action of pyridoxal sulfate with the unmodified apoenzyme. Thus, a free SH group on Cys-390 is not essential for the reaction.

Apoenzyme was allowed to react with the 5-carboxyethenyl analogue of pyridoxal phosphate as described under Experimental Procedures. When the resulting modified enzyme was digested with pepsin, a series of fluorescent peptides that exactly paralleled those obtained from pyridoxal sulfate were obtained. Pure samples of the tetrapeptide and the tripeptide were obtained and analyzed. Molar ratios for the tripeptide were as follows: chromophore 1.13; Asx 1.00; Lys 0.35; Ser 0.88; Glu 0.03; Gly 0.04.

The results presented here establish that the chromophores formed by the reaction of pyridoxal 5′-sulfate or the 5-carboxyethenyl analogue of pyridoxal phosphate with cytosolic aspartate aminotransferase are attached to the active site lysine-258. There is no cross-linking to other parts of the protein. Results presented in the preceding paper (Scott et al., 1982) show that for pyridoxal sulfate the chromophore formed is a substituted 2*H*-pyrrolo[3,4-*c*]pyridine as is shown in Scheme I.

Acknowledgments

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Structure–Function Relationships in *Escherichia coli* Translational Elongation Factor G: Modification of Lysine Residues by the Site-Specific Reagent Pyridoxal Phosphate†

Alfonso Giovane,‡ Ciro Balestrieri,‡ and Claudio Gualerzi*

ABSTRACT: Translational elongation factor G (EF-G) of *Escherichia coli* was modified with the selective, site-specific lysine reagent pyridoxal phosphate (PLP). The reaction results in the modification of a maximum of 12 lysine residues, one of which is essential for guanosine 5′-triphosphate (GTP) binding and whose modification is inhibited by the presence of GTP. Formation of a reversible adduct between 2,3-butanedione and an essential arginine similarly located in the GTP binding site [Rohrbach, M. S., & Bodley, J. W. (1977) *Biochemistry 16*, 1360–1363] also protects EF-G from PLP inactivation, suggesting that these two residues are spatially close to each other in the native factor. The essential lysine residue was found in the trypsin-resistant fragment T4 (M_r 41 000). In addition to the lysine essential for GTP binding, at least one further lysine was found to be important for EF-G function, since GTP-protected, PLP-modified EF-G molecules fully competent in binding to 50S ribosomal subunits showed decreased activity in 50S- and 70S-dependent GTP hydrolysis. It is likely that a PLP-modified lysine impairs the interaction of the factor with 30S ribosomal subunits and/or a conformational change of the factor required for the hydrolysis of GTP.

We are carrying out a systematic investigation of the phosphate-binding regions of various components of the protein synthetic machinery (Ohsawa & Gualerzi, 1981a,b) by use of the selective and site-specific lysine reagent pyridoxal phosphate (Rippa et al., 1967; Feeney et al., 1975; Glazer, 1976). In the present paper we report on the identification of an essential lysine residue in the guanine nucleotide binding site of elongation factor G (EF-G).

EF-G is one of the three protein factors required for protein biosynthesis and endowed with ribosome-dependent GTPase activity. [The others are IF2 and EF-Tu; for a recent review see Parmeggiani & Sander (1981).] The relationship between structure, mechanism of action, topographical location, and

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evolutionary origin of these GTPases remains an open and interesting problem; it is not unlikely that the GTP[1] binding sites of these proteins possess very similar structural features, possibly deriving from a common origin.

Materials and Methods

Materials. Pyridoxal 5′-phosphate (PLP) and 2,3-butanedione were purchased from Serva. [^{3}H]GTP and [^{3}H]GDP (10 Ci/mmol) and sodium boro[^{3}H]hydride were obtained from Amersham Buchler. TPCK-trypsin was purchased from Worthington. Fusidic acid was obtained from Leo Pharmaceutical Products. All other chemicals were of reagent grade. The following buffers were used: buffer I, which contained 10 mM Tris-HCl, pH 7.7, 10 mM magnesium acetate, 100 mM KCl, and 5 mM 2-mercaptoethanol; buffer II, which contained 20 mM triethanolamine hydrochloride, pH 7.8, 10 mM magnesium acetate, 30 mM KCl, and 5 mM 2-mercaptoethanol. Ribosomes and ribosomal subunits were prepared from *Escherichia coli* MRE600 as previously described (Risuleo et al., 1976).

Elongation factor G was purified to electrophoretic homogeneity from *E. coli* MRE600 by the method of Kaziro et al. (1969) with some modifications. The 40–65% ammonium sulfate fraction of the postribosomal supernatant was dissolved in 20 mM Tris-HCl, pH 7.5, 150 mM KCl, and 5 mM 2-mercaptoethanol and dialyzed against the same buffer. The solution was then loaded onto a DEAE-Sephadex A50 column (3.5 × 60 cm) and eluted with a 2-L linear gradient of 150–400 mM KCl in the same buffer. The fractions containing EF-G activity were pooled, dialyzed against 10 mM sodium phosphate (pH 7.0)–5 mM 2-mercaptoethanol, and loaded onto an hydroxylapatite column (2.5 × 40 cm) equilibrated with the same buffer. The column was washed with 50 mM sodium phosphate (pH 7), containing 5 mM 2-mercaptoethanol, until the absorption at 280 nm of the eluant was below 0.2. The column was then eluted with a 1-L gradient of 50–150 mM sodium phosphate. The active EF-G fractions were combined, dialyzed against buffer I, concentrated by batch elution from a small DEAE-cellulose column, and subjected to gel filtration on a Sephadex G-100 column (3 × 200 cm) equilibrated with the same buffer. The eluted fractions were checked for purity by $NaDodSO_4$ slab gel electrophoresis, combined, and concentrated as described above. EF-G was stored at −20 °C in buffer I containing 20% glycerol. The concentration of EF-G was determined spectrophotometrically at 280 nm by using an $A_{1cm}^{0.1\%}$ value of 0.84 (Rohrbach et al., 1975).

Reaction with Pyridoxal Phosphate. Solutions containing from 8 to 15 μM EF-G in buffer II were incubated at 10 °C with various PLP concentrations (ranging from 0.5 to 5 mM). Aliquots were removed during the time of incubation, and the reaction was stopped by the addition of either unlabeled $NaBH_4$ or NaB^3H_4. The reaction mixture was left for at least 10 min at 0 °C to ensure complete reduction by $NaBH_4$ before further processing. For determination of the number of PLP molecules incorporated into EF-G after the reaction, the samples were dialyzed against buffer I, and the concentration of PLP bound to EF-G was determined in a double-beam spectrophotometer (Perkin-Elmer 375) at 325 nm by using a molar extinction coefficient of 9710 for ε-pyridoxyllysine (Fischer et al., 1963).

[1] Abbreviations: GTP, guanosine 5′-triphosphate; Tris, tris(hydroxymethyl)aminomethane; TPCK, L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate.

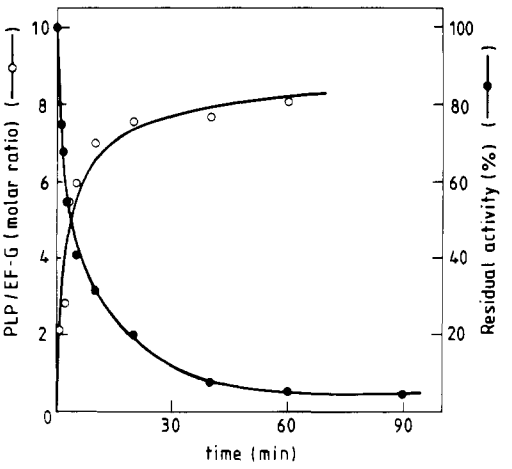

FIGURE 1: Time course of PLP incorporation into EF-G and of PLP-induced inactivation. The reaction mixture containing 12 μM EF-G and 4 mM PLP in 5 mL of buffer II was incubated at 10 °C. Aliquots (500 μL) were removed at the indicated times, and the reaction was stopped by addition of 100 mM $NaBH_4$ to a final concentration of 4 mM. The concentration of PLP bound to EF-G was determined spectrophotometrically as described under Materials and Methods. EF-G activity was assayed by measuring the formation of EF-G–50S–GDP–fusidic acid complex as described under Materials and Methods.

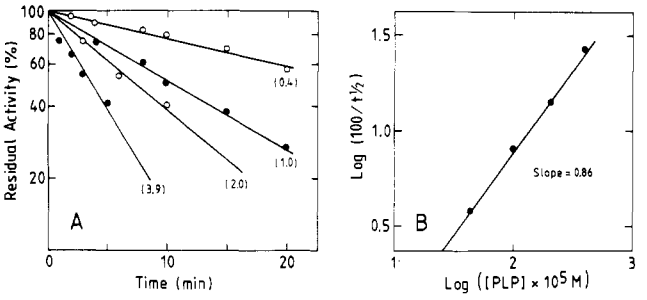

FIGURE 2: PLP concentration dependence of inactivation of EF-G. (A) Modification of EF-G was carried out as described in the legend to Figure 1 with the indicated concentrations of PLP. At the indicated times, aliquots containing 80 μg of EF-G were removed, mixed with 0.05 volume of 100 mM $NaBH_4$, and chilled to 0 °C. These samples were assayed for 50S–GDP–fusidic acid binding activity as described under Materials and Methods. (B) The reaction order was determined by plotting the inactivation half-times obtained above against the respective PLP concentrations in a log–log plot according to Levy et al. (1963).

Assay of EF-G Activity. The EF-G activity was tested either by the formation of the EF-G–[^{3}H]GDP–ribosome–fusidic acid complex on Millipore filters (Highland et al., 1971) or by [^{3}H]GTP hydrolysis (Rohrbach & Bodley, 1976).

Tryptic Digestion of EF-G. Limited tryptic digestion of EF-G was performed according to Alakhov et al. (1979). Samples containing 60–80 μg of EF-G were subjected to trypsin hydrolysis at 37 °C in 0.1 M Tris-HCl, pH 8.1, containing 5 mM 2-mercaptoethanol. The ratio of trypsin to EF-G was 1:50 by weight. The reaction was stopped after 8 min by addition of 0.5 M Tris-HCl, pH 6.8, 2% glycerol, and 5% 2-mercaptoethanol. After 5 min in a boiling water bath, the samples were analyzed by $NaDodSO_4$ gel electrophoresis in 12.5% polyacrylamide gels (Laemmli, 1970; Studier, 1973).

Results

When elongation factor G is incubated with PLP, its activity is progressively lost. After a 40-min incubation, approximately 90% of its biological activity—as measured by the binding to 50S ribosomal subunits in the presence of GTP and fusidic

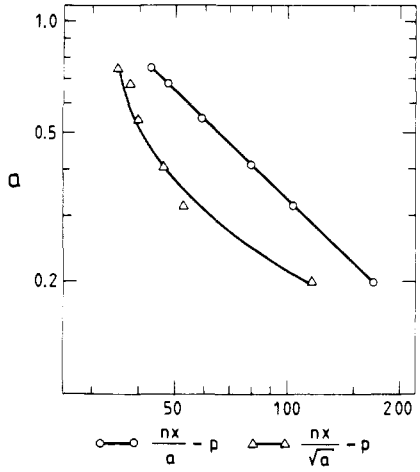

FIGURE 3: Determination of maximum number of PLP-reactive lysine residues in EF-G and of number of lysines essential for GTP-dependent binding to 50S ribosomal subunits. The data from Figure 1 were plotted according to Tsou (1962). See text for details. (○) Assuming one lysine essential, $i = 1$ and $p = 12.5$; (△) assuming two lysines essential, $i = 2$ and $p = 21$.

acid—is lost. At the same time, approximately 8 mol of PLP are incorporated per mol of EF-G (Figure 1).

The rate of EF-G inactivation was measured at various PLP concentrations (Figure 2A). As seen from the figure, the rate of loss of activity depends upon the PLP concentration, following apparent first-order kinetics at any given PLP concentration. In Figure 2B a secondary plot of these data is presented; the log–log plot of the half-times of the inactivation against the respective PLP concentrations yields a straight line with a slope of 0.86, indicating that the reaction is approximately first order with respect to PLP. EF-G contains 44 Lys residues (Ovchinnikow et al., 1982). To find out how many of these residues react with PLP and how many of them are essential for GTP-dependent 50S binding, we plotted the data of Figure 1 according to Tsou (1962) using the equation

$$nx = pa^{1/i} + (n - p)a^{\alpha/i} \qquad (1)$$

where n = the total number of Lys residues = 44, x = the molar fraction of Lys residues remaining unmodified after a given time of PLP reaction, a = the residual activity, p = the number of modifiable Lys residues, i = the number of Lys residues essential for activity, and α = the ratio of the rates of modification of nonessential vs. essential Lys residues. Equation 1 can be rewritten as

$$\log\left(\frac{nx}{a^{1/i}} - p\right) = \log(n - p) + \frac{\alpha - 1}{i}\log a \qquad (2)$$

We therefore plotted log $(nx/a^{1/i} - p)$ against log a and obtained a straight line for $i = 1$ and $p = 12.5$ (Figure 3). Furthermore, from the slope (0.97) it follows that $\alpha = 1.97$, indicating that the nonessential Lys residues react with PLP at an average rate that is approximately 2 times faster than that at which the essential residue reacts.

The maximal number of Lys residues amenable for reaction with PLP was also directly determined from the experiment shown in Figure 4. In this experiment, the formation of the Schiff base between PLP and Lys residues was followed spectrophotometrically. From the molar extinction coefficient of the Schiff base formed between lysine and pyridoxal phosphate (Ogawa & Fujioka, 1980), the number of modified Lys residues was calculated by plotting the reciprocal of the PLP concentration vs. the reciprocal of the Schiff base formed

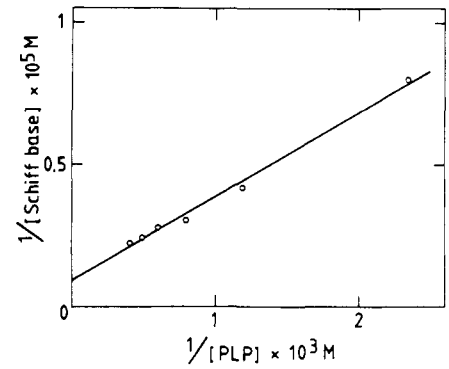

FIGURE 4: Quantitation of number of PLP-reactive lysines by spectrophotometric determination of Schiff-base formation. The reaction was started by addition of 3-μL aliquots of 50 mM PLP to cuvettes containing either 350 μL of 8.75 μM EF-G in buffer II or, as a reference, the same volume of buffer. Formation of the Schiff base was monitored at 428 nm until equilibrium was reached at 25 °C. Concentrations were calculated by using a molar extinction coefficient of 5600 (Ogawa & Fujioka, 1980).

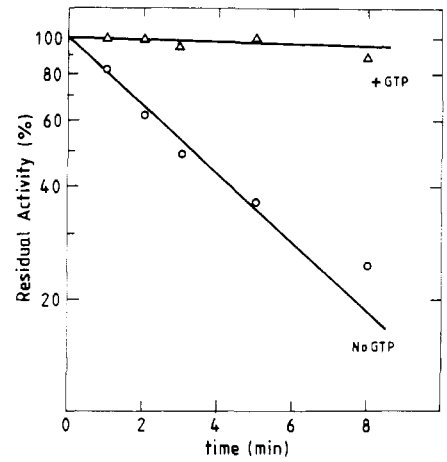

FIGURE 5: Protection of EF-G against PLP inactivation by GTP. The PLP reaction was carried out as described in the legend to Figure 1 in the absence of GTP (○) or in the presence of 1 mM GTP (△).

at each PLP concentration used. By extrapolation to infinite PLP concentration and from the known concentration of EF-G present in the reaction, the maximum number of modifiable Lys residues was found to be 12.03, in very good agreement with that calculated from the experiment shown above (Figure 3B).

The PLP-induced inactivation of the formation of the [^{3}H]GDP–fusidic acid–EF-G–50S complexes could stem from the inactivation either of the site interacting with the GTP molecule or of the site interacting with the 50S ribosomal subunits; alternatively, inactivation could be the result of a nonspecific alteration of the EF-G conformation brought about by the modification of a structurally critical Lys residue. Only in the first case, however, should GTP protect the EF-G from inactivation. That this is indeed the case is shown by the experiment presented in Figure 5. Complete protection of EF-G activity is obtained when the PLP reaction is carried out in the presence of GTP. The completeness of the protection shows that the GTP is very close to the lysine and probably in direct contact with it. Reaction with pyridoxal phosphate, in addition to being selective for the modification of Lys residues, also displays remarkable site specificity in modifying residues in phosphate binding sites of proteins [for review, see Feeney et al. (1975) and Glazer (1976)].

It is believed that PLP owes its site specificity in forming a Schiff base with lysines to the establishment of an electro-

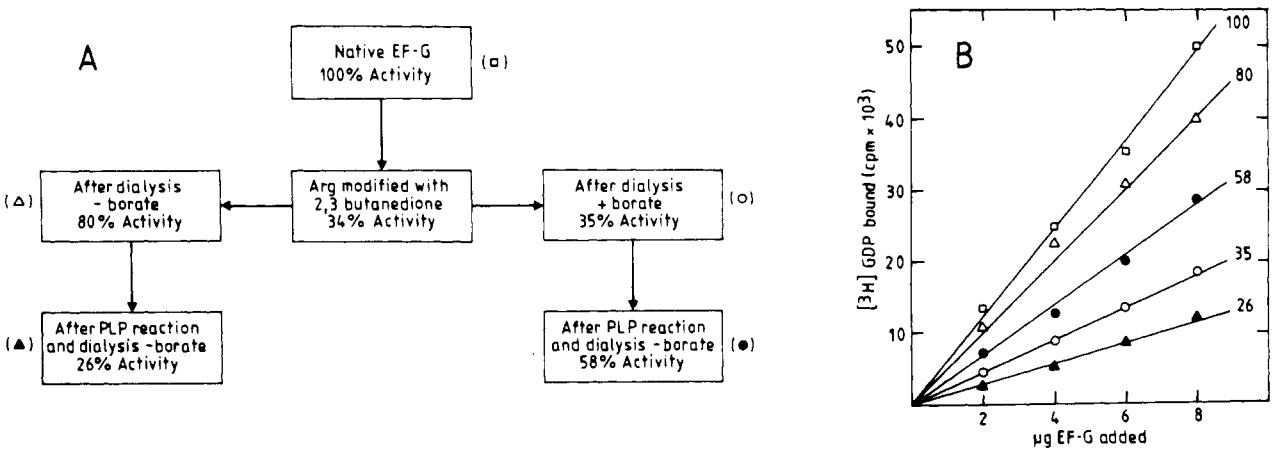

FIGURE 6: Protection of EF-G against PLP inactivation by prior reaction with 2,3-butanedione. The reaction of EF-G with 2,3-butanedione was performed essentially as described by Rohrbach & Bodley (1977). 2,3-Butanedione (Serva) was added (final concentration 10 mM) to a solution of EF-G (15 μM) in buffer II (pH 8.8) containing 50 mM sodium borate. After a 30-min incubation at 25 °C, the reaction mixture was divided into two portions that were exhaustively dialyzed at 2–4 °C against buffer II (pH 7.8) with and without 50 mM sodium borate. After dialysis, both EF-G samples were reacted with PLP (final concentration 5 mM) for 10 min at 10 °C and reduced by addition of 0.05 volume of sodium borohydride (100 mM). After reduction, both samples were dialyzed against buffer I. A scheme of the experiment is outlined on the left (A). The activity in GTP-dependent and fusidic acid dependent binding of EF-G to 50S ribosomal subunits (see Materials and Methods) was determined as a function of EF-G concentration at the five stages indicated (B).

static interaction between its phosphate group and a neighboring Arg or Lys residue (Milhausen & Levy, 1975; Minchiotti et al., 1981). The results shown in Figure 6 clearly demonstrate that a relationship between the essential Arg residue found by others (Rohrbach & Bodley, 1977) and the essential Lys residue found by us indeed exists. 2,3-Butanedione reacts selectively with Arg residues to form an adduct that is stable only in the presence of borate ions (Riordan, 1973; Rohrbach & Bodley, 1977). We found that if EF-G was incubated with butanedione and then dialyzed against borate-containing buffer to stabilize the Arg–butanedione adduct, its activity dropped to 35% in contrast to 80% if borate was not present [this result agrees with that of Rohrbach & Bodley (1977)]. If the butanedione-treated EF-G was incubated with PLP after the borate-free dialysis, then the activity fell to 26%. However, if butanedione-treated EF-G was dialyzed against borate to stabilize the Arg–butanedione adduct before incubation with PLP, then after a final borate-free dialysis, the residual activity was as high as 58%. The scheme of this experiment is presented in Figure 6A, while the actual experimental data are presented in Figure 6B.

In the next experiment we asked whether the PLP reaction has the same effect on the EF-G-dependent GTPase reaction as it has on the binding of the factor to the ribosome. As shown in Figure 7, we found that GTPase activity was also lost following reaction with PLP but that the inactivation was faster than the loss of ribosome binding capability. This finding suggested that the structural requirements for GTPase activity by EF-G are more stringent than for ribosome binding. This premise is further supported by the results of the experiment shown in Figure 8. In this experiment, in order to protect the GTP binding site while having the highest possible degree of modification of the other lysine residues, we treated EF-G with PLP following reaction with butanedione. After dialysis against buffer containing no borate to relieve butanedione inhibition, the EF-G molecules competent in ribosome binding were isolated by centrifugation of EF-G–50S complexes in the presence of GDP and fusidic acid. "Active" EF-G was then recovered from the complex and tested for its activity in 50S binding (Figure 8A) and for 50S-dependent (Figure 8B) and 70S-dependent (Figure 8C) GTPase activity. As seen from the figures, active EF-G isolated by 50S affinity was

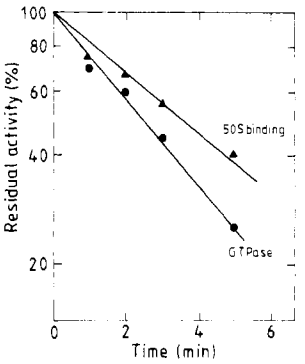

FIGURE 7: Comparison of PLP-induced inactivation of EF-G in 50S binding and 70S-dependent GTP hydrolysis. The reaction of EF-G with PLP was carried out for the indicated times as described in the legend to Figure 1. Binding to 50S ribosomal subunits (▲) and ribosome-dependent GTPase activity (●) were determined as described under Materials and Methods.

indistinguishable from native EF-G in its binding to 50S subunits but displayed about 50% less GTPase activity in the presence of 50 S (Figure 8B) and less than 30% activity in the presence of 70 S (Figure 8C). It follows that the PLP reaction modifies not only the lysine residue of the GTP binding site but also at least one additional lysine residue that affects the triggering of the GTPase activity without affecting the binding of the factor to the 50S ribosomal subunits. Furthermore, the greater inhibition observed in the case of 70S-dependent GTPase is probably the result of the failure of PLP-modified EF-G to interact with 30S ribosomal subunits, which strongly stimulate this activity (Parmeggiani & Sander, 1981).

To locate the essential lysine and thus, indirectly, to determine which part of the EF-G molecule contains the GTP binding site, we took advantage of the partial resistance of EF-G to trypsinization (Skar et al., 1975; Alakhov et al., 1979). In Figure 9 we present a typical electrophoretic pattern of tryptic fragments of EF-G; the pattern obtained by us is virtually identical with that previously published (Alakhov et al., 1979). After showing that the trypsinization was not grossly affected by PLP reaction, we measured the amount of PLP incorporated into each fragment and compared the extent of modification obtained when the reaction was carried

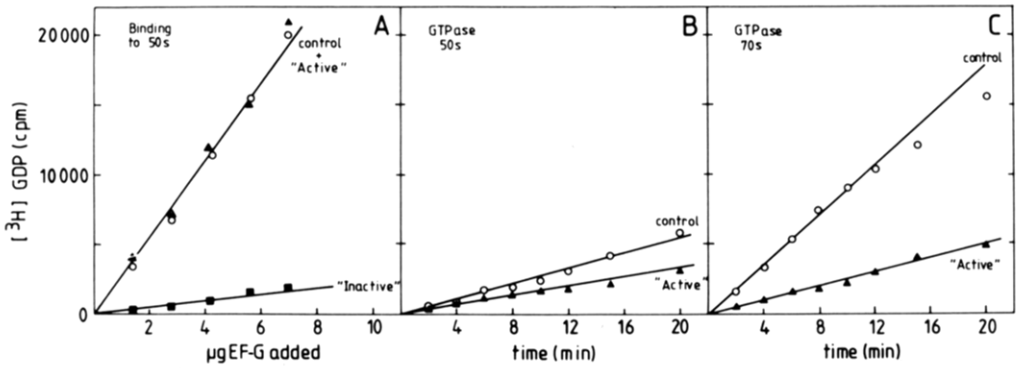

FIGURE 8: Differential effect of PLP reaction on 50S binding (A), 50S-dependent GTPase (B), and 70S-dependent GTPase (C) activity of EF-G. EF-G, protected by butanedione against PLP modification of the lysine in the GTP binding site, was prepared as described in the legend to Figure 6 except that PLP reaction was carried out for 15 min to ensure a higher degree of modification of the other lysines. The EF-G molecules active in binding to 50S ribosomal subunits were isolated by centrifugation (18 h at 40K rpm in the Spinco 50 Ti rotor) in the form of a quaternary complex with 50 S, GDP, and fusidic acid and separated from the inactive (■) molecules remaining in the supernatant. The pellet containing active EF-G was resuspended in buffer (10 mM Tris-HCl, pH 7.7, 800 mM KCl, and 5 mM 2-mercaptoethanol) and was purified by gel filtration through a Sephadex G-100 column (1 × 30 cm) equilibrated with the same buffer. The active EF-G molecules isolated in this way (▲) were tested in 50S binding and 50S and 70S ribosome dependent GTPase activity (see Materials and Methods) and compared to the binding of control EF-G molecules that had been subjected to the same isolation procedures (○). The [^{3}H]GDP (cpm) shown in the ordinate refers to either the GDP bound to Millipore filters in quaternary complexes with EF-G, fusidic acid, and ribosomes (A) or the GDP formed as a result of EF-G-dependent GTPase activity (B and C). The use of butanedione instead of GTP to protect the GTP binding site was prompted by the stability of the Arg–butanedione adduct vis-à-vis the prolonged PLP incubation time, the need to bypass a possible GTP-induced conformational change in EF-G that may affect the reactivity of the other lysines, and the desire to continue the correlation between the essential Arg, the essential Lys, and the GTP binding site.

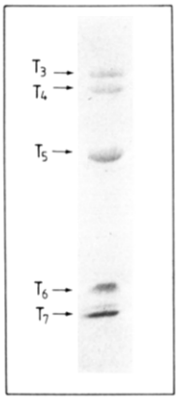

FIGURE 9: Electrophoretic separation of trypsin-resistant fragments of EF-G. The digestion, the electrophoretic conditions, and the nomenclature of the fragments (see also Tables I and II) are the same as previously described (Alakhov et al., 1979).

out in the presence or absence of GTP and with or without butanedione protection. In the first experiment EF-G with the essential arginine blocked by 2,3-butanedione was modified with PLP and nonradioactive sodium borohydride. After removal of the butanedione by dialysis, "active" and "inactive" EF-G fractions were obtained by 50S affinity purification essentially as described in Figure 8. The two EF-G fractions were then incubated with PLP, reduced with radioactive sodium borohydride, and subjected to tryptic hydrolysis, and the radioactivity associated with each fragment was determined. The results of two independent experiments, carried out with radioactive sodium borohydride having different specific activities, are presented in Table I.

In another experiment (Table II), EF-G was modified with PLP and radioactive borohydride in the presence and absence of GTP and the extent of modification of the various tryptic fragments determined as described above. From the results of these experiments (Tables I and II) we can conclude that the essential lysine residue protected by butanedione and GTP is located in fragment T4 (T3 is composed of T4 plus T7), which acquires more radioactivity after removal of butanedione (Table I) and which is less extensively labeled in the presence of the GTP ligand (Table II).

Table I: Effect of Arginine Blocking with 2,3-Butanedione on the Distribution of PLP among Different Tryptic Peptides[a]

		radioactivity (cpm) associated with peptides				
	samples	T3	T4	T5	T6	T7
expt 1	EF-G active	2 708	2 910	2 965	479	1 080
	EF-G inactive	1 710	1 965	2 894	475	1 003
expt 2	EF-G active	31 632	29 263	51 858	8 647	13 886
	EF-G inactive	22 543	20 639	51 157	8 198	13 208

[a] Active EF-G molecules isolated as described in Figure 8 were incubated with 4 mM PLP at 10 °C for 5 min. The reaction was stopped by addition of 0.1 volume of 100 mM NaB^3H_4. After exhaustive dialysis against 0.1 M Tris-HCl, pH 8.1, containing 5 mM 2-mercaptoethanol, the samples were subjected to limited trypsin hydrolysis and to $NaDodSO_4$ gel electrophoresis as described under Materials and Methods. The bands corresponding to EF-G fragments (Figure 9) were cut out and dissolved in 1 mL of soluene 350 (Packard), and the radioactivity was determined by scintillation counting.

Table II: Effect of GTP on the Distribution of PLP among Different Tryptic Peptides[a]

	radioactivity (cpm) associated with peptides				
samples	T3	T4	T5	T6	T7
EF-G	1086	2054	2783	285	688
EF-G plus 1 mM phosphate	1085	2013	2715	263	624
EF-G plus 1 mM GTP	771	1717	2691	229	599

[a] The reaction was carried out in buffer II as described in Figure 5. Where specified, the reaction mixture contained 1 mM GTP or 1 mM potassium phosphate. After a 5-min incubation at 10 °C, the reaction was stopped by addition of 0.1 volume of NaB^3H_4. The radioactivity associated with each trypsin-resistant fragment (Figure 9) was determined as described in Table I.

Discussion

The results presented in this paper show that an essential lysine residue is located in the GTP binding site of EF-G, close

to an essential Arg residue previously found in the same site by modification with 2,3-butanedione (Rohrbach & Bodley, 1977). Our conclusion concerning the proximity of these two residues is based on direct protection experiments in which EF-G was modified with PLP after blocking the essential arginine with butanedione. It is interesting to note that Rohrbach & Bodley (1977) found that the ionization of a group other than arginine, having a pK_a of 8.8 and present in the GTP binding site of the protein, facilitated the reaction of the essential arginine. It is possible that the ionizable group postulated by these authors is the lysine residue found in this study. Both the closeness implied by the complete protection of the lysine by GTP and the dependence upon ionization state suggest that the lysine is involved in GTP binding and/or hydrolysis rather than merely positioned near the GTP. The comparative slowness of the reaction of this lysine with PLP suggests its involvement in binding rather than as a catalytic nucleophile. As shown above (see Figures 1–4) several lines of evidence indicate that the total number of PLP-modifiable lysines in EF-G is 12 and that an average of about 8 mol of lysine are modified per mol of EF-G under standard reaction conditions resulting in ≥80% inhibition. Only one lysine, however, was found to be essential for GTP binding. Since GTP can protect EF-G from inactivation (Figure 5) and reduce somewhat the extent of PLP incorporation in fragment T4 (Table II), it is of interest to estimate the number of modified lysines in each EF-G fragment and to determine whether or not the extent of protection conferred by GTP on fragment T4 is compatible with the protection of a single lysine residue. Thus, taking into account that (a) under our conditions PLP modifies only lysine, (b) on the average eight lysines are modified per EF-G molecule under the conditions given in Table II, (c) the radioactivity associated with fragment T3 derives from both T7 and T4, and (d) fragment T7 (Alakhov et al., 1978) contains a single lysine residue (which we assume to be fully modified), we can estimate that the average distribution of PLP-reacted lysines among the EF-G fragments is the following: T4, 3–4; T5, 3–4; T6, <0.3; T7, 1. Furthermore, the extent of protection of the essential lysine in fragment T4 (including that present in T3) corresponds to ≥80% of an 80% modified lysine, in good agreement with the protection of activity shown in Figure 5.

Thus, the experiment shown in Table II (as well as that shown in Table I) indicates that the lysine in the GTP binding domain is localized in the trypsin-resistant fragment T4, the central domain of the EF-G molecule (Alakhov et al., 1979). According to a model proposed recently the N-terminal and C-terminal fragments of EF-G are joined by a hinge with the GTP binding center positioned in the space between the fragments (Kashparov et al., 1981). If the nucleotide binding site contains elements of more than one EF-G domain, it is not surprising that the lysine residue essential for GTP binding was found in fragment T4, while the target of EF-G photoaffinity labeling was in fragment T7 (Girshovich et al., 1978).

It is also clear from the results of this paper that the modification of one or more additional lysines, not involved in either GTP or 50S binding, impairs the ribosome-dependent GTPase activity of EF-G. Since inhibition of 70S-dependent GTPase activity is more sensitive than 50S-dependent GTPase to treatment with PLP, it is likely that this lysine is involved in the interaction between EF-G and 30S ribosomal subunits. It has indeed been suggested that EF-G contains a phosphate binding site for its interaction with 30S subunits (Parmeggiani & Sander, 1981). The inhibition of 50S-dependent GTPase, on the other hand, could stem from the PLP modification of either the same lysine or another lysine impairing a conformational change presumably required for GTP hydrolysis (Kashparov et al., 1981).

Finally, concerning the PLP binding site, it is known that this reagent acts as a cofactor for several enzymes while being a powerful inhibitor of others. It has recently been reported that striking similarities exist between the primary structure of the known PLP binding sites. Thus it was noticed that the PLP binding regions are rich in hydrophobic residues, correspond to regions where the protein structure is predicted to form a complete turn, and contain, in proximity to the reactive lysine, a basic or hydroxyl amino acid residue, supposedly an essential component of the anionic binding site (Minchiotti et al., 1981). The protection of lysine modification by blocking of the Arg residue, shown in this paper, not only characterizes the GTP binding site of EF-G as a typical PLP reactive site in this last respect but also offers direct evidence for the involvement of a nearby Arg in the anionic binding site.

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Time Dependence of Atomic Fluctuations in Proteins: Analysis of Local and Collective Motions in Bovine Pancreatic Trypsin Inhibitor†

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ABSTRACT: An analysis is made of the time dependence of the atomic motions obtained from two molecular dynamics simulations of the bovine pancreatic trypsin inhibitor; one simulation is for the protein in vacuum and the other for the protein in a van der Waals solvent with the atom size and density corresponding to those of water. Time series, correlation functions, and the time development of the mean square fluctuations are examined. A wide range of relaxation times is found for the displacement correlation functions (0.2–10 ps); the values of the relaxation times correlate with aspects of the structure and with the magnitude of the mean square displacements, as expected from the Langevin equation for an oscillator. It is shown that the atomic fluctuations which contribute to the temperature factor (thermal ellipsoid) can be separated into local oscillations superposed on motions with a more collective character. The former have a subpicosecond time scale; the latter, which can involve only a few neighboring atoms, a residue, or groups of many atoms in a given region of the protein, have time scales ranging from 1 to 10 ps or longer ($\bar{\nu} \simeq 3$–30 cm^{-1}). By following the time development of the atomic fluctuations from 0.2 to 25 ps, it is shown that the high-frequency oscillations, which contribute about 40% of the average root mean square fluctuations of main chain atoms, tend to be uniform over the structure. It is the longer time scale, more collective motions which introduce the variations in the fluctuation magnitudes that characterize different parts of the protein structure. These distributed modes are likely to be most sensitive to the external medium and to other environmental perturbations. Implications of these results for the function of proteins and analysis of anisotropic temperature factors are discussed.

For the interpretation of the structural and functional properties of proteins, it is essential to have a knowledge not only of the average positions of the atoms but also of the magnitudes and time scales of the fluctuations about the average positions (Karplus & McCammon, 1981). Recent X-ray diffraction studies of temperature factors for proteins have provided estimates of the magnitude of the fluctuations, expressed in terms of an isotropic and harmonic model (Frauenfelder et al., 1979; Artymiuk et al., 1979). The magnitudes of the mean square displacements calculated from molecular dynamics simulations have been shown to be in general agreement with the X-ray results (Northrup et al., 1980; van Gunsteren & Karplus, 1981, 1982a,b). Further, the simulation studies have demonstrated that the distribution functions for the atomic fluctuations tend to be highly anisotropic (Karplus & McCammon, 1979; Northrup et al., 1981, van Gunsteren & Karplus, 1981, 1982a,b) and somewhat anharmonic (Karplus & McCammon, 1979; Mao et al., 1982; van Gunsteren & Karplus, 1981, 1982a,b). X-ray estimates of the anisotropic character of the atomic motions have recently become available (Artymiuk et al., 1979; Konnert & Hendrickson, 1980; S. E. Phillips, unpublished experiments). By examining the temperature dependence of mean square displacements, Frauenfelder et al. (1979) have attempted to determine the anharmonic contributions to the local motions.

In the present paper, we focus on the time dependence of the atomic fluctuations that make the dominant contribution to the observed mean square displacements. These have been shown by simulation methods to occur on the picosecond time scale (McCammon & Karplus, 1980; Karplus & McCammon 1981). No data on the time scale of the motions are obtained from X-ray diffraction results, though other techniques (e.g., nuclear magnetic resonance, fluorescence depolarization, and Mössbauer spectroscopy) have shown that high-frequency fluctuations occur in proteins (Gurd & Rothgeb, 1979; Karplus & McCammon, 1981). We report here a detailed examination of the time dependence of the atomic fluctuations in the bovine pancreatic trypsin inhibitor (PTI), a small protein that has been used in a variety of motional studies (Karplus & McCammon, 1981). We analyze two simulations of this protein (van Gunsteren & Karplus, 1981, 1982a,b); the first simulation is for PTI in vacuum and the second for PTI in a van der Waals solvent composed of atoms with the size and particle density of water. The primary effect of the solvent

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