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Affinity labeling of GTP-binding proteins in cellular extracts

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GTP-binding proteins in cellular extracts from *Escherichia coli*, *Thermus thermophilus*, yeast, wheat germ or calf thymus were identified using in situ periodate-oxidized [α-^{32}P]GTP as affinity label. Site-specific reaction of individual GTP-binding proteins was achieved by cross-linking the protein-bound 2',3'-dialdehyde derivative of GTP with the single lysine residue of the conserved NKXD sequence through Schiff's base formation and subsequent cyanoborohydride reduction. Labeled GTP-binding proteins from prokaryotic or eukaryotic cell homogenates were separated by polyacrylamide gel electrophoresis and visualized by autoradiography. In addition cross-linking of [α-^{32}P]GTP with GTP-binding proteins was demonstrated in model systems using different purified GTPases, human c-H-*ras* p21, transducin from bovine retina, polypeptide elongation factor Tu (EF-Tu) from *T. thermophilus* and initiation factor 2 (IF2) from *T. thermophilus*. The described affinity labeling technique can serve as an analytical method for the identification of GTPases belonging to the classes of *ras*-proteins, elongation and initiation factors, and heterotrimeric signal transducing G-proteins.

GTP-binding protein; Affinity labeling; Peroxidase oxidation; NKXD; Consensus sequence

1. INTRODUCTION

GTP-binding proteins are members of a superfamily of GTPases whose biological activity is turned on by GTP and off by hydrolysing GTP to GDP [1]. This 'molecular switch' mechanism and the interaction with downstream effectors allows some GTP-binding proteins to play a regulatory role in processes like vesicular transport, membrane signal transfer, protein biosynthesis, visual transduction, and cell proliferation. Since some GTPases are specific targets of single point mutations or bacterial toxins, part of this protein species is of importance in the pathogenesis of cancer and infectious diseases.

Recent progress in the understanding of structure–function relationships in the GTPase superfamily is based on crystal structure analyses [2–4] and increasing amino acid sequence information [5]. For further biochemical investigations, efficient experimental tools are indispensable for rapid screening of GTP-binding proteins in cellular extracts of various organisms.

GTPases are usually identified by nitrocellulose-based nucleotide binding assays or tests monitoring the GTPase activity, signal transduction or modification with bacterial toxins, whereas genes of various GTPases can be identified by searching for consensus sequences. However, a general method for the fast and selective detection of GTPases on the level of protein chemistry is not available. X-ray structural analyses of nucleotide complexes of human c-H-*ras* p21 [4] or *E. coli* elongation factor Tu [2,3] revealed a similar architecture of the nucleotide binding site. In these proteins a conserved sequence motif, asparagine-lysine-X-aspartic acid (NKXD, where X stands for a variable amino acid residue), is typically involved in binding of GDP or GTP. The aliphatic side chain of the invariant lysine participates in hydrophobic interactions with the purine moiety of the nucleobase, whereas the ε-amino group of lysine is located in the vicinity of the ribose ring.

Recently we demonstrated, that in the case of *T. thermophilus* EF-Tu the ε-amino group of this conserved lysine^{137} residue reacts selectively with the aldehyde groups of periodate oxidized GDP or GTP [6]. To achieve a reaction of high specificity for the nucleotide binding site of EF-Tu it was essential to perform this labeling technique under kinetically controlled conditions omitting excess of oxidised nucleotide. In situ periodate oxidation of the EF-Tu·nucleotide complex followed by immediate reductive stabilization of the formed Schiff's base was therefore used. The same affinity labeling method was further applied to other GTPases, namely c-H-*ras* p21, bovine retina transducin and IF2 from *T. thermophilus*. The high selectivity by which these proteins could be labeled in their nucleotide binding site prompted us to explore the technique of periodate oxidation as a general tool for the identification of GTP-binding proteins containing NKXD consensus sequences. We report here the covalent modification of GTPases occurring in crude cellular extracts

Abbreviations: HEPES, (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]); Tricine, (*N*-tris[hydroxymethyl]-methylglycine; SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

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from *Escherichia coli*, *Thermus thermophilus*, yeast, wheat germ and calf thymus.

2. EXPERIMENTAL

2.1. *Materials and methods*

[α-^{32}P]GTP and [α-^{32}P]CTP (both 3,000 Ci/mmol) were from Amersham-Buchler (Braunschweig, Germany). $NaIO_4$, $NaCNBH_3$ and $NaBH_4$ were products from Serva (Heidelberg, Germany), acrylamide and *N,N'*-methylenebis(acrylamide) was obtained from BRL (Eggenstein, Germany). HEPES and Tricine were purchased from Sigma (Deisenhofen, Germany), all other salts, solvents and buffer substances (Merck, Darmstadt, Germany) were of highest purity available.

E. coli (strain DH5α) and *T. thermophilus* (strain HB8) bacteria were used. Commercial baker's yeast was obtained from Giegold (Schwarzenbach/Saale, Germany). Fresh calf thymus was purchased from the local slaughter house, untreated wheat germ was a gift of Grandel Keim-Diät (Augsburg, Germany). *T. thermophilus* EF-Tu·GDP was purified to homogeneity as described [7]. Purified heterotrimeric bovine retina transducin [8] was a gift of Dr. Ho, Chicago. c-H-*ras* p21 was isolated from an overproducing strain of *E. coli* [9]. Partially purified *T. thermophilus* initiation factor 2 was prepared essentially as described for *E. coli* IF2 using a ribosomal wash fraction, anion-exchange- and gel permeation-chromatography [10,11].

Protein concentration was determined using the commercial Bio-Rad Microassay (Bio-Rad, München, Germany). Autoradiography of the dried polyacrylamide gels was done with Kodak X-OMAT AR X-ray films.

2.2. *S-100 supernatants*

S-100 supernatants from different sources were isolated as follows.

E. coli and *T. thermophilus*: (a) cell disruption of microorganisms after lysozyme and DNase I treatment by nitrogen decompression, (b) centrifugation at 100,000 × *g*, and (c) 12 h dialysis of the supernatant at 4°C against 50 mM HEPES, 10 mM KCl, 10 mM $MgCl_2$, pH 7.6 (buffer A) [7].

Yeast: (a) mechanical disintegration of yeast cells with a glass bead mill, (b) centrifugation at 100,000 × *g*, and (c) dialysis against buffer A as described above [12].

Calf thymus: (a) homogenization of calf thymus tissue with a Potter-Elvehjem homogenizer and filtration through cheese cloth, (b) centrifugation at 100,000 × *g*, and (c) dialysis against buffer A as described [13].

Wheat germ: (a) mechanical disruption of wheat germ was performed with sea sand in a mortar [14], (b) centrifugation at 100,000 × *g*, and (c) dialysis as described above.

2.3. *Affinity labeling with in situ periodate-oxidized [α-^{32}P]GTP*

50 μl of extensively dialyzed S-100 supernatant or 50 μl of highly purified *T. thermophilus* EF-Tu·GDP, c-H-*ras* p21, bovine transducin or *T. thermophilus* IF2, which were adjusted to a protein concentration of 2 mg/ml in buffer A, were incubated with 5 μCi [α-^{32}P]GTP (or [α-^{32}P]CTP as a control) for 10 min at 37°C. In situ oxidation of protein-bound ^{32}P-labeled nucleotides was initiated by addition of 4 mM $NaIO_4$ and incubation for 1 min at 37°C, followed by reduction with 80 mM $NaCNBH_3$ for 1 min at 37°C and final treatment with 100 mM $NaBH_4$ for 1 h at 4°C. As a control, the S-100 supernatant or *T. thermophilus* EF-Tu·GDP were incubated with either [α-^{32}P]GTP or [α-^{32}P]CTP in the absence of $NaIO_4$, $NaCNBH_3$ and $NaBH_4$. Appropriate aliquots were withdrawn from the individual assays and analysed by SDS-PAGE [15].

3. RESULTS AND DISCUSSION

Covalent modification of the lysine residue in the conserved NKXD sequence of GTP-binding proteins was achieved by Schiff's base formation with periodate-oxidized GTP. The labile azomethine bond was stabilized by reduction of the corresponding *N*-alkyl-lysine (Fig. 1).

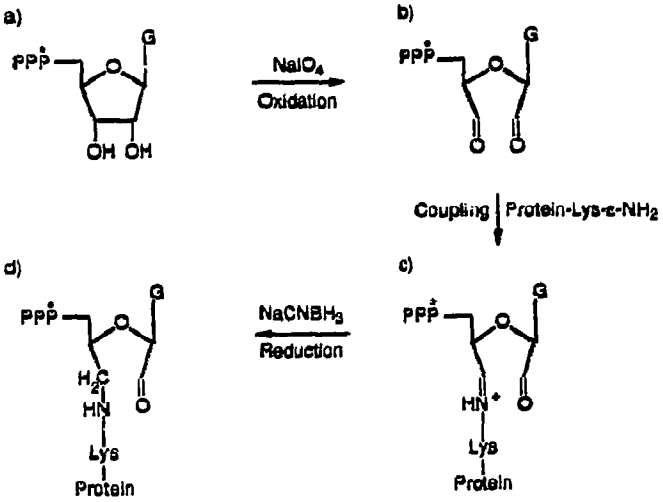

Fig. 1. Reaction scheme of cross-linking [α-^{32}P]GTP to the invariant NKXD sequence of GTP-binding proteins. (a) Protein-bound [α-^{32}P]GTP before and (b) after oxidation with sodium periodate; (c) initial azomethine reaction product after cross-linking to the ε-amino group of an active site lysine side-chain; (d) stabilization of the intermediate Schiff's base with sodium cyanoborohydride by conversion to the corresponding secondary amine.

For analytical purposes, the nucleotide binding site of GTPase first had to be occupied by a radioactive nucleotide. To achieve this after cell breakage and ultracentrifugation, the resulting homogenates were extensively dialyzed. The exclusion of free ribonucleotides, especially GTP, from the supernatant is indispensable for the yield and specificity of affinity labeling, since cytosolic free GTP will complete for exogenous [α-^{32}P]GTP in the coupling reaction (Fig. 1). Furthermore, the specificity of cross-linking is sensitive to the ratio of total [α-^{32}P]GTP to the overall amount of GTP binding sites. In the case of high [α-^{32}P]GTP, excess unbound nucleotide would be oxidized by periodate and increase the unspecific reaction with lysine residues located in areas of the protein other than the GTP binding site. In general, a stoichiometry of [α-^{32}P]GTP to GTP-binding proteins of less than one is recommended in order to suppress unspecific reactions. The use of freshly prepared S-100 supernatants is important, since the effective exchange of GDP for [α-^{32}P]GTP is dependent on the presence of exchange factors, e.g. elongation factor Ts in the case of prokaryotic EF-Tu.

It is essential to perform affinity labeling under kinetically controlled conditions. As evident from the three-dimensional structures of *E. coli* EF-Tu and c-H-*ras* p21 the *cis*-diol function of the bound nucleotide is exposed to the solvent and therefore accessible for modifying chemical reagents [2–4]. A brief in situ treatment of protein-bound [α-^{32}P]GTP with $NaIO_4$ for 1 min at

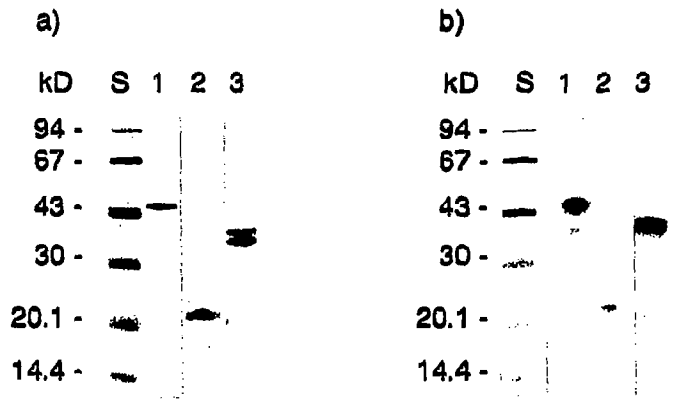

Fig. 2 Electrophoretic analyses of homogeneous GTP-binding proteins after cross-linking with [α-^{32}P]GTP. (a) Coomassie-stained SDS-PAGE of *T. thermophilus* EF-Tu (lane 1), c-H-*ras* p21 (lane 2) and transducin (lane 3, α- and β-subunit; the 8 kDa γ-subunit was eluted from the gel during the staining/destaining process). (b) Autoradiography of SDS-PAGE of *T. thermophilus* EF-Tu (lane 1), c-H-*ras* p21 (lane 2) and transducin (lane 3, only the α-subunit is labeled). Lanes S in Fig. 2a,b represent a mixture of standard proteins of molecular weight 94 kDa (phosphorylase b), 67 kDa (bovine serum albumin), 43 kDa (chicken egg white ovalbumin), 30 kDa (beef erythrocytes carboanhydrase), 20.1 kDa (soy bean trypsin inhibitor), and 14.4 kDa (cow milk α-lactalbumin).

37°C was in general sufficient for complete oxidation to the corresponding dialdehyde, [α-^{32}P]GTP$_{oxi}$. It is reported for *E. coli* and *B. stearothermophilus* EF-Tu that oxidized GDP has a much lower affinity to the GTPase as compared to the native substrate GDP [16]. As a consequence, dissociation of [α-^{32}P]GTP$_{oxi}$ from the active site will increase the probability of unspecific reactions with accessible lysine residues on the outer surface of the protein. Therefore, appropriate conditions for $NaCNBH_3$ reduction of the labile Schiff's base (1 min at 37°C with large excess of reagent) were chosen to stabilize the covalent intermediate. Short reaction times and pH values close to 7 are also advantageous to prevent β-elimination of the radioactively labeled phosphate residue of [α-^{32}P]GTP$_{oxi}$ [17]. In summary, a short in situ oxidation of the protein-bound nucleotide followed by fast stabilization of the Schiff's base will decrease the overall yield of cross-linking but contribute to increased specificity of labeling.

[α-^{32}P]GTP instead of [γ-^{32}P]GTP was used for labeling in order to prevent dephosphorylation of the nucleotide by cellular phosphatases or by the intrinsic GTPase activities of the GTP-binding proteins.

Affinity labeling of the isolated GTP-binding proteins EF-Tu·GDP from *T. thermophilus*, c-H-*ras* p21 and bovine transducin was performed as described in Section 2. Covalently bound radioactivity was detected by SDS-PAGE and autoradiography. As can be seen in Fig. 2 all of these proteins could be cross-linked by in situ oxidized [α-^{32}P]GTP. In the case of heterotrimeric bovine transducin only the GTP-binding α-subunit was specifically labeled. The small γ-subunit (about 8 kDa) could not be detected because it was eluted during the staining and destaining procedure (Fig. 2a and 2b, lane 3).

The results obtained from affinity labeling of GTPases in postribosomal supernatants from *T. thermophilus*, *E. coli*, yeast, wheat germ and calf thymus are depicted in Fig. 3. After reaction, the protein mixtures were separated by SDS-PAGE and stained by Coomassie blue (Fig. 3a). The protein bands carrying covalently bound ^{32}P radioactivity were identified by autoradiography (Fig. 3b,c). As internal standard homogeneous *T. thermophilus* EF-Tu·GDP was pretreated and labeled identically to the other samples (Fig. 3a,b, lane 1,2).

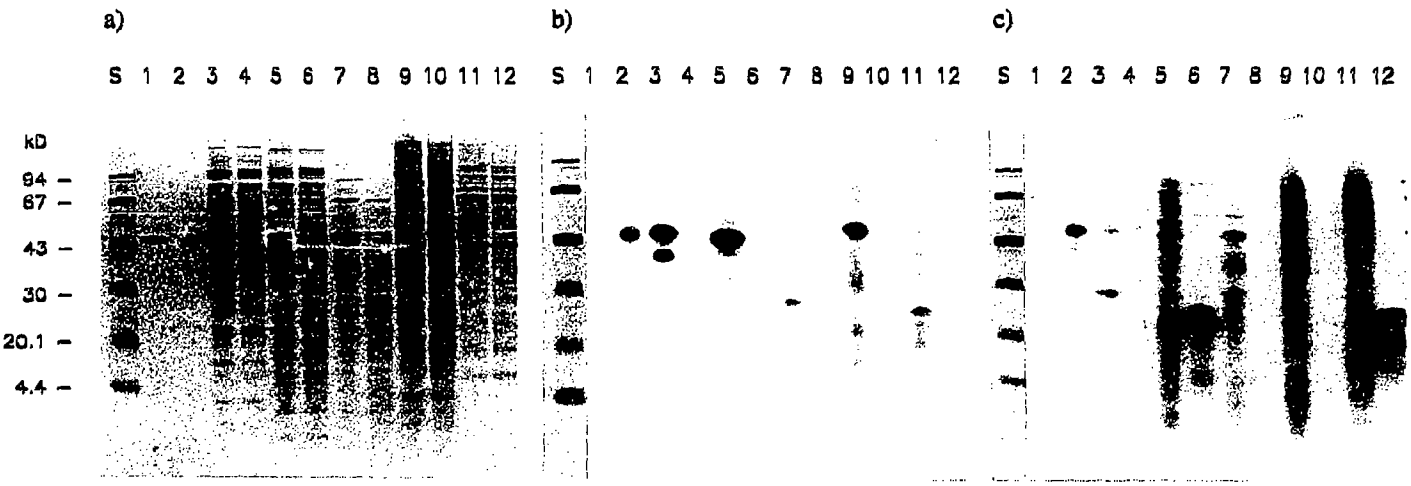

Fig. 3. Electrophoretic analyses of different cell extracts incubated with [α-^{32}P]GTP or [α-^{32}P]CTP. (a) Coomassie-stained SDS-PAGE of S-100 supernatants. 3 μg of *T. thermophilus* EF-Tu·GDP, 30 μg S-100 supernatants from *T. thermophilus*, *E. coli*, yeast, wheat germ and calf thymus incubated with [α-^{32}P]GTP (data with [α-^{32}P]CTP not shown) treated without (lanes 1,4,6,8,10 and 12) and with (lanes 2,3,5,7,9 and 11) $NaIO_4$, $NaCNBH_3$ and $NaBH_4$. (b) Autoradiography of SDS-PAGE of S-100 supernatants incubated with [α-^{32}P]GTP and (c) with [α-^{32}P]CTP. Lanes S are standard proteins as described in Fig. 2.

Efficient labeling of EF-Tu·GDP or S-100 supernatants with [α-^{32}P]GTP could not be achieved unless the initial Schiff's base intermediates were converted to stable secondary amines by cyanoborohydride reduction (Fig. 3b, lanes 2,3,5,7,9 and 11). No incorporation of ^{32}P radioactivity into authentic *T. thermophilus* EF-Tu·GDP or into S-100 supernatant GTP-binding proteins was observed when these proteins were treated under identical conditions but without periodate oxidation and cyanoborohydride reduction (Fig. 3b, lanes 1,4,6,8,10 and 12).

Although an identification of individual cross-linked proteins was not performed it is evident from Fig. 3b that proteins with a molecular weight of about 40–70 kDa were preferentially labeled. This size range would correspond to one of the most abundant cellular proteins, the polypeptide elongation factors (EF-Tu, of 45 kDa in prokaryotes, EF1α of about 50 kDa in eukaryotes). The absence of this 50 kDa band in the yeast S-100 extract could be due to its instability in buffers without glycerol [18]. The second prominent band which is radioactively labeled in all samples appeared in the molecular weight range of between 20 kDa and 30 kDa. Here the GTPases of the *ras*-family are expected [19].

Identification of the labeled amino acid residues was performed in the case of *T. thermophilus* EF-Tu cross-linked with [α-^{32}P]GTP, where as a major site of modification, the lysine-137 residue which is part of the conserved NKXD sequence, was identified [6]. A similar result was obtained by analysis of the labeled amino acid of c-H-*ras* p21 cross-linked with [α-^{32}P]GTP. Again the invariant lysine-117 residue of the conserved NKXD sequence motif was identified as a predominant reaction target [20] (data not shown).

The pronounced specificity for labeling isolated GTP-binding protein or S-100 supernatants with substoichiometric amounts of oxidized [α-^{32}P]GTP is in contrast to unspecific labeling of the same proteins with in situ oxidized [α-^{32}P]CTP, Fig. 3c. Under these reaction conditions the cross linking obviously does not occur in the putative CTP-binding site, but at accessible lysine residues on the outer surface of polypeptides. The labeled bands are distributed over a much broader range of molecular weights (100–10 kDa) (Fig. 3c). A control which was done with [α-^{32}P]CTP but without oxidation/ reduction showed intensive labeling of only a few bands (Fig. 3c, lanes 6 and 12). This is most probably the results of CMP incorporation into bulk tRNA by ATP(CTP)tRNA nucleotidyl transferase [21].

From Fig. 3c, lane 2 it is evident that isolated *T. thermophilus* EF-Tu·GDP can to some extent be labeled with [α-^{32}P]CTP by oxidation/reduction. From earlier experiments, however, it is known that pyrimidine nucleotides, e.g. UTP or CTP, do not compete with GDP and GTP for binding to EF-Tu and are as such no substrates for the elongation factor. The observed degree of labeling is therefore interpreted in terms of an unspecific side reaction of [α-^{32}P]CTP with lysine residues at the periphery of EF-Tu.

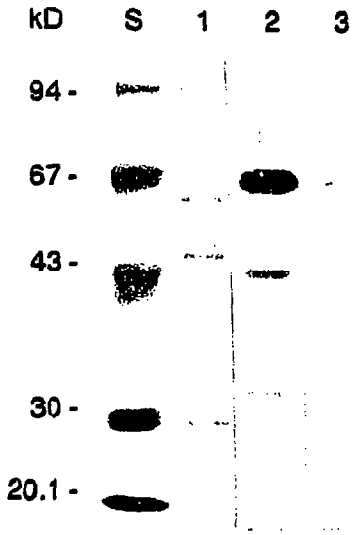

Fig. 4. Electrophoretic analyses of partially purified *T. thermophilus* IF2 after cross-linking with [α-^{32}P]GTP. Coomassie-stained SDS-PAGE (lane 1), autoradiography of SDS-PAGE (lane 2) and Western blot detection with anti-IF2 antibodies from *Bacillus stearothermophilus* (lane 3). Lane S is a mixture of standard proteins as given in Fig. 2.

Finally, another typical GTP-binding protein, the *T. thermophilus* initiation factor 2 was tested as a candidate for affinity labeling. A partially purified IF2 preparation (Fig. 4, lane 1) was treated with [α-^{32}P]GTP, oxidized and reduced and separated by SDS-PAGE. Autoradiographical analysis demonstrates intensive ^{32}P incorporation into a protein of about 67 kDa which corresponds to the molecular weight of wild-type *T. thermophilus* IF2 (Fig. 4, lane 2) as determined by SDS-PAGE. This finding was confirmed by a Western blot using anti-IF2 antibodies from *B. stearothermophilus* which revealed a positive signal of antibody binding at a molecular weight of 67 kDa and thus obviously coincides with the most intense band of ^{32}P incorporation (Fig. 4, lane 3).

It is shown in this study, that individual GTPases from crude cellular homogenates can be specifically labeled by oxidized [α-^{32}P]GTP as a cross-linking reagent applying an in situ periodate oxidation and reduction.

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