Shi, Y-B., Gamper, H., & Hearst, J. E. (1987) *Nucleic Acids Res. 15*, 6843-6854.
Siegfried, J. M., Sartorelli, A. C., & Tritton, T. R. (1983) *Cancer Biochem. Biophys. 6*, 137-142.
Sobell, H. M. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 5328-5331.
Staden, R. (1978) *Nucleic Acids Res. 5*, 1013-1015.
Straney, D. C., & Crothers, D. M. (1987) *Biochemistry 26*, 1987-1995.
Tinoco, I., Bover, P. N., Dengler, B., Levine, M. D., Uhlenback, O. C., Crothers, D. M., & Gralla, J. (1973) *Nature (London) New Biol. 246*, 40-41.
Valentini, L., Nicolella, V., Vannini, E., Menozzi, M., Penco, S., & Arcamone, F. (1985) *Farmaco, Ed. Sci. 40*, 376-390.
Van Dyke, M. W. (1984) Ph.D. Thesis, California Institute of Technology.
Van Dyke, M. W., Hertzberg, R. P., & Dervan, P. B. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 5470-5475.
von Hippel, P. H., Bear, D. G., Morgan, W. D., & McSwiggen, J. A. (1984) *Annu. Rev. Biochem. 53*, 389-446.
Wakelin, L. (1986) *Med. Res. Rev. 6*, 275-340.
Wakelin, L. P. G., & Waring, M. J. (1980) *J. Mol. Biol. 144*, 183-214.
Wakelin, L. P. G., Atwell, G. J., Rewcastle, G. W., & Denny, W. A. (1987) *J. Med. Chem. 30*, 855-861.
Waring, M. J., & Fox, K. R. (1983) in *Molecular Aspects of Anticancer Drug Action* (Neidle, S., & Waring, M. J., Eds.) pp 127-156, Macmillan, London.
Wilson, D. W., Grier, D., Reimer, R., Bauman, J. D., Preston, J. F., & Gabbay, E. J. (1976) *J. Med. Chem. 19*, 381-384.
Wilson, W. D., & Jones, R. L. (1981) *Adv. Pharmacol. Chemother. 18*, 177-222.

Affinity Labeling of the GDP/GTP Binding Site in *Thermus thermophilus* Elongation Factor Tu[†,‡]

Marcus E. Peter,[§] Brigitte Wittmann-Liebold,[∥] and Mathias Sprinzl*[,§]

Laboratorium für Biochemie, Universität Bayreuth, Postfach 10 12 51, D-8580 Bayreuth, FRG, and Max-Planck-Institut für molekulare Genetik, Ihnestrasse 8, D-1000 Berlin-Dahlem, FRG

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ABSTRACT: Elongation factor Tu from *Thermus thermophilus* was treated successively with periodate-oxidized GDP or GTP and cyanoborohydride. Covalently modified cyanogen bromide or trypsin fragments of the protein were isolated, and the position of their modification was determined. Lysine residues 52 and 137 were heavily labeled, lysine-137 being considerably more reactive in the GTP form as compared to the GDP form of the protein. These residues are in the proximity of the GDP/GTP binding site. Lys-325 was also labeled, but to a lower extent. The part of the EF-Tu containing residue 52 is missing in crystallized EF-Tu·GDP from *Escherichia coli* [Jurnak, F. (1985) *Science (Washington, D.C.) 230*, 32-36]. These results place the part of *T. thermophilus* EF-Tu corresponding to the missing fragment in *E. coli* EF-Tu in the vicinity of the nucleotide binding site and allow its role in the interaction with aminoacyl-tRNA and elongation factor Ts to be evaluated. Cross-linking of EF-Tu·GDP by irradiation at 257 nm showed that a sequence of 10 amino acids residues which is found in the *Thermus thermophilus* elongation factor Tu but not in other homologous bacterial proteins is located in the vicinity of the GDP/GTP binding site.

Elongation factor Tu (EF-Tu)[1] is a guanosine nucleotide binding protein which acts as a mediator of a new protein elongation cycle during protein biosynthesis (Miller & Weissbach, 1977). It interacts with GDP, GTP, elongation factor Ts, aminoacyl-tRNA, and ribosomes. The functional cycle of EF-Tu resembles that of other GTP binding proteins including transducin, the G-proteins in hormone receptor systems, and the poorly understood c-H-ras oncogene protein p21 (Gilman, 1987; Stryer & Bourne, 1986). Sequences of all these GTP binding proteins contain conserved sequences and domains pointing to similarities in their tertiary structures (Halliday, 1984; Gilman, 1987; Stryer & Bourne, 1986). For this reason, the elongation factor Tu from *Escherichia coli*, a GTP binding protein with a partially solved tertiary structure (Jurnak, 1985; la Cour et al., 1985), has become a model for the study of structure–function relationships of GTP binding proteins. This approach has, however, several disadvantages: First, the elongation factor Tu from *E. coli* is an unstable protein. It is susceptible to rapid thermal denaturation and is sensitive toward proteolytic cleavage. Second, this protein cannot be prepared in nucleotide-free form without rapid loss of its activity (Ohta et al., 1977) which seriously hampers structural and biochemical investigation. Third, although the high-resolution three-dimensional structure has been solved for the G binding domain of *E. coli* EF-Tu (Jurnak, 1985; la Cour et al., 1985), this was achieved by using a partially degraded protein which lacked a fragment of 14 amino acids (Jurnak, 1985).

To circumvent some of these problems, we have investigated the elongation factor Tu from the extreme thermophilic bacterium *Thermus thermophilus* (Degryse et al., 1978). This GTP binding protein is considerably more stable than EF-Tu from *E. coli* (Nakamura et al., 1978; Arai et al., 1978) and can be prepared in nucleotide-free form. These features fa-

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* To whom correspondence should be addressed.
[§] Universität Bayreuth.
[∥] Max-Planck-Institut für molekulare Genetik.

[1] Abbreviations: EF-Tu and EF-Ts, elongation factors Tu and Ts, respectively; aa-tRNA, aminoacyl transfer RNA; HPLC, high-performance liquid chromatography; PTH, phenylthiohydantoin.

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cilitate the investigation of its biochemical properties. Recently, the sequence of *T. thermophilus* EF-Tu has been determined in two laboratories (Seidler et al., 1987; Kushiro et al., 1987), revealing 70% identical amino acids to EF-Tu from *E. coli*, plus an additional sequence of 10 amino acids which are not present in EF-Tu from other bacteria.

In the present work, we investigated the structures of EF-Tu·GDP and EF-Tu·GTP from *Thermus thermophilus* by mapping the nucleotide binding site with affinity labeling techniques. The results show that the "missing" oligopeptide in the three-dimensional structure of *E. coli* EF-Tu (Jurnak, 1985) is localized in the protein GDP/GTP binding site. The sequence of 10 additional amino acids, which is present in *T. thermophilus* EF-Tu but not in other native EF-Tu proteins, is also located close to the guanosine moiety. Together with the recently published work on the three-dimensional structure of the c-H-ras oncogene protein p21 (de Vos et al., 1988), these results indicate a high structural and functional homology of the GTP binding proteins.

Materials and Methods

[^{3}H]CDP (17.1 Ci/mmol), [^{3}H]GDP (11.3 Ci/mmol), and [U-^{14}C]GTP, (575 mCi/mmol) were purchased from Amersham–Buchler (Braunschweig, FRG). [^{32}P]Orthophosphate (9000 Ci/mmol) was obtained from Du Pont/New England Nuclear (Bad Nauheim, FRG). [γ-^{32}P]ATP and [β-^{32}P]GDP (1000 Ci/mmol each) were prepared as described elsewhere (Johnson & Walseth, 1979). $NaIO_4$, $NaCNBH_3$, and $NaBH_4$ were obtained from Serva (Heidelberg, FRG); acrylamide and *N,N'*-methylenebis(acrylamide) were from BRL (Eggenstein, FRG). TPCK-treated trypsin was from Worthington (Freehold, NJ). EF-Tu·GDP was purified from *T. thermophilus* cells, strain HB8, as described by Leberman et al. (1980). Nucleotide-free EF-Tu was prepared as previously described (Seidler et al., 1987). The protein had a GDP binding activity of 21 000 units/mg (Arai et al., 1972).

Affinity Labeling with Periodate-Oxidized Nucleotides. Five hundred microliter of 20 μM nucleotide-free EF-Tu, 10 mM $MgCl_2$, 50 mM KCl, and 50 mM sodium borate, pH 7.5, was added to 5 nmol of lyophilized [U-^{14}C]GTP. To label EF-Tu with [U-^{14}C]GDP_{oxi}, the reaction mixture (EF-Tu·[U-^{14}C]GTP) was incubated for 2 h at 37 °C to convert GTP to GDP. Hydrolysis of GTP was followed by thin-layer chromatogaphy on PEI–cellulose sheets with 0.8 M LiCl/1 M formic acid as solvent. For labeling of EF-Tu with CDP_{oxi}, 10 μM [^{3}H]CDP (17.1 Ci/mmol) [for labeling with ATP_{oxi}, 10 μM [γ-^{32}P]ATP (50 Ci/mmol)] was added to 20 μM EF-Tu·GDP in the above buffer. In all cases, oxidation was performed in situ with 1 mM $NaIO_4$ for 1 min at 37 °C. The reduction with 20 mM $NaCNBH_3$ was done at 37 °C as described in the legend to Figure 1. The reaction was stopped by addition of 25 mM $NaBH_4$. After 10 min at room temperature, 500 μL of 10% aqueous formic acid was added, and the mixture was desalted on a NAP-10 column (Pharmacia, Uppsala, Sweden) equilibrated with 5% formic acid and lyophilized.

Photoaffinity labeling and peptide analysis on SDS–urea–polyacrylamide gels were done as described previously (Seidler et al., 1987). Nucleotides which were not covalently bound were removed from chemically labeled EF-Tu by a gel permeation chromatography step on Superose 12 HR 10/30 (Pharmacia) using 70% aqueous formic acid and a flow rate of 0.4 mL/min. The polypeptide was cleaved with CNBr as described (Seidler et al., 1987), and the CB fragments were separated on the same column under identical conditions. The fractions containing the fragments CB1 and CB2 were pooled and evaporated to dryness. The material was dissolved in 100 μL of 100 mM *N*-methylmorpholine acetate, pH 8.1, digested with 100 μg/mL TPCK-treated trypsin for 4 h at 37 °C, and lyophilized.

The labeled oligopeptides were purified by reverse-phase HPLC using a Vydac C_4/300 Å (the Vydac material was from "The Separation Group", Hisperia) or a LiChrospher 100 RP-18 5-μm column (Merck, Darmstadt, FRG) (4.6 × 250 mm).

Microsequence Analysis. A solid-phase sequencer (LKB Model 4020) was used for automated Edman degradation of pooled and dried radioactive peaks. Cyanogen bromide fragments were coupled directly by their terminal homoserine lactone residue (Laursen, 1977). Tryptic peptides were coupled by using the carbodiimide coupling procedure as described elsewhere (Salnikow et al., 1981). In this case only, the amount of radioactivity released at each step was measured. For sequence determinations, automated Edman degradation was carried out on a gas-phase sequencer (Applied Biosystems Model 477A). PTH-amino acids were identified by reverse-phase HPLC on an Applied Biosystems PTH C_{18} 5-μm column (2.1 × 220 mm) using an on-line amino acid analyzer (Applied Biosystems Model 120A).

Results

Affinity Labeling of EF-Tu. Treatment of EF-Tu·GDP or EF-Tu·GTP with sodium periodate results in in situ oxidative cleavage of the 2',3'-carbon bond of the protein-bound nucleotide leading to formation of two aldehyde groups. These groups can react with nearby ϵ-amino groups of EF-Tu lysine residues by Schiff base formation (Lowe & Beechey, 1982). The Schiff base can be stabilized by reduction with sodium cyanoborohydride which preferentially reduces iminium systems at neutral pH and reacts only slowly with aldehydes (Borch et al., 1971). Since GTP_{oxi} and GDP_{oxi} are true analogues of GTP and GDP in bacterial protein biosynthesis (Hamel, 1975), they are suitable for specific affinity labeling of EF-Tu.

Specificity of affinity labeling by this approach strongly depended on the conditions used. This was demonstrated by labeling of EF-Tu from *Thermus thermophilus* with radioactive periodate-oxidized GDP (GDP_{oxi}) and electrophoretic analysis of the peptides formed by cyanogen bromide (CB) cleavage. Table I lists the CB fragments and their properties (Seidler et al., 1987). Separation of the CB fragments originating from the affinity-labeled *T. thermophilus* EF-Tu is shown in Figure 1. Six peptides were visible after Coomassie blue staining of the 12.5% SDS–urea–polyacrylamide gel (lane 1), belonging to the fragments CB1–CB7 (CB5 and CB6 are comigrating) as established by sequence analysis (Seidler et al., 1987). Smaller peptides (CB8–CB12; Table I) were eluted from the gel under these conditions. Lanes 2–4 in Figure 1 represent the autoradiograms of CB peptides of EF-Tu after treating them with guanosine nucleotides under different conditions. Lanes 2 and 3 belong to peptides originating from EF-Tu·GDP and EF-Tu·GTP, respectively. In both cases, the labeling of CB1 (major intensity) and CB2 (minor intensity) was detectable. When the reduction was carried out over a longer time (lane 4), there was a higher yield of labeled EF-Tu, but the specificity of the reaction was reduced. CB2 was labeled to a greater extent, and additional radioactive peptides appeared in addition to CB1 and CB2. CDP and ATP cannot replace GDP in the nucleotide binding site of EF-Tu under the conditions used (Arai et al., 1978). CDP_{oxi} and ATP_{oxi} were therefore used as controls to test the specificity of EF-Tu affinity labeling with oxidized guanine nucleotides. Re-

Table I: Peptides Obtained by Cleavage of *T. thermophilus* EF-Tu with Cyanogen Bromide[a]

peptide	mol wt	no. of amino acid residues	no. of lysines	position in sequence
CB1	10102	92	7	1–92
CB2	9807	89	5	273–361
CB3	5220	49	2	224–272
CB4	4988	41	3	183–223
CB5	4434	42	2	364–405
CB6	3399	30		153–182
CB7	2564	22		114–135
CB8	1386	14		100–113
CB9	1314	12		141–152
CB10	690	7		93–99
CB11	605	5	1	136–140
CB12	248	2		326–363

[a] According to Seidler et al. (1987). The lower part of the table contains the oligopeptides which could not be identified by SDS–urea–PAGE (see text).

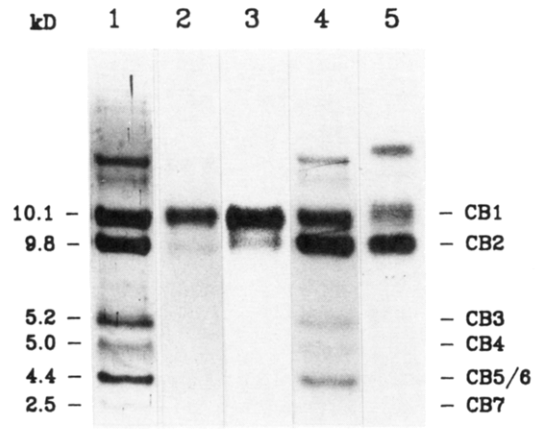

FIGURE 1: Electrophoresis of cyanogen bromide fragments obtained from *T. thermophilus* elongation factor Tu on 12.5% SDS–urea–polyacrylamide gels. Lane 1, Coomassie blue stained CB fragments of the native protein. Lanes 2–5, autoradiography of the cyanogen bromide fragments from EF-Tu labeled with [U-^{14}C]GTP_{oxi} (reduction time 1 min) (lane 2), [U-^{14}C]GDP_{oxi} (reduction time 1 min) (lane 3), [U-^{14}C]GDP_{oxi} (reduction time 60 min) (lane 4), and [^{3}H]CDP_{oxi} (reduction time 1 min) (lane 5).

placement of GDP_{oxi} for CDP_{oxi} in the reaction mixture led, as expected, to increased nonspecific reaction especially in the region of EF-Tu corresponding to CB2 (lane 5). The slow-migrating radioactive bands in lanes 4 and 5 correspond to partially cleaved protein.

The radioactivity of fragments CB1 and CB2 was in the ratio of about 4:1 if the labeling was performed with GTP_{oxi} or GDP_{oxi} (in situ). CDP_{oxi} labeled these fragments in a ratio 1:2 (Figure 1, lanes 2, 3, and 5). Thus, GDP_{oxi} and GTP_{oxi} show an 8 times higher preference for CB1 labeling as compared to CDP_{oxi}. This shows that there is a certain degree of unspecific reaction of CDP_{oxi} with CB1 which could be due to a modification including Lys-52.

CB1 and CB2 contain seven and five lysine residues, respectively. The reactivity of these residues in *E. coli* EF-Tu toward lysine-specific reagents was studied by Antonsson and Leberman (1984). The most reactive lysines were in a region which corresponds to fragment CB2 in *T. thermophilus* EF-Tu. This agrees with the observed yield of unspecific reaction with CDP_{oxi} (lane 5) where CB2 is considerably more reactive than CB1. With the specific reagent GDP_{oxi}, however, the yield of CB1 modification is higher than that of fragment CB2.

The affinity of GDP_{oxi} for EF-Tu from *E. coli* is 58 times (for EF-Tu from *Bacillus stearothermophilus*, 50 times) lower than GDP (Wittinghofer et al., 1977). In order to eliminate

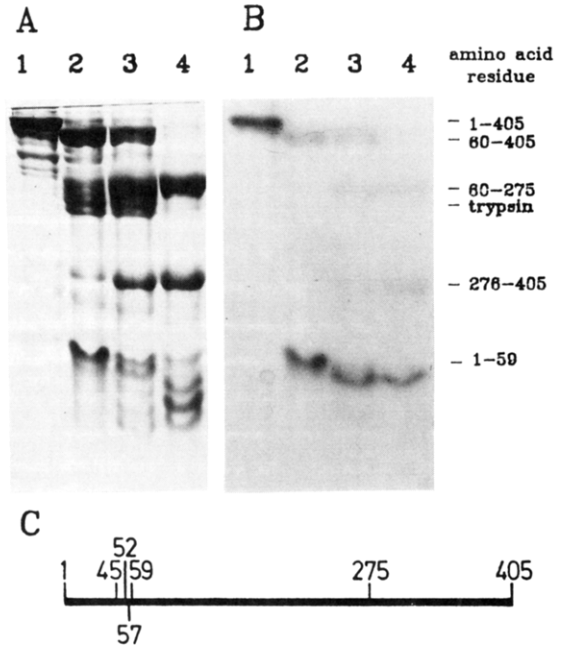

FIGURE 2: SDS–urea–PAGE of [β-^{32}P]GDP_{oxi}-labeled EF-Tu from *T. thermophilus*. Coomassie blue staining (A) and autoradiography (B). Lane 1, electrophoresis of the labeled polypeptide; lanes 2, 3, and 4, electrophoresis of the labeled polypeptide after 10-s, 1-min, and 2-h treatment with trypsin, respectively. 4 nmol of EF-Tu was labeled 1 min with [β-^{32}P]GDP_{oxi} as described under Materials and Methods. After treatment with $NaBH_4$, 100 μg of trypsin was added. Digestion was performed at 37 °C. Reactions were stopped by adding the same volume of formic acid, and the mixtures were lyophilized. (C) Schematic representation of tryptic cleavage sites on the polypeptide chain of *T. thermophilus* EF-Tu.

the possibility of GDP_{oxi} dissociation from the binding site, which could lead to unspecific labeling (Figures 1, lane 4), we performed the reaction under conditions (total reaction time of 2 min) where the possibility of GDP_{oxi} dissociation from EF-Tu is reduced. The yield of modification of lysine-52 was considerably increased when it occurs from in situ oxidized GDP_{oxi} located in the binding site of the protein as compared to a free oxidized nucleotide, GDP_{oxi} or CDP_{oxi}.

For further analytical work on affinity labeling, conditions leading to specific reaction (in situ oxidation and short reaction times; Figure 1, lanes 2 and 3) were employed. Under these conditions, the yield of labeling was about 0.1 nmol of covalently bound nucleotide per nanomole of EF-Tu.

Limited tryptic hydrolysis (Figure 2) demonstrates the specificity of EF-Tu affinity labeling with GDP_{oxi}. The tryptic cleavage sites of *T. thermophilus* EF-Tu are very similar to those of *E. coli* EF-Tu (Wittinghofer et al., 1980). Trypsin cleaves the native polypeptide chain initially behind residue 59, producing fragments 1–59 and 60–405 (lane 2). Autoradiography of lane 1 and lane 2 (Figure 2B) demonstrates the strong preferential labeling of the N-terminal 1–59 polypeptide. Further digestion of this fragment by shortening from the C-terminus leads to the peptide 1–57 (lanes 3 and 4). No cleavage occurs at the next position, Lys-52, since this residue is modified by GDP_{oxi} (see below). None of the fragments with a length shorter than 1–57 residues which are visible by Coomassie blue staining are modified (Figure 2A, lanes 3, and 4).

Site of Affinity Labeling with GDP_{oxi} and GTP_{oxi}. Since small CB peptides (CB8–CB12; lower part of Table I) could not be detected by PAGE, they were isolated by gel permeation chromatography (GPC) (Figure 3). From GPC of CB fragments obtained from affinity-labeled EF-Tu·GTP, two

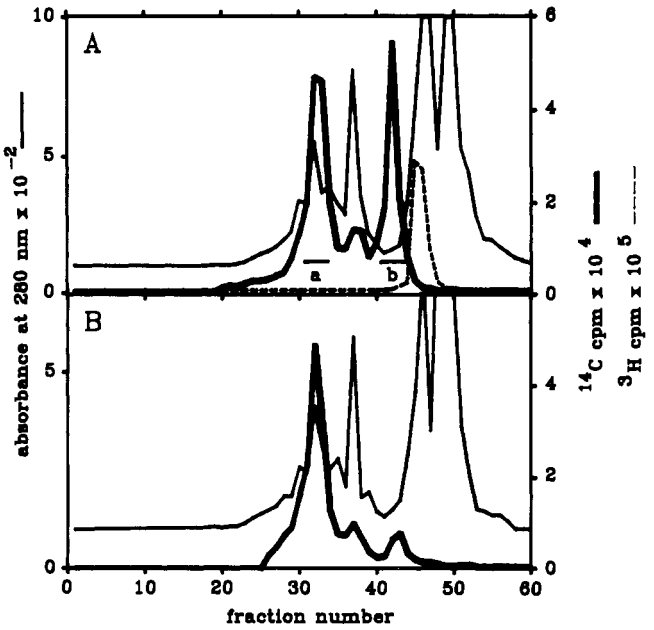

FIGURE 3: Gel permeation chromatography of cyanogen bromide fragments obtained by cleavage of EF-Tu labeled with [U-^{14}C]GTP$_{oxi}$ (A) and [U-^{14}C]GDP$_{oxi}$ (B). Samples (10 nmol of each) of labeled polypeptide [158 nCi in (A) plus 0.1 nmol of [^{3}H]GDP (1.13 μCi) as a standard; 110 nCi in (B)] were separated on a Superose 12 column (Pharmacia) using 70% aqueous formic acid as eluant. Fractions of 400 μL were collected at a flow rate of 0.4 mL/min.

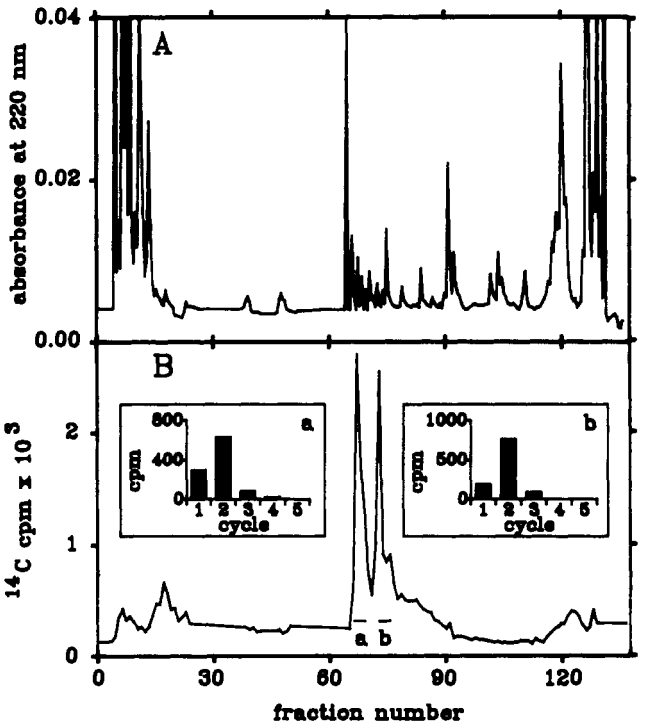

FIGURE 4: Separation of [U-^{14}C]GTP$_{oxi}$-labeled peptides from peak b in Figure 3A. Solvent A was 0.1% aqueous trifluoroacetic acid and solvent B 0.1% aqueous trifluoroacetic acid in 70% 2-propanol. 27 nCi was applied on a Merck LiChrospher RP-18 column using a linear gradient of 0–10% B in 60 min and 10–65% B in 80 min. The flow rate was 0.5 mL/min and the fraction size 400 μL. (A) UV absorbance; (B) radioactivity profile. Insets in (B) show the radioactivity appearing in the soluble phase during Edman degradation of pools a and b on a solid-phase sequencer.

main radioactive peaks appeared (Figure 3A). The peak containing the small fragments is well separated from radioactive monomeric nucleotides (Figure 3A). Rechromatography of this low molecular weight fraction on a reverse-phase

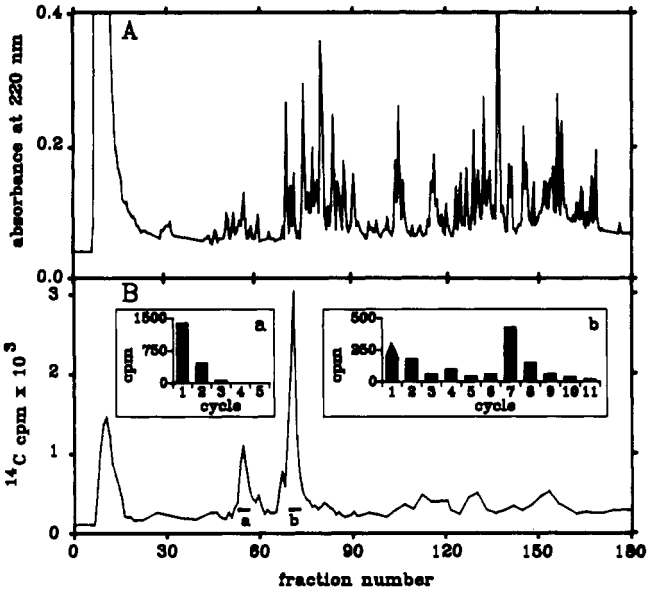

FIGURE 5: Separation of peptides obtained by trypsin treatment of [U-^{14}C]GTP$_{oxi}$-labeled CB1 and CB2 containing peak a from Figure 3A (10 nmol; 38 nCi). 50 nmol of cold material prepared in an identical way was added. Chromatography was performed on a Vydac C_4 300-Å column using the same solvent as in Figure 4 with a linear gradient from 0–15% B to 15–55% B (90 min each). Fractions of 0.4 μL were collected at a flow rate of 0.4 mL/min. (A) Absorbance; (B) radioactivity profile. Insets in (B) show the radioactivity of the corresponding peptides to pooled peaks a and b on a solid-phase sequencer. The breakthrough of radioactivity at the first cycle in inset b is due to an incomplete covalent attachment of the labeled peptide using the carbodiimide coupling procedure. Using a preceding washing step with 100% trifluoroacetic acid before starting the Edman degradation, it could be avoided. For details, see Materials and Methods.

HPLC column gave two peaks (Figure 4) originating from peptides which carry the radioactivity on the second amino acid from the N-terminus (inset of Figure 4B). There are two cyanogen bromide fragments in EF-Tu with lysine in the second position from the N-terminus. Since CB1 was shown to be exclusively modified at Lys-52, the only remaining CB fragment is CB11. Furthermore, as seen in Table I, CB11 is the only low molecular weight peptide which contains a lysine. The sequence of CB11 is Asn(136)-Lys-Val-Asp-Met(140) (Seidler et al., 1987), corresponds with the low molecular weight of the isolated peptide. We conclude that Lys-137 is labeled in this peptide. The appearance of two radioactive peaks in Figure 4B is therefore probably due to partially deamidation of Asn-136 in this peptide.

The peak belonging to the fast eluting peptide of high molecular weight (Figure 3, fraction a) was digested with trypsin, and the resulting fragments were separated on a reverse-phase HPLC column (Figure 5). Two radioactive fractions appeared. Edman degradation on a gas-phase sequencer of fraction a revealed the sequence Xaa-Glu-Glu-Gly-Gly (Table II). In parallel, this fraction was sequenced by using a solid-phase sequencer, and the radioactivity in the soluble fraction was determined after each degradation step (inset a, Figure 5). The result suggests that the modified peptide has a lysine residue at its N-terminus. Together, these results are consistent with the modification occurring at residue 325, which is a part of the CB2 fragment. The partial sequence in the vicinity of Lys-325 is Leu(323)-Lys-Lys-Glu-Glu-Gly-Gly-Arg(330). Since Lys-325 is modified, trypsin has only cleaved after lysine-324 and then at arginine-330, leading to peptide Xaa-Glu-Glu-Gly-Gly-Arg (Table II).

Edman degradation of the peptide from fraction b in Figure 5 on a gas-phase sequencer provided the sequence Asp-Tyr-

Table II: Sequence Analysis of Labeled Peptides[a]

cycle	amino acid	yield of PTH (pmol)	residue no. in sequence of EF-Tu
		Peptide a	
1	Xaa	nd[b]	325
2	Glu	54	326
3	Glu	75	327
4	Gly	48	328
5	Gly	48	329
		Peptide b	
1	Asp	133	46
2	Tyr	213	47
3	Gly	176	48
4	Asp	124	49
5	Ile	90	50
6	Asp	148	51
7	Xaa	nd	52
8	Ala	107	53
9	Pro	nd	54
10	Glu	63	55
11	Glu	75	56

[a] Peptides a and b derived from peaks a and b in the profile of Figure 5B. Only the fraction of each peak with the maximum radioactivity was subjected to Edman degradation in a gas-phase sequencer. [b] nd: The amount of phenylthiohydantoin-proline (PTH-proline) could not be determined because the corresponding peak overlapped with the peak for diphenylthiourea. The amount of PTH-Xaa was not determined due to the lack of a corresponding standard. The peak of PTH-Xaa was identified at the end of the HPLC profiles, with a longer retention time than PTH-Leu, the last peak in a separation of standard PTH-amino acids.

Table III: Yield[a] of EF-Tu Labeling with GTP_{oxi} and GDP_{oxi} as the Percent of the Total Radioactivity Bound to the Protein

residue	method	
	GTP_{oxi}	GDP_{oxi}
Lys-52	41	56
Lys-325	10	22
Lys-137	38	11
sum of other 17 lysines	11	11
total	100	100

[a] Autoradiograms (Figure 1, lanes 2 and 3) were scanned by using a Beckman DU8 UV/VIS spectrophotometer equipped with a scanning device. Exposed X-ray films were cut to appropriate stripes and scanned at 550 nm, and areas above the blank were integrated. As a blank, an X-ray film exposed over a line without radioactivity was taken. Additionally, the radioactivity profiles of Figures 3–5 were used for evaluation.

Gly-Asp-Ile-Asp-Xaa-Ala-(Pro)-Glu-Glu (Table II). This peptide corresponds to amino acid residues 46–56 in the sequence of *T. thermophilus* EF-Tu. The radioactivity determined in the appropriate soluble fractions during stepwise Edman degradation of this fragment on a solid-phase sequencer is in agreement with this result, since, as expected, most of the radioactivity appeared during the seventh cycle (inset, Figure 5). It is obvious that the affinity labeling occurred at lysine residue 52 of EF-Tu.

Identification of the lysine residues which react with oxidized G nucleotides allows a quantitative evaluation of the autoradiograms shown in lanes 2 and 3 of Figure 1 and the radioactivity profiles of Figure 3A and Figure 5B. The data are summarized in Table III. A difference in the distribution of radioactivity originating from GDP_{oxi} and GTP_{oxi} among the different lysine residues is obvious. GDP_{oxi} reacts preferentially with lysine residue 52 and hardly labels lysine-137, while GTP_{oxi} modifies both lysine residues with about the same efficiency. The absolute yield of lysine-325 modification is higher with GDP_{oxi} than with GTP_{oxi}. After the site of labeling

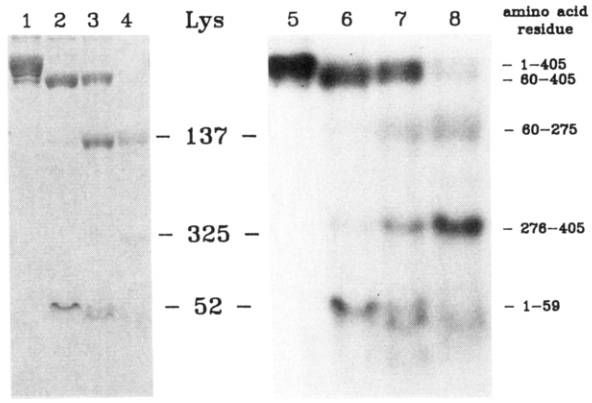

FIGURE 6: Limited tryptic digestion of EF-Tu from *T. thermophilus*, labeled with [U-^{14}C]GTP_{oxi} or [γ-^{32}P]ATP_{oxi}. 4 nmol of EF-Tu was labeled with 2 nmol of [U-^{14}C]GTP_{oxi} (lanes 1–4) or 2 nmol of [γ-^{32}P]ATP_{oxi} (lanes 5–8) as described under Materials and Methods. The tryptic digestion was performed as described in the legend to Figure 2. Lanes 1 and 4, autoradiography of an SDS–urea–PAGE analysis of the labeled polypeptide; lanes 2 and 6, after 10 s; lanes 3 and 7, after 1 min; lanes 4 and 8, after 2-h treatment with trypsin, respectively. Tryptic fragments which contain lysine residues 52, 137, and 325 are labeled by the corresponding numbers.

with GDP_{oxi} and GTP_{oxi} at Lys-52 and Lys-137 was established, the specificity and the yield of the labeling reaction could be easily monitored by limited tryptic digestion of the reacted protein (Figure 6). In the case of GTP_{oxi}, the peptides containing residues 52 and 137 were labeled with about equal intensity (Figure 6, lanes 1–4). This is in agreement with the gel filtration experiment shown in Figure 3A. GDP_{oxi} labeled Lys-52 preferentially as shown in Figure 2. ATP, which does not replace bound GDP under the experimental conditions, mainly labeled after oxidation residues in fragment 276–405 different from residues 52 and 137. This was clearly demonstrated in Figure 6 (lanes 5–8).

Photoaffinity Labeling of EF-Tu. UV irradiation of EF-Tu·[β-^{32}P]GDP or EF-Tu·[β-^{32}P]GTP complexes at 257 nm led to covalent attachment of the nucleotides to the protein. The site of attachment was analyzed by cleavage of the protein with cyanogen bromide, separation of the CB fragments on SDS–urea–polyacrylamide gel electrophoresis, and autoradiography. A major reaction site was found on fragment CB4 (Figure 7, lane 1) which carried 78% of the radioactivity compared to 10, 4, and 8% on fragments CB1, CB2, and CB3, respectively. No significant differences were observed when EF-Tu·GDP or EF-Tu·GTP was irradiated (data not shown). Attempts to isolate the labeled CB4 by gel permeation chromatography for sequencing studies were not successful due to instability of the covalent nucleotide–protein linkage. The same property was observed by others (Havron & Sperling, 1977). The theoretical possibility exists that the labeling of CB4 is a result of higher stability of a cross-link in this part of the protein and not the result of a specific reaction. This was, however, excluded by a control experiment with ATP and comparison of the yield of cross-linking before and after cyanogen bromide cleavage.

Discussion

Elongation factor Tu from *Thermus thermophilus* was labeled with oxidized guanosine nucleotides on lysine residues 52, 137, and 325. On the basis of high (70%) sequence homology of this elongation factor with EF-Tu from *E. coli* (Seidler et al., 1987), the three-dimensional structures of these bacterial EF-Tu's are expected to be very similar. In Figure 8, a 3D model of the *E. coli* EF-Tu G domain is presented (Jurnak, 1985) with numbering corresponding to the *T.*

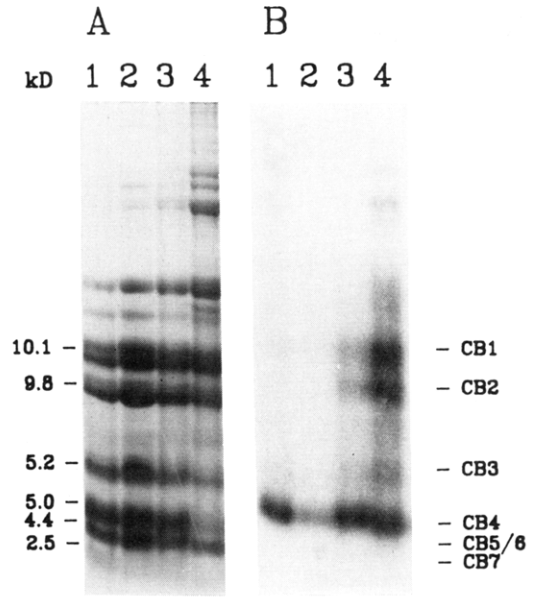

FIGURE 7: Analysis of CB fragments of photolabeled *T. thermophilus* EF-Tu. 1 nmol of EF-Tu was irradiated in the presence of [β-^{32}P]GDP with a laser at 257 nm as described previously (Seidler et al., 1987). Different intensities were used: lane 1, 5 mW/cm^2 for 5 min; lane 2, 15 mW/cm^2 for 1 min; lane 3, 15 mW/cm^2 for 5 min; lane 4, 30 mW/cm^2 for 5 min. Labeled fragments were analyzed by 12.5% SDS–urea–PAGE (A), and the gel was applied to autoradiography (B). For details, see Materials and Methods.

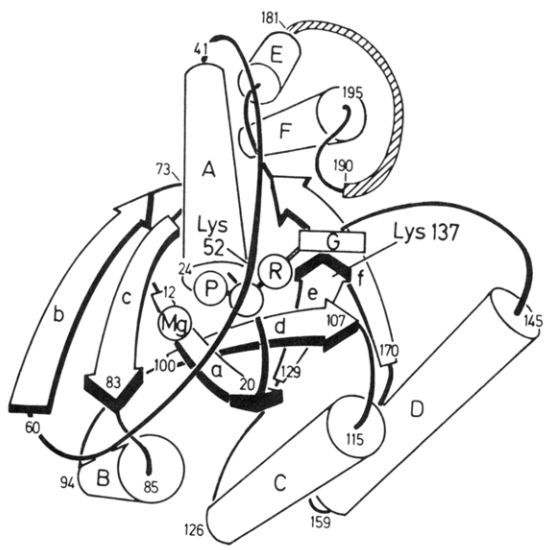

FIGURE 8: Model of the GDP binding domain of EF-Tu from *T. thermophilus*, which was derived from the crystallographic structure of *E. coli* EF-Tu·GDP (Jurnak, 1985; Jurnak, personal communication). Positions equivalent to residues Lys-52 and Lys-137 which are located next to the GDP binding site are indicated. The numbering is adapted to *T. thermophilus* EF-Tu. The hatched loop corresponds to 10 additional amino acid residues.

thermophilus protein. Residue 52 is part of a region in *E. coli* EF-Tu which is absent from the crystallizing protein (Jurnak, 1985; residues 45–59). Consequently, the location of this loop in the 3D structure of EF-Tu is not known. Very recent work of Miyazaki et al. (1988) on the homologous eucaryotic elongation factor 1α of two yeast species shows that a leucine residue which corresponds to the neighboring position of the affinity-labeled Lys-52 in *T. thermophilus* EF-Tu is protected against chymotryptic digestion by addition of GDP or GTP. The proximity of this region to the GDP/GTP binding site is additionally supported by the finding that the methylation of Lys-56 in EF-Tu of *E. coli* leads to a decrease in the

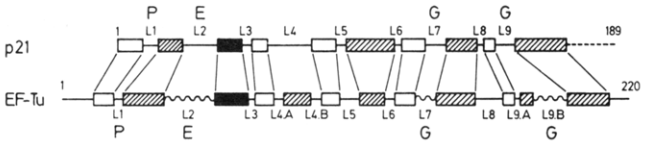

FIGURE 9: Comparison of the secondary structure elements of the G domain from *T. thermophilus* EF-Tu with p21. Hatched boxes indicate α-helices and open boxes β-strands, and shaded boxes correspond to the only antiparallel β-strand in each protein. The loops are numerated from L1 to L9 according to de Vos et al. (1988). P, phosphoryl binding loop; E, effector loop; G, guanine base binding regions. Affinity-labeled loops are marked by sinuous lines.

tRNA-stimulated GTPase activity (van Noort et al., 1986).

The sequence of the c-H-ras oncogene protein p21 has, as a GTP binding protein, a high degree of homology with the GDP binding domain of *E. coli* EF-Tu (Halliday, 1984). Available three-dimensional structures of the EF-Tu G binding domain (la Cour et al., 1985; Jurnak, 1985) and p21 (de Vos et al., 1988), together with the results from this work, allow a comparison of the structural domains of both GTP binding proteins. In addition to sequence homology, a homology in the secondary and tertiary structure of both proteins emerges (Figure 9). In p21, four loops are essential for GDP binding (de Vos et al., 1988). Affinity labeling has now identified three of these four loops as a GDP binding site in *T. thermophilus* EF-Tu. The fourth loop, which is located in the proximity of the nucleotide phosphate groups, would not be able to react either with the photoactivated guanine ring or with the oxidized ribose of GDP/GTP for steric reasons.

The loop, consisting of amino acid residues 45–60 in *T. thermophilus* EF-Tu, has some interesting properties. Using site-directed mutagenesis, Sigal et al. (1986) localized a so-called "effector loop" in p21, which is mainly responsible for interaction with adenylate cyclase. Considering the structural homology of both proteins, the region around amino acid residues 45–60 of *T. thermophilus* EF-Tu would correspond to this "effector" region in p21 (Halliday, 1984; de Vos et al., 1988). Indeed, this part of *E. coli* EF-Tu was implicated in interaction with aa-tRNA (Laursen et al., 1981) which is the effector in the case of the EF-Tu cycle (Bourne, 1986). The basic amino acid residues in this loop are protected from proteolysis by the binding of aa-tRNA (Jacobson & Rosenbusch, 1977).

We previously noticed (Gulewicz et al., 1981) that EF-Tu·GDP from *T. thermophilus* does not interact with EF-Ts when cleaved at position 59. Since EF-Ts enhances the rate of GDP dissociation from EF-Tu (Ruusala et al., 1982), GDP and EF-Ts may bind to the same site on EF-Tu. The region around amino acid residue 59 could fulfill this function. Recent work of Möller et al. (1987) is in agreement with this suggestion. Furthermore, the accessibility of the 45–60 loop for trypsin cleavage decreases in the order EF-Tu·GTP > EF-Tu·EF-Ts > EF-Tu·GDP (Douglass & Blumenthal, 1979).

In all EF-Tu's, the loop 45–60 contains a so-called PEST region, rich in proline, glutamic acid, serine, threonine, and basic amino acids. This region is believed to promote cleavage of the polypeptide chain (Rogers et al., 1986) and to regulate the turnover of the proteins in eucaryotic cells. EF-Tu molecules are also often found cleaved in this region (Möller et al., 1987).

Lysine-137, which is labeled to a high extent with GTP_{oxi} but little with GDP_{oxi}, is an invariable residue present at this position in all GTP binding proteins (Halliday, 1984; Gilman, 1987). The ϵ-amino group of the homologous lysine residue in *E. coli* is believed to interact with the endocyclic 4′-oxygen of the guanosine ribose ring (la Cour, personal communica-

tion). The low yield of labeling from GDP at this position may reflect a rigid fixation of GDP_{oxi} in the active site of the protein. This is not the case for GTP_{oxi}, where the 2′(3′)-aldehyde groups can efficiently react with the more mobile ϵ-amino group of lysine-137. Lys-137 corresponds to Lys-117 in p21 (Halliday, 1984). Replacement of this residue decreases GTP binding to a much higher extent (5000-fold) than replacement of the neighboring invariable residues Asn-116 (10-fold) or Asp-119 (100-fold) (Der et al., 1986), both of which are considered to interact with the guanine moiety of GDP (la Cour et al., 1985; Jurnak 1985). This finding underlines the importance of this Lys residue for nucleotide binding.

The high reactivity of lysine-137 with GTP_{oxi} demonstrates again the specificity of the affinity labeling directed to the nucleotide binding site. In an earlier study (Antonsson & Leberman, 1984), only 4 of 23 residues were left unlabeled, using an unspecific lysine reagent, [^{14}C]ethyl acetimidate, for competitive labeling of *E. coli* EF-Tu·GTP and EF-Tu·GTP·aa-tRNA. One of these unlabeled residues was Lys-136, which corresponds to Lys-137 in EF-Tu from *T. thermophilus*. These results are also in agreement with an earlier report of Kraal and Hartley (1978), who used formaldehyde for reductive methylation of EF-Tu·GDP and EF-Tu·GTP from *E. coli*.

Residue 325 is a lysine which reacted both with GDP_{oxi} and with GTP_{oxi}. It belongs to domain III of EF-Tu (Jurnak, 1985) and is present in all EF-Tu sequences determined up to now. Whether the higher reactivity of the GTP_{oxi} as compared to GDP_{oxi} toward Lys-325 is due to higher flexibility of the nucleotide or to a conformational change of the protein cannot be decided at the present. It is interesting to emphasize that the neighboring Lys-324 was not modified by GDP_{oxi} or by GTP_{oxi}.

Elongation factor Tu from *T. thermophilus* has a stretch of 10 additional amino acids which were not found in any sequenced bacterial EF-Tu molecule (Jones et al., 1980; Ohama et al., 1987; Lechner & Böck, 1987) including EF-Tu from *E. coli*. A comparable loop has only been found in EF-Tu from *Euglena gracilis* chloroplasts (Montandon & Stutz, 1984). This region is confined to residues 181–190 of the *T. thermophilus* EF-Tu sequence.

Photoaffinity labeling of the *T. thermophilus* elongation factor resulted in covalent attachment of guanosine nucleotides to cyanogen bromide fragment 4 consisting of amino acids 183–223. Cyanogen bromide fragment 11 which was cross-linked to the oxidized ribose of GTP did not carry any radioactivity. With [γ-^{32}P]ATP as a photoaffinity probe for labeling of EF-Tu·GDP, no modification of CB4 but only of CB1 and CB2 was detectable (data not shown). Residues 181–196 can form a loop between the E and F helices in the corresponding *E. coli* EF-Tu three-dimensional structure (Figure 8) and could easily be located in the vicinity of the G-nucleotide binding site. Therefore, this region is the most probable candidate for photoaffinity modification since residues beyond residue 196 are part of an inaccessible helix F (196–200) or are in domain II (201–223). We therefore place the sequence 181–190 over the nucleotide binding site (Figure 8) and suggest that this region is the site of covalent linkage between protein and nucleotide obtained by photoirradiation of EF-Tu·GDP or EF-Tu·GTP. This model receives further support from the fact that this loop contains the sequence Lys-Thr-Arg (residues 187–189) which is also present in the sequence of p21 (residues 147–149) (Dhar et al., 1982), being a part of the second guanine binding loop of the G-binding site (Figure 9) (de Vos et al., 1988; McCormick et al., 1985). A very recent report shows that the replacement of Ala-146 by valine in the sequence of p21 leads to >1000-fold increased rate of nucleotide exchange, confirming that this region should be involved in the binding of the nucleotide (Feig & Cooper, 1988).

Finally, these results derived from chemical and photochemical labeling of EF-Tu from *T. thermophilus* show that only one of the four cross-linking site reveals a striking difference in its reactivity to GDP and GTP. This site, which is refered to as the G region (Halliday, 1984), is strongly conserved in all GTP binding proteins. Moving this loop relative to the binding site could lead to a structural change of GTP binding proteins which is responsible for their different functions in the GDP and GTP forms.

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Registry No. GDP, 146-91-8; GTP, 86-01-1; L-Lys, 56-87-1.

REFERENCES

Antonsson, B., & Leberman, R. (1984) *Eur. J. Biochem. 141*, 483–487.

Arai, K.-I., Kawakita, M., & Kaziro, Y. (1972) *J. Biol. Chem. 247*, 7029–7037.

Arai, K.-I., Ota, Y., Arai, N., Nakamura, S., Henneke, C., Oshima, T., & Kaziro, Y. (1978) *Eur. J. Biochem. 92*, 509–519.

Borch, R. F., Bernstein, M. D., & Dupont Durst, H. (1971) *J. Am. Chem. Soc. 93*, 2897–2904.

Bourne, H. R. (1986) *Nature (London) 321*, 814–816.

Degryse, E., Glandsdorff, N., & Pierard, A. (1978) *Arch. Microbiol. 117*, 189–196.

Der, C. J., Pan, B.-T., & Cooper, G. M. (1986) *Mol. Cell. Biol. 6*, 3291–3294.

De Vos, A. M., Tong, L., Milburn, M. V., Matias, P. M., Jancarik, J., Noguchi, S., Nishimura, S., Miura, K., Ohtsuka, E., & Kim, S.-H. (1988) *Science (Washington, D.C.) 239*, 888–893.

Dhar, R., Elis, R. W., Oroszlan, S., Shapiro, B., Maizel, J., Lowy, D., & Scolnick, E. (1982) *Science (Washington, D.C.) 217*, 934–936.

Douglass, J., & Blumenthal, T. (1979) *J. Biol. Chem. 254*, 5383–5387.

Feig, L. A., & Cooper, G. M. (1988) *Mol. Cell. Biol. 8*, 2472–2478.

Gilman, A. G. (1987) *Annu. Rev. Biochem. 56*, 615–649.

Gulewicz, K., Faulhammer, H. G., & Sprinzl, M. (1981) *Eur. J. Biochem. 121*, 155–162.

Halliday, K. R. (1984) *J. Cyclic Nucleotide Protein Phosphorylation Res. 9*, 435–448.

Hamel, E. (1975) *Biochim. Biophys. Acta 414*, 326–340.

Havron, A., & Sperling, J. (1977) *Biochemistry 16*, 5631–5635.

Jacobson, G. R., & Rosenbusch, J. P. (1977) *Eur. J. Biochem. 77*, 409–417.

Johnson, R. A., & Walseth, T. F. (1979) *Adv. Cyclic Nucleotide Res. 10*, 136–167.

Jones, M. D., Petersen, T. E., Nielsen, K. M., Magnusson, S., Sottrup-Jensen, L., Gausing, K., & Clark, B. F. C. (1980) *Eur. J. Biochem. 108*, 507–526.

Jurnak, F. (1985) *Science (Washington, D.C.) 230*, 32–36.

Kraal, B., & Hartley, B. S. (1978) *J. Mol. Biol. 124*, 551–564.
Kushiro, A., Shimizu, M., & Tomita, K.-I (1987) *Eur. J. Biochem. 170*, 93–98.
La Cour, T. F. M., Nyborg, J., Thirup, S., & Clark, B. F. C. (1985) *EMBO J. 4*, 191–219.
Laursen, R. A. (1977) *Methods Enzymol. 47*, 277–289.
Laursen, R. A., L'Italien, J. J., Nagarkatti, S., & Miller, D. L. (1981) *J. Biol. Chem. 256*, 8102–8109.
Leberman, R., & Egner, U. (1984) *EMBO J. 3*, 339–341.
Leberman, R., Antonsson, B., Giovanelli, R., Guariguata, R., Schumann, R., & Wittinghofer, A. (1980) *Anal. Biochem. 104*, 29–36.
Lechner, K., & Böck, A. (1987) *Mol. Gen. Genet. 208*, 523–528.
Lowe, P. N., & Beechey, R. B. (1982) *Bioorg. Chem. 11*, 55–71.
McCormick, F., Clark, B. F. C., la Cour, T. F. M., Kjeldgaard, M., Norskov-Lauritsen, L., & Nyborg, J. (1985) *Science (Washington, D.C.) 230*, 78–82.
Miller, D. L., & Weissbach, H. (1977) in *Molecular Mechanisms of Protein Biosynthesis* (Weissbach, H., & Pestka, S., Eds.) pp 323–373, Academic, New York.
Miyazaki, M., Uritani, M., Fujimura, K., Yamakatsu, H., Kageyama, T., & Takahashi, K. (1988) *J. Biochem. (Tokyo) 103*, 508–521.
Möller, W., Schipper, A., & Amons, R. (1987) *Biochimie 69*, 983–989.
Montandon, P.-E., & Stutz, E. (1984) *Nucleic Acids Res. 12*, 2851–2859.
Nakamura, S., Ohta, S., Arai, K.-I., Arai, N., Oshima, T., & Kaziro, Y. (1978) *Eur. J. Biochem. 92*, 533–543.
Ohama, T., Yamao, F., Muto, A., & Osawa, S. (1987) *J. Bacteriol. 169*, 4770–4777.
Ohta, S., Nakanishi, M., Tsuboi, M., Arai, K.-I., & Kaziro, Y. (1977) *Eur. J. Biochem. 78*, 599–608.
Rogers, S., Wells, R., & Rechsteiner, M. (1986) *Science (Washington, D.C.) 234*, 364–368.
Ruusala, T., Ehrenberg, M., & Kurland, C. G. (1982) *EMBO J. 1*, 75–78.
Salnikow, J., Lehmann, A., & Wittmann-Liebold, B. (1981) *Anal. Biochem. 117*, 433–442.
Seidler, L., Peter, M., Meissner, F., & Sprinzl, M. (1987) *Nucleic Acids Res. 15*, 9263–9277.
Sigal, I. S., Gibbs, J. B., D'Alonzo, J. S., & Scolnick, E. M. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 4725–4729.
Stryer, L., & Bourne, H. R. (1986) *Annu. Rev. Cell Biol. 2*, 391–419.
Van Noort, J. M., Kraal, B., Sinjorgo, K. M. C., Persoon, N. L. M., Johanns, E. S. D., & Bosch, L. (1986) *Eur. J. Biochem. 160*, 551–561.
Wittinghofer, A., Warren, W. F., & Leberman, R. (1977) *FEBS Lett. 75*, 241–243.
Wittinghofer, A., Frank, R., & Leberman, R. (1980) *Eur. J. Biochem. 108*, 423–431.

^{1}H NMR Studies of Human C3a Anaphylatoxin in Solution: Sequential Resonance Assignments, Secondary Structure, and Global Fold†

Walter J. Chazin,*,‡ Tony E. Hugli,§ and Peter E. Wright*,‡
Departments of Molecular Biology and Immunology, Research Institute of Scripps Clinic, 10666 North Torrey Pines Road, La Jolla, California 92037

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ABSTRACT: The spin systems that comprise the ^{1}H nuclear magnetic resonance (NMR) spectrum of the complement fragment C3a (M_r 8900) have been completely identified by an approach which integrates data from a wide range of two-dimensional NMR experiments. Both relayed and multiple quantum experiments play an essential role in the analysis. After the first stage of analysis the spin systems of 60 of the 77 residues were assigned to the appropriate residue type, providing an ample basis for subsequent sequence-specific assignments. Elements of secondary structure were identified on the basis of networks of characteristic sequential and medium-range nuclear Overhauser effects (NOEs), values of $^3J_{HN\alpha}$, and locations of slowly exchanging backbone amide protons. Three well-defined helical segments are found. Gradients of increasing mobility in distinct segments of the C3a polypeptide are observed, with very high mobilities for several residues near the C- and N-termini, including the complete C-terminal receptor binding site pentapeptide LGLAR. The NMR data, combined with known disulfide linkages and a small number of critical long-range NOEs, provide the global folding pattern of C3a in solution. Identical solution structures were found for both the intact active protein and the largely inactive physiologic product des-Arg^{77}-C3a. Differences between the solution and crystal structures of C3a are observed, particularly in the N-terminal region. The relevance of these new observations is discussed with respect to physiologic responses that are elicited by the "local hormone-like" anaphylatoxin molecule.

Anaphylatoxin C3a is a small protein fragment (77 amino acids, M_r 8900) of complement component C3 released into the blood upon activation of the complement cascade. The C3a molecule is characterized biologically as a spasmogen capable of inducing smooth muscle contraction, increasing vascular permeability, and causing a skin wheal and flare reaction when injected intradermally. Many of the biologic responses are mediated via tissue mast cells that are activated either directly or indirectly by the anaphylatoxin. The protein has been studied by a variety of biochemical techniques [re-

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* To whom correspondence should be addressed.
‡ Department of Molecular Biology.
§ Department of Immunology.

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