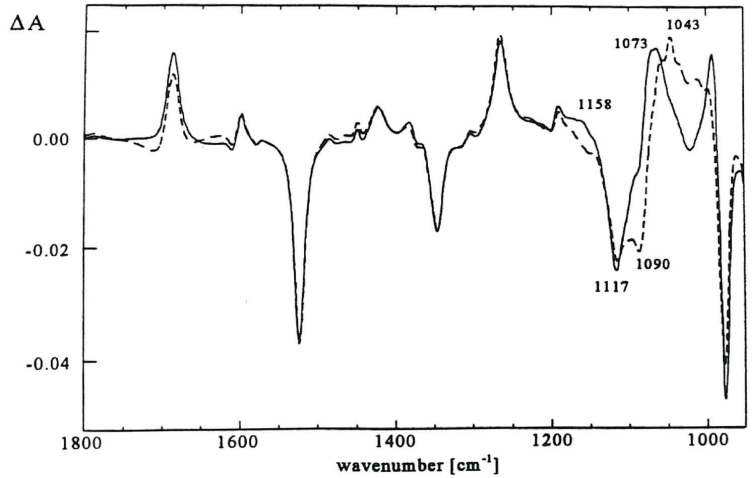


FIG. 4. Photolysis spectra of caged phosphate with Mg^{2+} (dashed line) and without Mg^{2+} (solid line): 50 m*M* caged phosphate, (100 m*M* $MgCl_2$), 200 m*M* HEPES, pH 7.0.

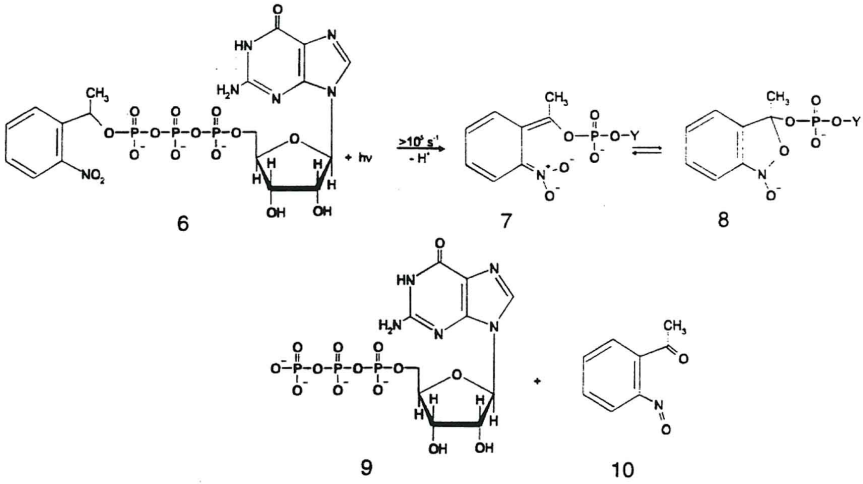
Figure 4 shows the photolysis spectrum of caged phosphate at pH 8.0 in the presence and absence of magnesium.

The positive band at 1073 cm^{-1}, which belongs to the orthophosphate, is shifted to 1043 cm^{-1}. This shift to lower wavenumbers is probably caused by the decrease of the partial double bond character of the P–O bonds involved in magnesium coordination. The band broadening in the orthophosphate region could also be due to a mixture of different magnesium orthophosphate complexes, e.g., $Mg_3(PO_4)_2$ and $MgHPO_4$.[24]

The small positive band at 1158 cm^{-1} missing in the presence of magnesium is due to a slightly higher concentration of $H_2PO_4^-$ in the absence of magnesium, which shifts the equilibrium of the orthophosphate species toward HPO_4^{2-}.

In the absence of magnesium the negative band belonging to the caged phosphate moiety at 1117 cm^{-1} has a shoulder at 1090 cm^{-1}. In the presence of magnesium a clearly resolved negative band is observed at 1090 cm^{-1}. The appearance of this band is probably caused by the shift of the positive band from 1073 to 1043 cm^{-1}. Therefore, it seems reasonable to assign this band to the degenerate stretching vibration of caged phosphate, which is masked by the positive band in the absence of magnesium. No further

[24] A. W. Taylor, A. W. Frazier, and E. L. Gurney, *Trans. Faraday Soc.* **59,** 1585 (1963).



SCHEME 2. Reaction scheme for the photolysis of caged GTP.[9]

influence of magnesium on the bands corresponding to caged phosphate is detected.

Caged GTP

Further important compounds for the investigation of biological systems are caged nucleotides such as caged GTP and ATP.[4,5,9,13] The photolysis reaction of caged GTP is shown in Scheme 2 (Structures **6–10**).

The photolysis spectrum of caged GTP (Fig. 5, solid line) shows bands due to the 1-(2-nitrophenyl)ethyl moiety (1688, 1525, and 1346 cm^{-1}, as already discussed) and phosphate bands below 1300 cm^{-1}. Bond cleavage between the caged moiety and the leaving group GTP gives rise to the negative band at 1252 cm^{-1}. It is caused by the asymmetric stretching vibration of the γ (PO_2^-) group connected to the caging group. During photolysis this group is converted to the terminal γ (PO_3^{2-}) group of the released GTP. The degenerate stretching vibration of the γ (PO_3^{2-}) group leads to the positive band at 1118 cm^{-1}. Generally, the spectral region between 1050 and 1200 cm^{-1} is determined by a superposition of the degenerate γ (PO_3^{2-}) and the symmetric (PO_2^-) stretching modes.[25–27]

[25] H. Takeuchi, H. Murata, and I. Harada, *J. Am. Chem. Soc.* **110,** 392 (1988).
[26] A. Barth, W. Kreutz, and W. Mantele, *Biochim. Biophys. Acta* **1194,** 75 (1994).
[27] A. Barth, W. Mantele, and W. Kreutz, *Biochim. Biophys. Acta* **1057,** 115 (1991).

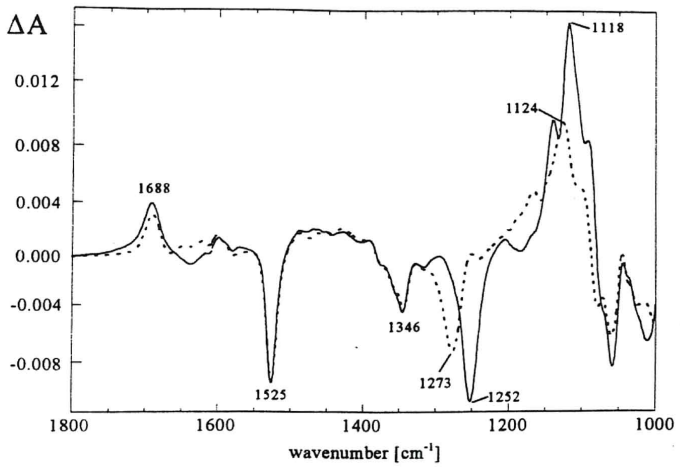


FIG. 5. Photolysis spectra of caged GTP with Mg^{2+} (dashed line) and without Mg^{2+} (solid line): 20 m*M* caged GTP (50 m*M* $MgCl_2$), 100 m*M* HEPES, pH 7.0.

Influence of Magnesium on Caged GTP

The formation of complexes among caged GTP, GTP, or other nucleotides with divalent cations, such as Ca^{2+}, Mg^{2+}, and Mn^{2+}, has a strong effect on FTIR spectra.[25,28]

The comparison of caged GTP spectra with and without Mg^{2+} (Fig. 5) shows no significant effect above 1300 cm^{-1}. The negative band at 1252 cm^{-1} in the absence of Mg^{2+} is less intensive and is shifted upward to 1273 cm^{-1} in the presence of the cation. This can be explained by an increase of the partial double bond character of the P–O bonds not directly involved in magnesium coordination. The positive band around 1118 cm^{-1} is shifted to 1124 cm^{-1} in the Mg^{2+}-bound case.

The similarity of caged GTP and caged ATP photolysis (Fig. 6) shows, as expected, that FTIR differences spectra are dominated by changes derived from the 1-(2-nitrophenyl)ethyl group and the phosphate chain.

Time-Resolved Measurements

Various FTIR techniques have been developed to collect time-resolved absorbance data. They are the rapid scan,[29] the stroboscopic,[30] and the

[28] H. Brintzinger, *Biochim. Biophys. Acta* **77,** 343 (1963).
[29] K. Gerwert, G. Souvignier, and B. Hess, *Proc. Natl. Acad. Sci. U.S.A.* **87,** 9774 (1990).
[30] G. Souvignier and K. Gerwert, *Biophys. J.* **63,** 1393 (1992).

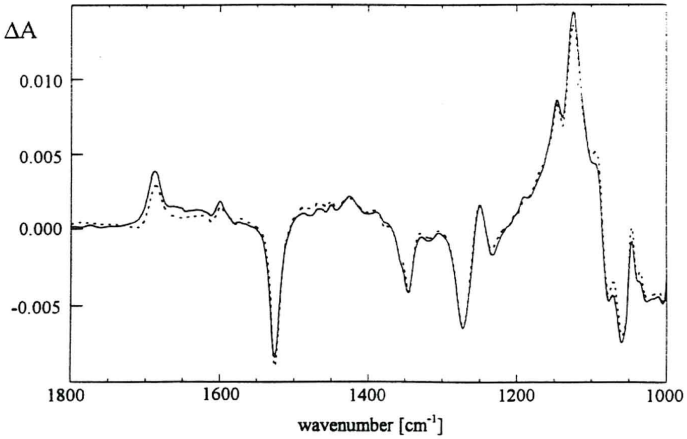


FIG. 6. Caged GTP (solid line), caged ATP (dashed line), 40 m*M* caged GTP/ATP, 200 m*M* $MgCl_2$, 500 m*M* HEPES/NaOH, pH 7.5.

step-scan techniques.[2,31,32] (and citations therein). Here we use the rapid scan technique with a 12-msec time resolution to record the IR difference spectra of the intermediate (Scheme 2, **7, 8**) and the photolysis products of caged GTP. Conditions close to physiological values, pH 7.5 and the presence of $MgCl_2$, are used for the measurements. The presence of dithiothreitol (DTT) is essential for the biological application of several caged compounds because the photolysis of the 1-(2-nitrophenyl)ethyl group yields 2-nitrosoacetophenone (Scheme 2, **10**). This compound can react with the cysteines in proteins and therefore inactivate or modify the biological system.[4] These complications can be overcome by the addition of thiols, such as DTT, which scavenge this nitrosoketone.[33]

Figure 7 compares photolysis spectra of caged GTP with and without DTT. The main bands are the same in the two spectra, which demonstrates the relatively small influence of DTT on the spectrum. The largest difference is the missing band at 1688 cm^{-1} in the measurement with DTT. This shows that the carbonyl group of the 2-nitrosoketone is absent in the final products, which can be explained by the reaction of 2-nitrosoketone with DTT. The lower part of the spectra shows small differences in the intensities at 1250,

[31] W. Uhmann, A. Becker, C. Taran, and F. Siebert, *Appl. Spectrosc.* **45**, 390 (1991).
[32] R. A. Palmer, J. L. Chao, R. M. Ditmar, V. G. Gregoriou, and S. E. Plunkett, *Appl. Spectrosc.* **47**, 1297 (1993).
[33] J. H. Kaplan, B. Forbush III, and J. F. Hoffman, *Biochemistry* **17**, 1929 (1978).

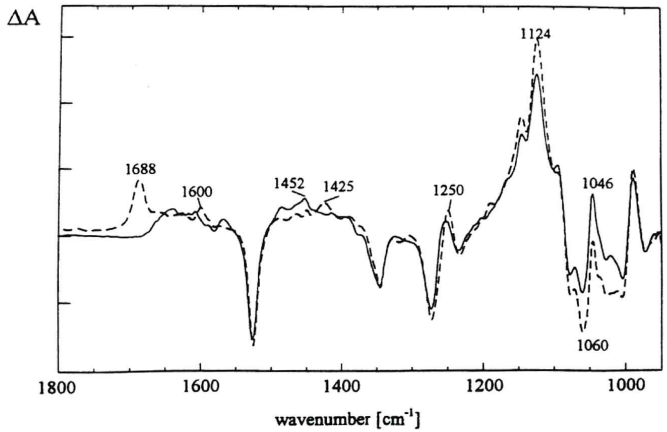


FIG. 7. Photolysis spectra of caged GTP with DTT (solid line, 50 m*M* caged GTP, 250 m*M* $MgCl_2$, 250 m*M* DTT, 500 m*M* HEPES/NaOH, pH 7.5, 10°, resolution 8 cm^{-1}) and without DTT (dashed line, 20 m*M* caged GTP, 50 m*M* $MgCl_2$, 100 m*M* HEPES/NaOH, pH 7.5, 20°, resolution 4 cm^{-1}).

1124, 1060, and 1046 cm^{-1} and a few smaller bands at 1600, 1452, and 1425 cm^{-1}.

In order to determine the IR difference spectra of the intermediate, spectra obtained between 1 and 26 msec, after a single laser flash are averaged (Fig. 8A). The region between 1600 and 1700 cm^{-1} shows a decreased signal-to-noise (*S/N*) ratio because of the large water background absorption. The product difference spectrum (Fig. 8B), obtained between 86 and 105 sec after the flash, shows the IR differences between caged GTP and the final products of the caged GTP photolysis, i.e., GTP and the reaction products of 2-nitrosoacetophenone with DTT.

As determined by a global fit analysis,[34] the intermediate decays exponentially with a decay constant of 8 sec^{-1} to the final products. Thus the average intermediate concentration in the spectrum (Fig. 8A) is 96%. Barth *et al.*[15] have reported the IR spectrum of an intermediate during the photolysis of caged ATP. In general, the difference spectra shown in A agree nicely with their published one, even though slightly different conditions (different buffer, pH 8.5, no magnesium) were used. Several bands, e.g., at 1379, 1328, 1244, and 1181 cm^{-1}, which they assign to the *aci*-nitro anion (Scheme 2, **7**), can also be found in the spectrum (Fig. 8A). Barth *et al.*[15] could make

[34] B. Hessling, G. Souvignier, and K. Gerwert, *Biophys. J.* **65,** 1929 (1993).

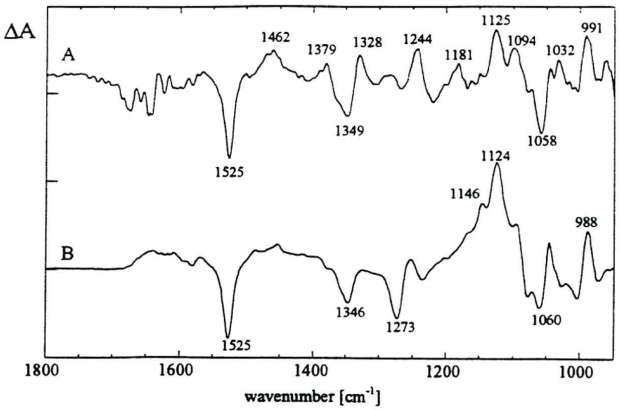


FIG. 8. Fourier transform infrared difference spectra of laser pulse photolysis of caged GTP, 50 m*M* caged GTP, 250 m*M* $MgCl_2$, 250 m*M* DTT, 500 m*M* HEPES/NaOH, pH 7.5, 10°, resolution 8 cm^{-1}. (A) Obtained 1–26 msec after a single laser flash; (B) obtained 86–105 sec after a single laser flash.

specific band assignments by the use of ^{13}C, ^{15}N, and ^{18}O isotopomers of caged ATP. Deviations to the difference spectra of Barth *et al.*[15] are observed for the disappearing asymmetric (NO_2) stretching vibration. They reported a shift of this educt band between the intermediate and the product difference spectrum. However, no difference at 1525 cm^{-1} is observed between spectra A and B. These differences could arise from the fact that Barth *et al.* used a different buffer that has a large background absorption in this spectral region.

Raman Spectroscopy

Raman spectroscopy, like infrared spectroscopy, probes the vibrational properties of a molecule. The selection rules between these two are different, however. This difference is a useful tool for the assignment of bands. Asymmetric vibrations give stronger bands in the IR spectrum, whereas bands due to symmetric vibrations are stronger in the Raman spectrum.

Figure 9 shows the solution Raman spectrum of caged GTP (A) and GTP (B). The Raman spectrum of the solvent was subtracted. The strongest band in the Raman spectrum of caged GTP is the symmetric (NO_2) stretching vibration at 1346 cm^{-1},[14] whereas the asymmetric (NO_2) stretching vibration gives rise only to a small band in the spectrum at 1525 cm^{-1}. By

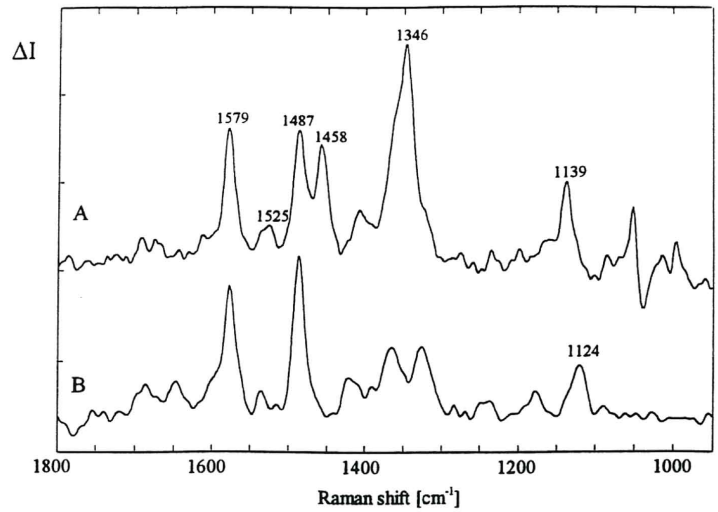


FIG. 9. Solution Raman spectrum. (A) Caged GTP: 100 m*M* caged GTP, 250 m*M* $MgCl_2$, 250 m*M* DTT, 500 m*M* HEPES/NaOH, pH 7.5, 4°. (B) GTP: 20 m*M* GTP, 60 m*M* $MgCl_2$, pH 7.5, 4°. The Raman spectrum of the solvent was subtracted.

comparison with the Raman spectrum of GDP of Weng *et al.*[35] the band at 1579 cm^{-1} can be assigned to the N3–C4 and C4–N9 stretching motions of the ring of the purine base. The band at 1487 cm^{-1} can be assigned to the N7=C8 and C4=C5 stretching vibration of the purine base. Another strong band is at 1458 cm^{-1}, which can tentatively be assigned to the phenyl ring of the 2-nitrophenyl group of caged GTP. The band at 1139 cm^{-1} can be caused by the symmetric (PO_2^-) vibration of the α-, β-, and γ-phosphate groups of caged GTP. The strong buffer scattering between 1000 and 1100 cm^{-1} causes a subtraction artifact at 1050 cm^{-1} and leads to a decreased S/N ratio in this spectral region. For Mg^{2+}-complexed GTP (Fig. 9B) the Raman band at 1124 cm^{-1} is assigned, as for magnesium complexed ATP,[25] to the in-phase symmetric (PO^{2-}) mode.

To demonstrate the different selection rules for IR and Raman spectroscopy, the Raman difference spectrum between GTP and caged GTP (Fig. 10A) and the IR difference spectrum of photolyzed caged GTP (Fig. 10B) are compared. In order to subtract the two Raman spectra (Figs. 9A and 9B), spectra are normalized using the band at 1579 cm^{-1}.

[35] G. Weng, C. X. Chen, V. Balogh-Nair, R. Callender, and D. Manor, *Protein Sci.* **3**, 22 (1994).

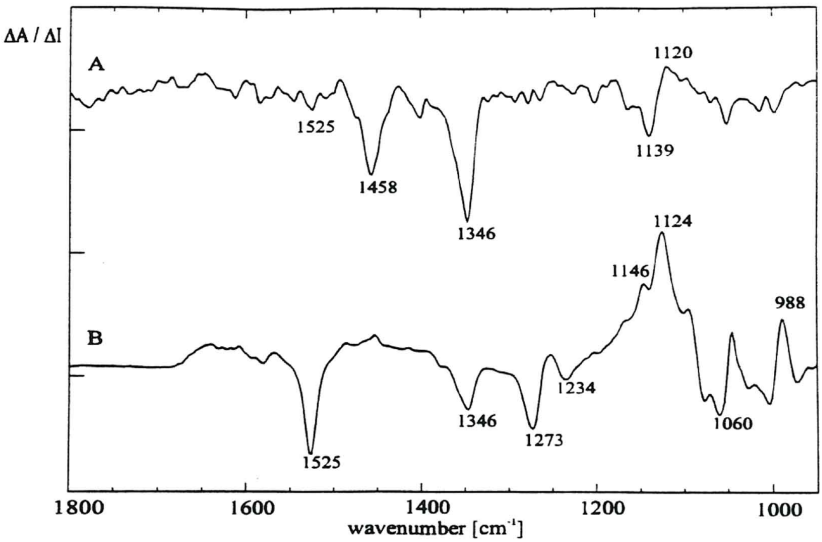
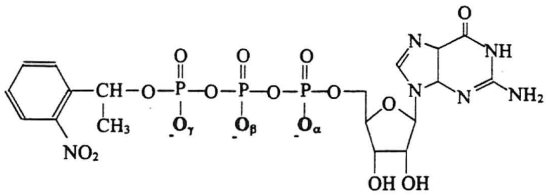


FIG. 10. (A) NIR-FT-Raman difference spectra of GTP (Fig. 9B) minus caged GTP (Fig. 9A); (B) FTIR difference spectra of laser pulse photolysis of caged GTP; 50 m*M* caged GTP, 250 m*M* $MgCl_2$, 250 m*M* DTT, 500 m*M* HEPES/NaOH, pH 7.5, 10°, obtained 86–105 sec after a single laser flash, resolution 8 cm^{-1}.

In contrast to FTIR difference spectra, Raman difference spectra between caged GTP and GTP show no large differences. Spectra show nicely that the symmetric vibration of the (NO_2) group at 1346 cm^{-1} [14] gives a much larger signal in the Raman spectrum, whereas the asymmetric (NO_2) stretching vibration at 1525 cm^{-1} is much stronger in the IR difference spectrum. The negative band at 1458 cm^{-1} is tentatively assigned to the phenyl ring of the 2-nitrophenyl group of caged GTP. The negative band at 1139 cm^{-1} is probably caused by the in-phase mode of the three (PO_2^-) groups of caged GTP. By comparison with the solution Raman spectrum of GTP (Fig. 9A), a small band at 1120 cm^{-1} can be visualized. This band is assigned to the in-phase mode of the (PO_2^-) groups of GTP.

Band Assignments of Caged GTP

For further band assignment, we synthesized caged GTP isotopomers that contain one ^{18}O isotope selectively in the α, β, and γ position, respec-



SCHEME 3. Position of the ^{18}O labels introduced. All oxygens the ^{16}O isotope **(1)**; α: ^{18}O isotope, β and γ: ^{16}O isotope **(2)**; β: ^{18}O isotope, α and γ: ^{16}O isotope **(3)**; γ: ^{18}O isotope, α and β: ^{16}O isotope **(4)**.

tively. Photolysis spectra of the four caged GTP isotopomers (Scheme 3, unlabeled and ^{18}O labeled) are shown in Fig. 11. To normalize spectra, the symmetric (NO_2) vibration at 1346 cm^{-1} is used (Fig. 11, dashed line).

The comparison of the four photolysis spectra shows downshifts for each of the ^{18}O-labeled compounds. Shifting of caged GTP bands is indicated by a light shading and shifting of GTP bands by a dark shading (Fig. 11, line 1). The black area symbolizes a shift that is observed only in the spectrum of the γ-^{18}O-labeled caged GTP. Spectra are termed by the numbers according to the respective caged GTP isotopomers in Scheme 3. The wave-

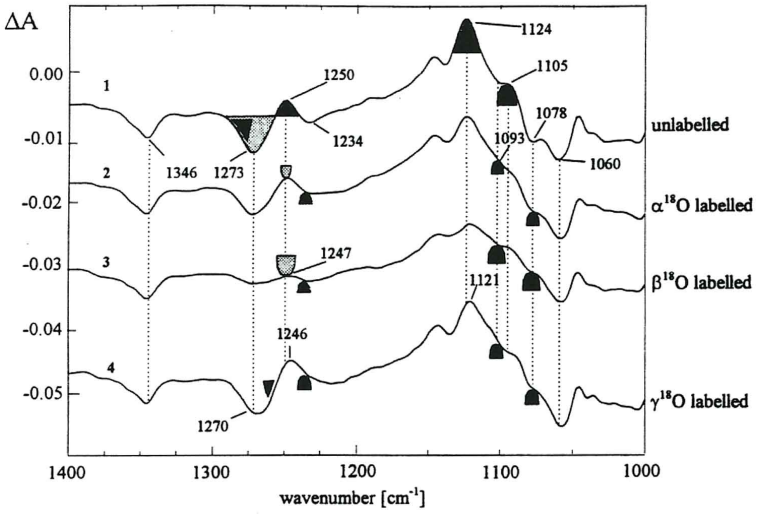


FIG. 11. Caged GTP photolysis spectra; 20 m*M* caged GTP, 50 m*M* $MgCl_2$, 100 m*M* HEPES, pH 7.5. **(1)** Unlabeled; **(2)** α-^{18}O-labeled; **(3)** β-^{18}O-labeled; and **(4)** γ-^{18}O-labeled.

numbers to which the bands are shifted in the spectra of the labeled compounds are indicated by the same shadings referring to line 1.

The negative band at 1273 cm^{-1}, representing the asymmetric modes of the three (PO_2^-) groups of caged GTP, is sensitive to all three labels. The intensity of this band is decreased by α- and even more by β-^{18}O-labeling, and the band is shifted to 1250 cm^{-1}. This is indicated by the light shading on lines 2 and 3. γ-^{18}O-Labeling causes a downshift of the band at 1273 to 1270 cm^{-1} but does not change its intensity (black area on line 4, Fig. 11). This interpretation is supported by the double differences (Fig. 12). Here the photolysis difference spectra of the labeled compounds are subtracted from that of the unlabeled one. For α- and β-labeled caged GTP, a shift can be detected from 1273 to 1253 and 1251 cm^{-1}, respectively. In contrast, caged GTPγ^{18}O shows a specific shift from 1277 to 1261 cm^{-1}.

The positive band at 1250 cm^{-1} represents the asymmetric stretching vibration of the α and β (PO_2^-) group of the free GTP (Fig. 11). Due to α, β, and γ labeling, the band at 1250 cm^{-1} is downshifted to around 1240 cm^{-1} (dark shading, Fig. 11). This becomes clearer in double difference spectra (Fig. 12). For the α, β, and γ label a shift is observed from 1253 to 1238, from 1251 to 1236, and from 1261 to 1240 cm^{-1}, respectively. For GTPβ^{18}O the shift is less obvious due to the masking shift of the band at 1273 cm^{-1}.

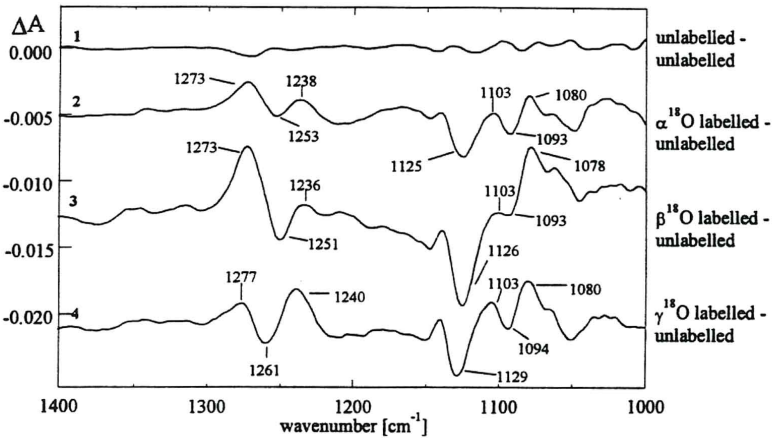


FIG. 12. Double differences of caged GTP photolysis spectra: 20 m*M* caged GTP, 50 m*M* $MgCl_2$, 100 m*M* HEPES, pH 7.5. **(1)** Unlabeled; **(2)** α-^{18}-O-labeled; **(3)** β-^{18}O-labeled; and **(4)** γ-^{18}-O-labeled.

TABLE II
ASSIGNMENTS OF CHANGING PHOSPHATE VIBRATIONS DURING CAGED GTP PHOTOLYSIS REACTION[a]

Caged GTP	Wavenumber (cm^{-1})	Comment	GTP	Wavenumber (cm^{-1})	Comment
α-(PO_2^-)	1273	Coupled with β	α-(PO_2^-)	1253	α, β, and γ coupled
				1125	α, β, and γ coupled
				1093	α, β, and γ coupled
β-(PO_2^-)	1273	Coupled with α	β-(PO_2^-)	(1251)	α, β, and γ coupled
				1126	α, β, and γ coupled
				1093	α, β, and γ coupled
γ-(PO_2^-)	1277		γ-(PO_3^{2-})	1261	
				1129	α, β, and γ coupled
				1094	α, β, and γ coupled

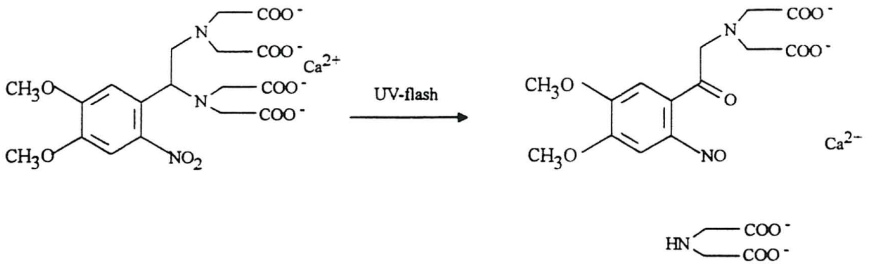
[a]Results are obtained by the use of the isotopomers 1 to 4 (see Scheme 3).

After photolysis the γ-phosphate is converted to a nonbridging (PO_3^{2-}) group. The degenerate stretching vibration of the γ (PO_3^{2-}) group should absorb below 1150 cm^{-1}. Furthermore, below 1150 cm^{-1} phosphate bands due to symmetric stretching vibrations of (PO_2^-) groups are superimposed.[15] The most intensive positive band is located at 1124 cm^{-1} (Fig. 11). It is downshifted by α-^{18}O-labeling. A better insight into the shifts is again provided by double difference (Fig. 12).[25] In principle, two different shift patterns are possible. One possibility is a downshift from 1125 to 1080 cm^{-1} (Fig. 12), but the maximal downshift due to ^{18}O-labeling in a diatomic molecule is 43 cm^{-1}. Therefore, it is more likely that the band is downshifted from 1125 to about 1103 cm^{-1} and a second shift is superimposed from 1093 to 1080 cm^{-1}. In this case, the shifted bands at 1103 and 1093 cm^{-1} cancel each other. Similar shifts are seen due to β-^{18}O-labeling from 1126 to 1103 and 1093 to 1078 cm^{-1} (Fig. 12). γ-^{18}O-Labeling seems to cause shifts from 1129 to 1103 and 1094 to 1080 cm^{-1}. The bands at 1124 and 1093 cm^{-1} seem to represent α and β (PO_2^-) that are coupled to the γ (PO_3^{2-}) vibrations. The observed sensitivity of the bands to the ^{18}O-labeling can be explained by a strong vibration coupling among all three phosphate groups (Table II).

DM-Nitrophen: A Photolabile Chelator of Divalent Cations

Another important group of caged compounds are photolabile derivatives of cation chelating reagents such as DM-nitrophen.[33,36–39] DM-Ni-

[36] J. H. Kaplan and G. C. R. Ellis-Davies, *Proc. Natl. Acad. Sci. U.S.A.* **85,** 6571 (1988).
[37] G. C. Ellis-Davies, J. H. Kaplan, and R. J. Barsotti, *Biophys. J.* **70,** 1006 (1996).



SCHEME 4. Photolysis of DM-nitrophen.

trophen contains an EDTA molecule and has a high affinity for divalent cations in the unphotolyzed state [K_D (Ca^{2+}) $\approx 5 \times 10^{-9}$ *M*. K_D (Mg^{2-}) $\approx 2.5 \times 10^{-6}$ *M*].[36] During the photolysis of DM-nitrophen, a C–N bond is broken and iminodiacetic acid is released (Scheme 4). Thus the K_D for the cations decreases to the millimolar range.[36,40]

This chelator has been used for investigating exocytosis,[41] muscle fibers,[42] and FTIR measurements on sarcoplasmic reticulum (SR) Ca^{2+}-ATPase.[18,40] Time-resolved FTIR difference spectroscopy has been applied to measure the kinetics of calcium binding to the SR Ca^{2+}-ATPase.[40]

A typical FTIR photolysis spectrum of DM-nitrophen is shown in Fig. 13. As is the case with the other caged compounds, the band at 1525 cm^{-1} is assigned to the asymmetric stretching vibration of (NO_2). This band is taken as reference to rescale spectra from different measurements when necessary. It could be expected that the bands due to the carboxylic groups are changed when different divalent cations are used.[43,44] As shown in Fig. 13, the wavenumber of the negative band at around 1585 cm^{-1} is different in the presence of Ca^{2+} (1585 cm^{-1}), Mg^{2+} (1588 cm^{-1}), and without divalent cation (1583 cm^{-1}). This band can be assigned to the asymmetric stretching vibration of the (COO^-) groups of the EDTA

[38] A. L. Escobar, F. Cifuentes, and J. L. Vergara, *FEBS Lett.* **364,** 335 (1995).
[39] J. A. McCray, N. Fidler-Lim, G. C. Ellis-Davies, and J. H. Kaplan, *Biochemistry* **31,** 8856 (1992).
[40] A. Troullier, K. Gerwert, and Y. Dupont, *Biophys. J.* **71,** 2970 (1996).
[41] T. D. Parsons, G. C. Ellis-Davies, and W. Almers, *Cell Calcium* **19,** 185 (1996).
[42] J. R. Patel, G. M. Diffee, and R. L. Moss, *Biophys. J.* **70,** 2333 (1996).
[43] M. Nara, M. Tasumi, M. Tanokura, T. Hiraoki, M. Yazawa, and A. Tsutsumi, *FEBS Lett.* **349,** 84 (1994).
[44] G. B. Deacon, R. J. Phillips, *Coord. Chem. Rev.* **33,** 227 (1980).

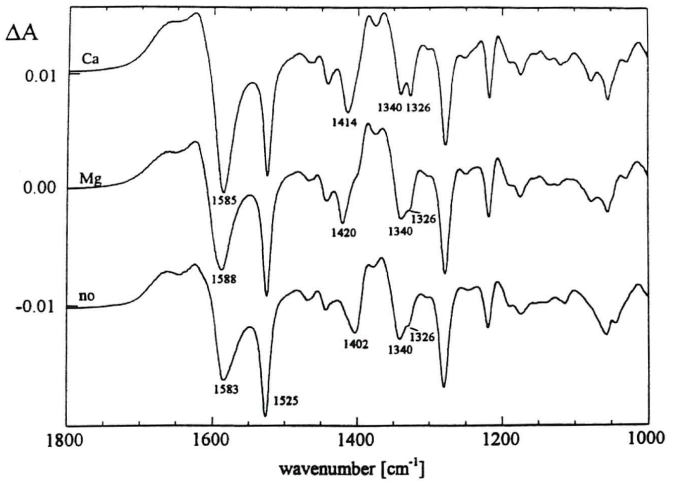


FIG. 13. 20 m*M* DM-nitrophen, 20 m*M* DTT, 10 m*M* Ca^{2+} (Mg^{2+} or without divalent cation), respectively, 0.3 *M* MOPS, pH 7.0, resolution 2 cm^{-1}.

moiety of DM-nitrophen.[16,17,45] Similar variation is observed for the band assigned to the symmetric carboxylate mode in the presence of Ca^{2+} (1414 cm^{-1}), Mg^{2+} (1420 cm^{-1}), and without divalent cation (1402 cm^{-1}). The symmetric vibration of arylic (NO_2) is usually found in the range of 1357 to 1318 cm^{-1}.[17] This vibration has been assigned to a broad negative band at 1332 cm^{-1} observed in the photolysis spectrum of DM-nitrophen.[18] We also observe a large band in this range that shows, in fact, two resolved peaks (Fig. 13): a band at 1340 cm^{-1} that is not modified in the absence or presence of divalent cations and a band at 1326 cm^{-1} that is sensitive to the presence of divalent cation. The last one is also present in EDTA absorbance spectra and is assigned to the (COO^-) moiety.[45] Therefore, only the band at 1340 cm^{-1} in the photolysis spectrum of DM-nitrophen should be assigned to the aromatic symmetric stretching vibration of the (NO_2) moiety.

Its ability to release a variety of cations by flash photolysis makes DM-nitrophen a useful compound for FTIR measurements on many biological systems.

[45] D. T. Sawyer and P. J. Paulsen, *J. Am. Chem. Soc.* **80**, 1597 (1958).

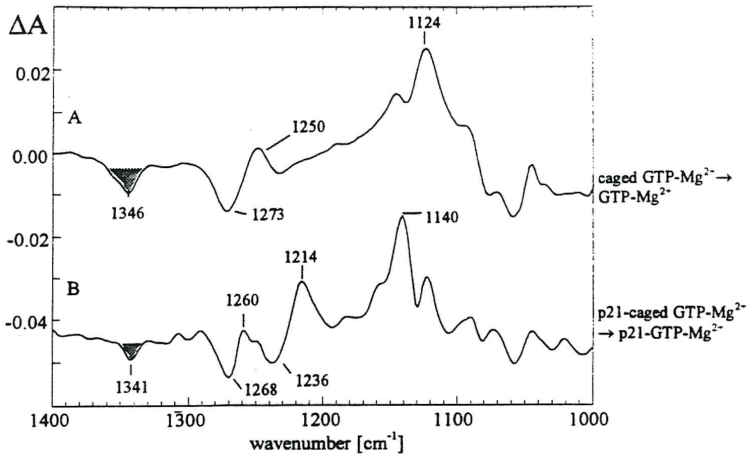


FIG. 14. (A) Photolysis spectrum of 20 m*M* caged GTP, 50 m*M* $MgCl_2$, 100 m*M* HEPES, pH 7.5 (B) Photolysis spectrum of 10 m*M* H-*ras* p21–caged GTP, 25 m*M* $MgCl_2$, 10 m*M* DTT, 50 m*M* HEPES, 1% glycerol, pH 7.5.

Biological Application: Fourier Transform Infrared Studies of GTPase Mechanism of H-*ras* p21

Caged GTP can be used as a substrate for H-*ras* p21. It is tightly bound similarly to GTP in close proximity to magnesium, but it is not hydrolyzed.[46] The photolysis spectrum of H-*ras* p21–caged GTP is shown in Fig. 14.

The shape of the H-*ras* p21–caged GTP photolysis spectrum (Fig. 14B) is remarkably different from the spectrum without protein (Fig. 14A). The difference bands of the free nucleotide (1273/1250 cm^{-1}) are split into two negative bands at 1268 and 1236 cm^{-1} and two positive bands at 1260 cm^{-1} and 1214 cm^{-1} in the enzyme-bound form. The broad positive band pattern originally around 1124 cm^{-1} is shifted to 1140 cm^{-1}. Also, below 1050 cm^{-1} small changes are observed. Compared to the photolysis without H-*ras* p21, the phosphate bands gain intensity and are shifted to higher wavenumbers. Because the degrees of freedom of the nucleotide are reduced by binding to the protein the bands appear sharper.[47,48]

[46] I. Schlichting, G. Rapp, J. John, A. Wittinghofer, E. F. Pai, and R. S. Goody, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 7687 (1989).

[47] K. Gerwert, V. Cepus, A. J. Scheidig, and R. S. Goody, *in* "Proceedings of Time-Resolved Vibrational Spectroscopy VI" (A. Lau, F. Siebert, and W. Werncke, eds.), p. 185, Springer Verlag, Berlin, 1994.

[48] V. Cepus, R. S. Goody, and K. Gerwert, submitted (1998).

The GTPase reaction takes place after the photolytic liberation of GTP.[46,47] Fourier transform infrared spectra were then recorded from 30 sec to 2 hr 10 min. The difference calculation was referred to the last recorded spectrum (Fig. 15). Therefore, the positive bands represent the H-*ras* p21–GTP state and the negative ones the H-*ras* p21–GDP state and inorganic phosphate. At 1260 cm^{-1} a strong positive band is detected that is characteristic for the asymmetric stretching vibration of the GTP (PO_2^-) groups. The negative band at 1100 cm^{-1} represents the strongest GDP band. It is dominated by the degenerate stretching vibration of the terminal β (PO_3^{2-}) group.[25] Results show that kinetic analysis of the GTPase reaction of H-*ras* p21 can be performed with FTIR difference spectroscopy. Thus, a new experimental approach, time-resolved FTIR difference spectroscopy, is established that monitors p21 GTPase reaction with high structural and high time resolution.

Summary

Time-resolved FTIR difference spectroscopy is a powerful tool for investigating molecular reaction mechanisms of proteins. In order to detect, beyond the large background absorbance of the protein and the water, absorbance bands of protein groups that undergo reactions, difference spectra have to be performed between a ground state and an activated state of the sample. Because the absorbance changes are small, the reaction has to be started *in situ*, in the apparatus, and in thin protein films. The use of caged compounds offers an elegant approach to initiate protein reactions with a nanosecond UV laser flash. Here, time-resolved FTIR and

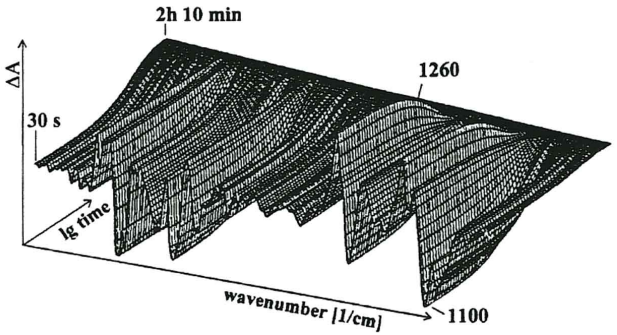


FIG. 15. Three-dimensional global fit representation of the GTPase reaction of H-*ras* p21; 10 m*M* H-*ras* p21–GTP, 25 m*M* $MgCl_2$, 10 m*M* DTT, 50 m*M* HEPES, pH 7.5, 1% glycerol.

FT–Raman photolysis studies of the commonly used caged compounds, caged P_i, caged ATP, caged GTP, and caged calcium are presented. The use of specific isotopic labels allows us to assign the IR bands to specific groups. Because metal ions play an important role in many biological systems, their influence on FTIR spectra of caged compounds is discussed. The results presented should provide a good basis for further FTIR studies on molecular reaction mechanisms of energy or signal transducing proteins. As an example of such investigations, the time-resolved FTIR studies on the GTPase reaction of H-*ras* p21 using caged GTP is presented.

Acknowledgments

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