Proc. Natl. Acad. Sci. USA
Vol. 86, pp. 7687–7690, October 1989
Biochemistry

Biochemical and crystallographic characterization of a complex of c-Ha-*ras* p21 and caged GTP with flash photolysis

(time-resolved structure)

ILME SCHLICHTING, GERT RAPP, JACOB JOHN, ALFRED WITTINGHOFER, EMIL F. PAI, AND ROGER S. GOODY

Max-Planck-Institut für Medizinische Forschung, Abteilung Biophysik, Jahnstr. 29, 6900 Heidelberg, Federal Republic of Germany

Communicated by David C. Phillips, June 30, 1989

ABSTRACT **The GTP binding domain of the c-Ha-*ras* protooncogene product (p21'c) and the corresponding region from an oncogenic mutant form of the protein in which glycine at position 12 has been replaced by valine [p21'(G12V)] have been crystallized with P^3-1-(2-nitro)phenylethylguanosine 5'-*O*-triphosphate (caged GTP) at their active sites. The crystals give x-ray diffraction patterns to a resolution of better than 0.3 nm. Photolysis can be achieved in the crystal, after which GTP hydrolysis takes place at the rate expected from solution studies. Complete x-ray data sets have been obtained for the starting caged-GTP state and the final GDP state after photolysis and hydrolysis, demonstrating the feasibility of time-resolved structural investigations of the process of GTP hydrolysis.**

p21 proteins (M_r 21,000; 189 amino acid residues) are products of the c-Ha-*ras* genes. Single-point mutations in these genes have been found in some acute transforming animal viruses and in a high percentage of human tumors (see ref. 1 for a review). The p21 proteins bind guanine nucleotides with high affinity and specificity and exhibit a low intrinsic GTPase activity. They resemble other guanosine nucleotide binding proteins (such as the signal-transducing G proteins) in that they appear to be active (in the sense of signal transmission) when in the guanosine triphosphate (GTP)-bound form and inactive in the guanosine diphosphate (GDP)-bound form, although it is not yet known in which signal transmission pathway they are involved. To evaluate structural differences between the p21·GTP and p21·GDP complexes and to analyze the mechanism of hydrolysis of the GTPase reaction in crystalline proteins, we have crystallized the GTP binding domain of the cellular form of p21 (p21'c) and the same region from a transforming mutant form [p21'(G12V)] complexed with P^3-1-(2-nitro)phenylethylguanosine 5'-*O*-triphosphate (caged GTP; Fig. 1), a photolabile precursor of GTP that is stable to hydrolysis by the protein. We report here the characterization of these crystals.

MATERIALS AND METHODS

p21 and its mutants were prepared as described by using an *Escherichia coli* expression system (2). The truncated forms of the p21 proteins, which lack 23 amino acids at the carboxyl terminus, were used (3). Caged GTP was prepared by the reaction of caged phosphate (4) with GDP that had been activated with diimidazole carbonyl by the method of Hoard and Ott (5). GDP at the active site of p21 was replaced quantitatively by caged GTP by a procedure to be described elsewhere (unpublished data). The complex between p21 and caged GTP was obtained by using a very small molar excess of analog over protein, and excess nucleotide was removed by gel filtration. Crystallization of the p21·caged GTP complex was as described for other p21 nucleotide complexes (6), except that significantly higher protein (20–24 mg/ml) and dithioerythritol (20 mM) concentrations were used. It also was found advantageous to use polyethylene glycol 400 (Serva), rather than 1450 as in ref. 6, at 23–24% (wt/vol) for p21'c and 26–29% for p21'(G12V). This gave crystals with a longest axis of >400 μm. Large crystals of p21'c could only be obtained by seeding with microcrystals. Crystallization was done at room temperature in the dark. Growth was checked under the microscope with a UV and heat filter (Spindler and Hoyer, Göttingen, F.R.G.).

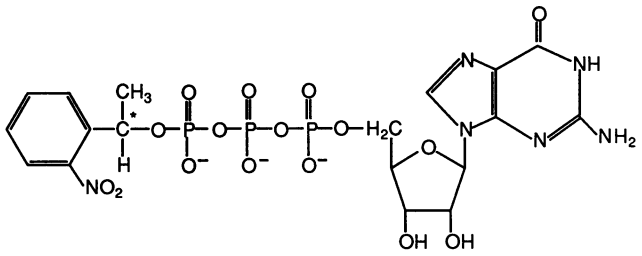

FIG. 1. Structure of caged GTP. The substance exists as two diastereomers because of the chiral center (*).

Flash photolysis was achieved in a 1-mm diameter quartz capilliary with a Xenon flash lamp (Rapp Optoelektronik, Dossenheim, F.R.G.; model JML, which includes lamp, power supply, and optical arrangement appropriate for these studies). The crystal was at the focus of the convergent beam from this system. To preserve the high-resolution x-ray diffraction pattern and GTPase activity of the crystals, it was found to be essential to photolyze the crystals while they were still well bathed in mother liquor and to use a filter (Scott UG 11) that allows radiation in the range of 300–400 nm to pass and that had been subjected to dielectric coating to prevent passage of residual radiation in the 700-nm range, which is characteristic of the UG 11 filter. Failure to take these precautions resulted in significant loss of resolution in the x-ray diffraction period (to *ca.* 0.5–0.6 nm). There is probably a certain degree of warming of the sample after the flash, and this may be partly counteracted by having the crystal soaked in a relatively large volume of mother liquor.

Analysis of the state of the nucleotide bound to p21 both in solution and in crystals was achieved by using reversed-phase HPLC (Shandon C_{18}) under ion-pairing conditions as described (6) and, for detection of caged GTP, by using reversed-phase chromatography on the same column with a gradient of acetonitrile in 50 mM potassium phosphate (pH 6.5).

RESULTS AND DISCUSSION

Time-dependent crystallographic studies on enzymes have been achieved by simple diffusion of substrates into crystals

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviation: caged GTP, P^3-1-(2-nitro)phenylethylguanosine 5'-*O*-triphosphate.

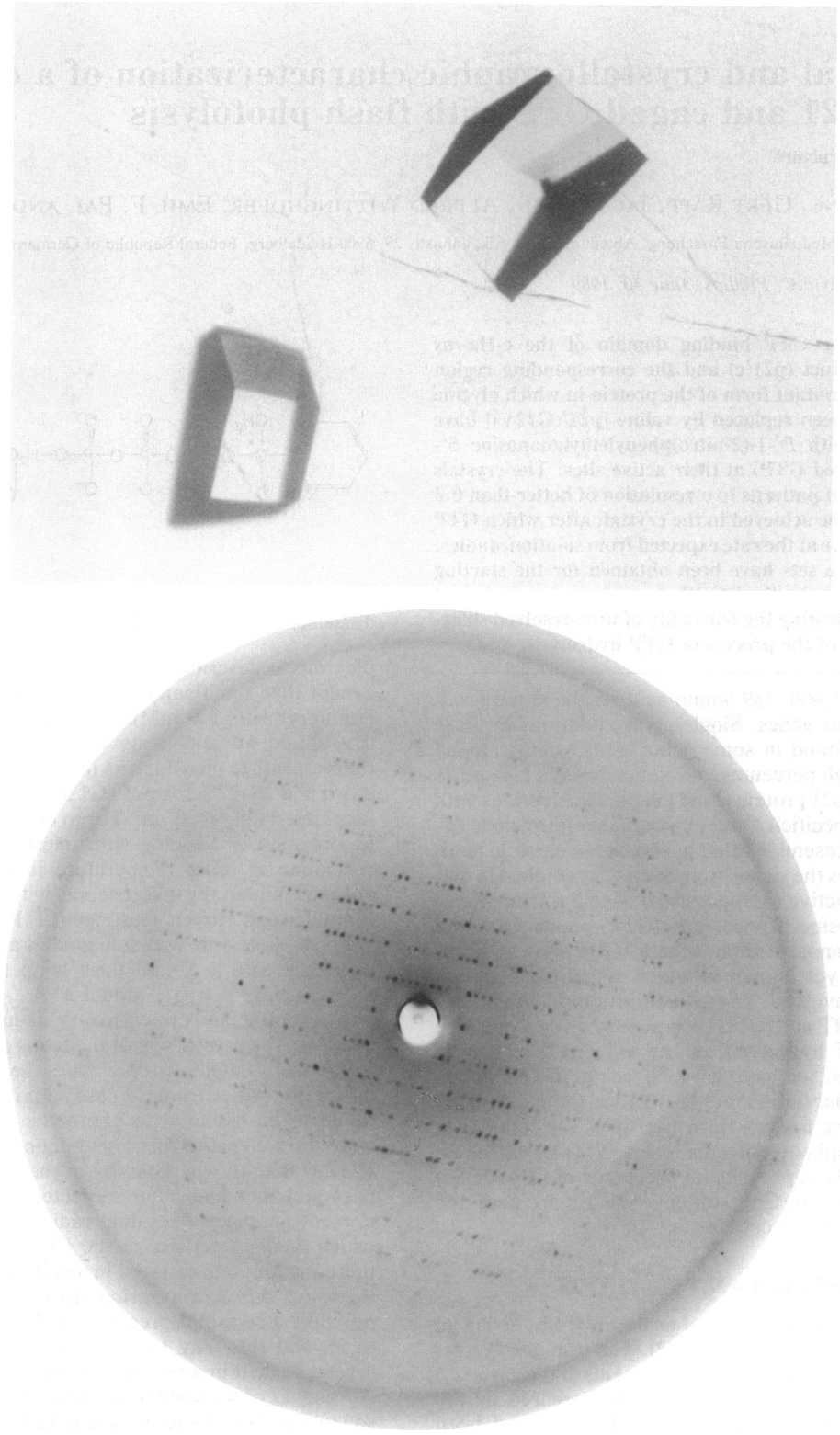

FIG. 2. (*Upper*) Crystals of p21'c complexed with caged GTP. The longest axis is *ca.* 400 μm. (*Lower*) X-ray diffraction pattern from a crystal as shown in *Upper*. The precession angle was 15°.

followed by rapid collection of x-ray diffraction data with a synchrotron source (7). This was possible in the case of glycogen phosphorylase *b*, since the protein can be crystallized in the absence of a ligand at the active site and the diffusion times of substrates into the crystals are relatively short (several minutes) compared to enzymatic reaction times that are much longer under appropriate conditions (7). This approach is not possible for p21 proteins because they are obtained with a strongly bound molecule of GDP at the active site. The half-life for the release of GDP under conditions required for the GTPase activity (i.e., in the presence of Mg^{2+} ions) of the proteins is on the order of hours—i.e., in the same time range as the GTPase reaction. The bound GDP can be removed, but the nucleotide-free protein is

relatively unstable (8). Thus, a different approach was needed, and the most suitable appeared to be the use of photolabile precursors of GTP.

Caged nucleotides (i.e., nucleotides made inert by a photolabile protecting group) have been used for a variety of investigations in recent years. The best documented example is in studies of muscle contraction using caged ATP (4, 9, 10). In these cases, there was no (or insignificant) interaction of the protected derivative with the system under investigation before photolysis. In the case of p21 proteins, it seemed likely that GTP analogs protected at the γ-phosphate group would still show significant interaction with the active site of the protein, since the most important requirement for strong binding to p21 appears to be an unmodified guanine base (unpublished work) in contrast to myosin and actomyosin, where an intact triphosphate moiety is the most important requirement (11).

The affinity of caged GTP for p21 was determined by monitoring the displacement by caged GTP of GDP from its complex with p21, as described for other nucleotides by Tucker *et al.* (2) and Feuerstein *et al.* (12). By using the previously measured value of 6×10^{10} M^{-1} for the binding constant of GDP to p21 at 0°C (12), a value of 6×10^{8} M^{-1} can be calculated for the corresponding constant for caged GTP. Thus, although the affinity is lower than that for GDP by a factor of 100, it is still easily high enough to allow production of a stoichiometric complex with the protein without using a large excess of analog.

The 1:1 complex between p21′c and caged GTP was prepared as described elsewhere for other p21·nucleotide analog complexes (unpublished data). It could be crystallized under conditions similar to those used for the crystallization of other nucleotide complexes with p21 (6). The crystals (Fig. 2 *Upper*) diffracted to better than 0.3 nm (Fig. 2 *Lower*) and had the space group $P3_121$ or its enantiomorph $P3_221$ with cell parameters $a = b = 4.10$ nm and $c = 16.48$ nm, which are very similar to the corresponding data for crystals of the p21′c·GTP(β,γ-NH) complex [$P3_221$; $a = b = 4.03$ nm, $c = 16.22$ nm, (6)]. After the crystals were redissolved, sodium dodecyl sulfate/PAGE showed the presence of p21′ (Fig. 3 *Upper*), and HPLC (Fig. 3 *Lower*) proved that mainly caged GTP (both diastereomers in approximately equal amounts) was present as the bound nucleotide in the crystals.

Photolysis in the crystals was achieved with a powerful Xenon flash lamp. Since a single flash of duration *ca.* 2 msec from this lamp was not sufficient to convert all of the caged GTP to GTP (*ca.* 30% per flash could be obtained under optimal conditions), at least 10 flashes were used to obtain essentially quantitative conversion, as shown by reversed-phase HPLC after the crystals were dissolved. After photolytic removal of the protecting group, hydrolysis of GTP to GDP took place in the crystal. This was shown by using a series of crystals to obtain single datum points. The half-life for hydrolysis at room temperature was 43 min, corresponding to a rate constant of 1.6×10^{-2} min^{-1} (Fig. 4). p21′c hydrolyzes GTP with a rate constant in solution of 3.7×10^{-2} min^{-1} at 37°C (3), which is slightly higher than the corresponding constant for the full-length protein. Thus, the kinetics of GTP hydrolysis do not appear to be affected significantly by the crystal lattice. After GTP hydrolysis was complete, the crystals still diffracted to better than 0.3 nm. Complete data sets from the caged GTP form and from the GDP form after photolysis and hydrolysis have been collected in the case of p21′c.

A potential problem with the approach described here stems from the fact that the GTP derivative used exists as a pair of diastereomers because of the chiral center in the protecting group. It is apparent from the HPLC results that both can bind to p21 and that the crystals contain an approximately 1:1 mixture of the two isomers. They also appear to

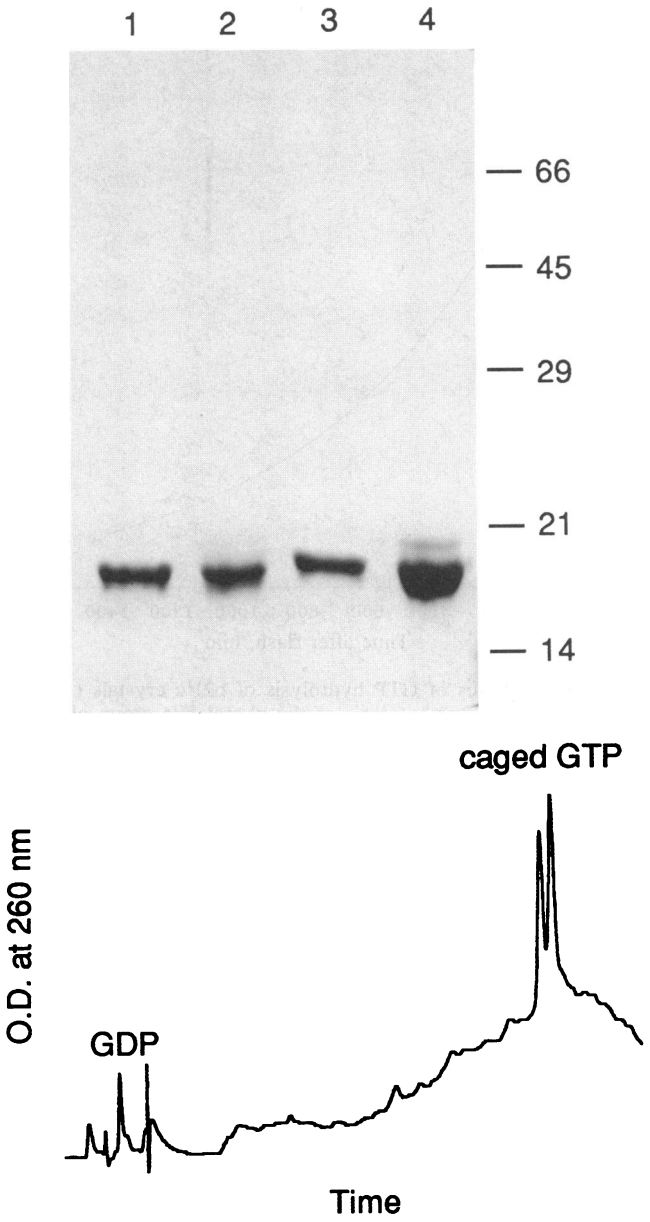

FIG. 3. (*Upper*) Sodium dodecyl sulfate/PAGE of redissolved crystals of p21′c (lane 2; lane 1 shows the preparation before crystallization) and of p21′(G12V) (lane 4; lane 3 shows the preparation before crystallization) complexed with caged GTP. (*Lower*) Reversed-phase chromatography of a redissolved crystal of the p21′(G12V)·caged GTP complex after standing for 2 months at room temperature in the dark (HPLC conditions as in *Materials and Methods*). The two diasteromers of caged GTP were eluted near the end of the chromatogram.

be equally well photolyzed at the active site, as also shown by further HPLC results (data not shown). It is possible, however, that the quality of the crystals as reflected in the diffraction data is limited by this lack of homogeneity. Recently we have succeeded in obtaining crystals of p21′ with a single diastereomer at the active site.

Similar experiments were also carried out with a truncated oncogenic mutant form of p21 [p21′(G12V)]. As expected from solution measurements, this mutant cleaves GTP in the crystals more slowly than the cellular form (rate constant = *ca.* 1.6×10^{-3} min^{-1} at room temperature; compare 3×10^{-3} min^{-1} in solution at 37°C from ref. 3).

The experiments reported here provide the basis for time-resolved studies on the mechanism of GTP hydrolysis and on

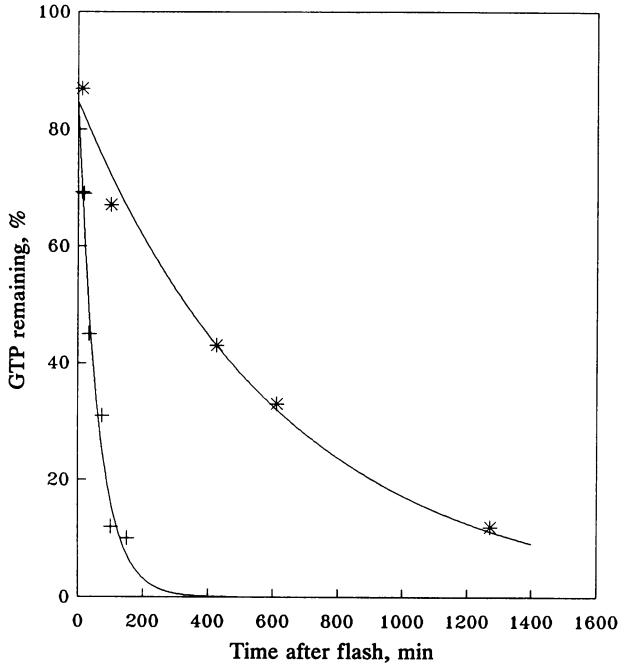

FIG. 4. Kinetics of GTP hydrolysis of p21'c crystals (+) and p21'(G12V) (*) after photolysis of the photolabile group of caged GTP. The relative amounts of GTP and GDP were determined by dissolving the crystals followed immediately by HPLC. The curves do not extrapolate to 100% GTP at time = 0, probably because of transient heating of the crystals during photolysis. Preliminary experiments suggest that this problem can be avoided by using a frequency-doubled ruby laser (λ = 347 nm).

the resulting structural changes in p21. By using the Laue diffraction method at a synchrotron x-ray source, time resolution within the required range should be easily attainable, as recently demonstrated for glycogen phosphorylase *b* by Hajdu *et al.* (13). These authors also showed that the problem of interpretation of Laue diffraction data can be solved. Thus, in the case of p21, it should be possible to provide a series of snapshots of the protein structure during GTP hydrolysis. By using slowly hydrolyzing mutants, perhaps in combination with the very slowly hydrolyzed but well-recognized GTP analog guanosine 5'-[γ-thio]triphosphate (initially in its caged form), it might be possible to obtain complete data sets at the start of and during the GTPase reaction by conventional crystallographic methods.

We thank M. Isakov and A. Scherer for technical assistance and K. C. Holmes for encouragement. This work was supported in part by the European Community (Contract ST000042).

1. Barbacid, M. (1987) *Annu. Rev. Biochem.* **56,** 779–827.
2. Tucker, J., Sczakiel, G., Feuerstein, J., John, J., Goody, R. S. & Wittinghofer, A. (1986) *EMBO J.* **5,** 1351–1358.
3. John, J., Schlichting, I., Schiltz, E., Rösch, P. & Wittinghofer, A. (1989) *J. Biol. Chem.* **264,** 13086–13092.
4. McCray, J. A., Herbette, L., Kihara, T. & Trentham, D. R. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7237–7241.
5. Hoard, D. E. & Ott, D. G. (1965) *J. Am. Chem. Soc.* **87,** 1785–1788.
6. Scherer, A., John, J., Linke, R., Goody, R. S., Wittinghofer, A., Pai, E. F. & Holmes, K. C. (1989) *J. Mol. Biol.* **206,** 257–259.
7. Hajdu, J., Acharya, K. R., Stuart, D. I., McLaughlin, P. J., Barford, D., Oikonomakos, N. G., Klein, H. & Johnson, L. N. (1987) *EMBO J.* **6,** 539–546.
8. Feuerstein, J., Goody, R. S. & Wittinghofer, A. (1987) *J. Biol. Chem.* **262,** 8455–8458.
9. Goldman, Y. E., Hibberd, M. G. & Trentham, D. R. (1984) *J. Physiol. (London)* **354,** 577–604.
10. Rapp, G., Poole, K. J. V., Maeda, Y., Güth, K., Hendrix, J. & Goody, R. S. (1986) *Biophys. J.* **50,** 993–997.
11. Goody, R. S., Hofmann, W. & Mannherz, H. G. (1977) *Eur. J. Biochem.* **78,** 317–324.
12. Feuerstein, J., Kalbitzer, H. R., John, J., Goody, R. S. & Wittinghofer, A. (1987) *Eur. J. Biochem.* **162,** 49–55.
13. Hajdu, J., Machin, P. A., Campbell, J. W., Greenhough, T. J., Clifton, I. J., Zurek, S., Gover, S., Johnson, L. N. & Elder, M. (1987) *Nature (London)* **329,** 178–181.