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Proton Nuclear Magnetic Resonance Study on the Roles of Histidine Residues in the Binding of Polypeptide Chain Elongation Factor Tu from *Thermus thermophilus* with Aminoacyl Transfer Ribonucleic Acid and Guanine Nucleotides†

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ABSTRACT: Proton nuclear magnetic resonance (^{1}H NMR) spectra were measured of the polypeptide chain elongation factor Tu (EF-Tu) from an extreme thermophile, *Thermus thermophilus* HB8 [Nakano, A., Miyazawa, T., Nakamura, S., & Kaziro, Y. (1979) *Arch. Biochem. Biophys. 196*, 233–238], in order to elucidate the environment around functionally important histidine residues. In the present study, the behavior of five histidine C_2 proton signals was studied in more detail. A hydrogen–deuterium exchange experiment was carried out on the histidine C_2 protons of free EF-Tu, and the previous assignments of C_2 proton signals were revised in part. An analysis of the ^{1}H NMR spectra of EF-Tu photooxidized under various conditions indicates that a histidine residue is located in the aminoacyl-tRNA binding site and is probably essential for the binding with aminoacyl-tRNA. A solvent-accessible histidine residue is found to lie near the aminoacyl-tRNA binding site. Furthermore, the effect of paramagnetic hexacyanochromate(III) ion on the ^{1}H NMR spectra of free EF-Tu suggests that another histidine residue lies near the guanine nucleotide binding site.

The polypeptide chain elongation factor Tu (EF-Tu)[1] promotes the GTP-dependent binding of aminoacyl-tRNA (aa-tRNA) to the A site of ribosomes [for reviews, see Miller & Weissbach (1977) and Kaziro (1978)]. EF-Tu has at least two active sites, one for binding with GDP or GTP and the other for interaction with aa-tRNA. The conformation around the aa-tRNA binding site is altered upon ligand substitution from GDP to GTP, and only EF-Tu·GTP, but not EF-Tu·GDP, can bind aa-tRNA to form a ternary aa-tRNA·EF-Tu·GTP complex. Upon binding of the ternary complex to the A site of ribosomes, GTP is hydrolyzed and then EF-Tu·GDP is released. For EF-Tu from *Escherichia coli*, various spectroscopic studies (Arai et al., 1974a, 1975, 1976; Crane & Miller, 1974; Wilson et al., 1978) have shown that the conformational transitions induced by the ligand change in fact occur near the cysteine residue in the aa-tRNA binding site. Recently, the complete amino acid sequence of *E. coli* EF-Tu has been determined (Arai et al., 1980) and the tertiary structure of the modified form of *E. coli* EF-Tu·GDP has been reported by X-ray analyses at 6.0-Å (Kabsch et al., 1977) and 2.6-Å (Morikawa et al., 1978) resolution. According to Morikawa et al. (1978), the molecule of *E. coli* EF-Tu consists of two or three domains and tracing of the main chain is difficult in the loose domain(s) which corresponds to ~60% of the whole molecule, probably because the local conformation in the loose domain(s) is more disordered than in the tight domain.

Nuclear magnetic resonance (NMR) spectroscopy provides important information about dynamic structure–function re-

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[1] Abbreviations used: EF-Tu, polypeptide chain elongation factor Tu; aa-tRNA, aminoacyl transfer ribonucleic acid.

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lationships of biomolecules and is especially useful for studying the structure of such a flexible molecule as EF-Tu. In the previous paper (Nakano et al., 1979), we have reported that EF-Tu (M_r 49000) from an extreme thermophile, *Thermus thermophilus* HB8 (Arai et al., 1978a,b; Nakamura et al., 1978), is much more stable than *E. coli* EF-Tu and is far more suitable for intensive NMR studies. From the analysis of ^{1}H NMR spectra of *T. thermophilus* EF-Tu and photooxidation experiments of histidine residues in EF-Tu, we have found that histidine residues are involved in the binding of *T. thermophilus* EF-Tu with both guanine nucleotides and with aa-tRNA and ribosomes. From the result of a hydrogen–deuterium exchange experiment, in the present study, we have partly revised the previous assignments of histidine C_2 proton signals. Furthermore, an analysis of the ^{1}H NMR spectra of EF-Tu photooxidized under various conditions has shown that a histidine residue is located in the aa-tRNA binding site and is probably essential for the binding with aa-tRNA. The microenvironments around some other histidine residues have also been elucidated.

Experimental Procedures

Materials. EF-Tu·GDP was purified from *T. thermophilus* HB8 by the method of Arai et al. (1978a). EF-Tu·GTP was prepared from EF-Tu·GDP by incubation with phosphoenolpyruvate and pyruvate kinase (Arai et al., 1974b). Nucleotide-free EF-Tu was prepared as described previously (Nakano et al., 1979). aa-tRNA was prepared by charging unfractionated *E. coli* tRNA (Schwarz/Mann) with 20 amino acids in the presence of ATP and the *E. coli* S-100 fraction (Arai et al., 1974b).

Pyruvate kinase and phosphoenolpyruvate were purchased from Boehringer. [8-^{3}H]GDP was from the Amersham Radiochemical Centre. All nucleotides were purified by Dowex 1 column chromatography (Kaziro et al., 1972). L-[U-^{14}C]-Phe-tRNA was also prepared as described previously (Arai et al., 1974b). Rose bengal was from Wako Pure Chemical Industries. NaO^2H, ^2HCl, and 99.75% ^2H_2O were obtained from E. Merck. Potassium hexacyanochromate(III), prepared by Bigelow's method (Fernelius, 1946), was a kind gift from K. Watanabe of the University of Tokyo.

Assays of EF-Tu. The [^{3}H]GDP binding activity of EF-Tu was measured by the nitrocellulose membrane filter procedure (Arai et al., 1972). The activity for promoting the binding of [^{14}C]Phe-tRNA to ribosomes was assayed as described before (Arai et al., 1974a).

^{1}H NMR Measurements. 270-MHz ^{1}H NMR spectra of EF-Tu were obtained on a Bruker WH270 pulse FT spectrometer at 50 °C. At least 1024 free induction decays were accumulated over 8192 data points, and the convolution difference technique (Campbell et al., 1973) was used to obtain well resolved spectra (Nakano et al., 1979). Sodium 2,2-dimethyl-2-silapentane-5-sulfonate was used as an internal reference for chemical shifts. A ^2H_2O solution of EF-Tu, containing 0.1 M NaCl and 0.1 mM dithiothreitol, was prepared as described before (Nakano et al., 1979). pH was adjusted by addition of 0.1 M ^2HCl or 0.1 M NaO^2H and was measured at 50 °C with a Radiometer PHM26 pH meter equipped with a Nisshin Rika CE-103 electrode. The direct meter readings are quoted in this paper. NMR spectra were measured only in the pH range from 5.5 to 8.0 because of the instability of samples out of this pH range (Nakano et al., 1979).

^{1}H NMR of Photooxidized EF-Tu. EF-Tu was photooxidized by using rose bengal as a photosensitizer (Takahashi, 1970; Nakano et al., 1979). In a typical large-scale experiment, 3 mL of a reaction mixture containing 0.2 mM free EF-Tu or EF-Tu·GDP and 0.1 mM rose bengal in 20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 5 mM 2-mercaptoethanol, and 0.1 M NH_4Cl was ice cooled, aerated by continuous stirring, and illuminated by a 375-W tungsten lamp from a distance of 15 cm. Aliquots were withdrawn at appropriate intervals, and the activity of EF-Tu to promote Phe-tRNA binding to ribosomes was measured. After the activity decreased sufficiently, the photooxidation was stopped by turning out the lamp. Then Norit was added to the solution and, after a few minutes, removed together with rose bengal by glass fiber filtration. In a large-scale photooxidation of the ternary aa-tRNA·EF-Tu·GTP complex, 15 mL of a reaction mixture contained 46 μM EF-Tu·GTP, 20 μM rose bengal, 89 μM aa-tRNA, 610 μM tRNA, and 100 μM excess GTP (with its regenerating system: pyruvate kinase and phosphoenolpyruvate) in 50 mM Tris-HCl (pH 7.5), 8 mM magnesium acetate, 10 mM 2-mercaptoethanol, and 0.15 M NH_4Cl. After the reaction, the dye was removed and solid ammonium sulfate was added to the solution to 75% saturation. The precipitate was collected by centrifugation and redissolved in 5 mL of 20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 5 mM 2-mercaptoethanol, and 0.1 M NH_4Cl. Then, with a large excess of GDP, the solution was incubated for 10 min at 37 °C to dissociate the ternary complex. The protein, now in the form of EF-Tu·GDP, was again collected by ammonium sulfate precipitation and dialyzed extensively against 20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 5 mM 2-mercaptoethanol, and 0.12 M NH_4Cl. It was then applied on a DEAE-Sephadex A-50 column (2.5 × 30 cm) equilibrated with 20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 5 mM 2-mercaptoethanol, and 0.1 M NH_4Cl. EF-Tu·GDP was eluted with 20 mM Tris-HCl (pH 7.5), 10 mM magnesium acetate, 5 mM 2-mercaptoethanol, and 0.3 M NH_4Cl and was completely separated from aa-tRNA, tRNA, and excess nucleotides. A control experiment was carried out in the same manner as above, except that EF-Tu·GTP and excess GTP (with its regenerating system) were replaced by EF-Tu·GDP and excess GDP. Another control experiment was also carried out, where aa-tRNA was replaced by deacylated tRNA in addition to the above replacement.

Results

pH Titration. The low-field region of the ^{1}H NMR spectra of EF-Tu is already shown in Figure 1 of the previous paper (Nakano et al., 1979). The pH titration data as obtained previously for free EF-Tu, EF-Tu·GDP, and EF-Tu·GTP are plotted in Figure 1, where the assignments for histidine C_2 proton signals are revised in part. Among the three figures (parts A–C of Figure 1), the C_2 proton signals with the same numerals are assigned to the same histidine residues. The last small letters, f, d, and t, denote *free* EF-Tu, EF-Tu·*GDP*, and EF-Tu·*GTP*, respectively. The asterisked signals are not affected by pH change and their intensities appreciably vary from one preparation to another, so that these signals probably arise from impurities which are not eliminated in the purification procedure of EF-Tu.

We suggested in the previous paper (Nakano et al., 1979) that a histidine residue in *T. thermophilus* EF-Tu was directly involved in the GDP binding on the assumption that signals H5f and H1d (in the notation of this paper) arose from the same histidine residue. However, this assumption was not proved because the exchange rate of bound GDP was extremely slow and it was only possible to show that the signal intensity of H5f was reduced while that of H1d was raised by

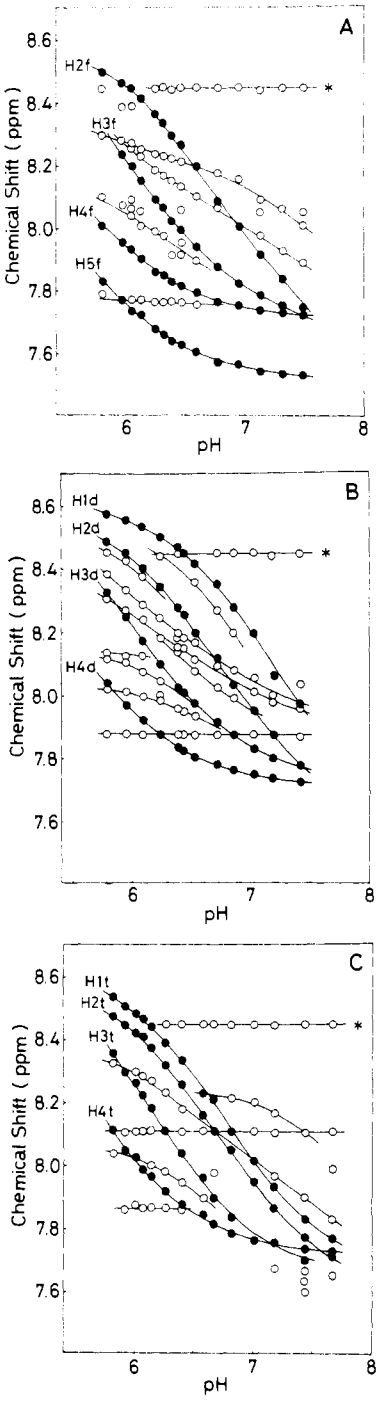

FIGURE 1: ^1H NMR titration curves of free EF-Tu (A), EF-Tu·GDP (B), and EF-Tu·GTP (C) in ^2H_2O solution at 50 °C. The same data as in the previous paper (Nakano et al., 1979) are plotted, and filled circles represent relatively well-defined titration curves. Signals with the same numerals are assigned to the same histidine residues, and the last small letters, f, d, and t, denote *free* EF-Tu, EF-Tu·*GDP*, and EF-Tu·*GTP*, respectively.

the addition of GDP. Accordingly, there was still an alternative possibility that these two signals, H5f and H1d, arose from different histidine residues. In the present study, the hydrogen–deuterium exchange experiment provided important data for solving this problem.

Hydrogen–Deuterium Exchange. A hydrogen–deuterium exchange experiment of histidine C_2 protons [see Markley (1975)] was carried out by incubating a ^2H_2O solution of free EF-Tu at pH 6.8, 50 °C (Figure 2). The exchange rates of signals H4f and H5f were slower than those of signals H2f and H3f; signals H4f and H5f were still observed after the

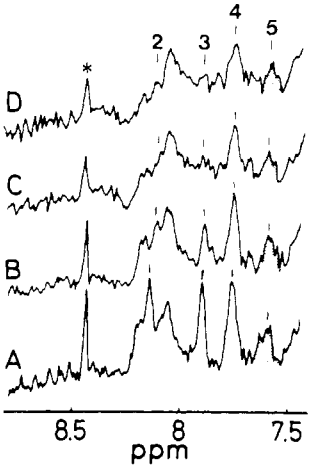

FIGURE 2: Effect of hydrogen–deuterium exchange on the ^1H NMR spectra of free EF-Tu in the histidine C_2 proton region. A ^2H_2O solution of 1 mM free EF-Tu containing 0.1 M NaCl and 0.1 mM dithiothreitol was incubated at pH 6.8 and 50 °C for 0 (A), 16 (B), 40 (C), and 64 h (D). The asterisked peak is from impurity.

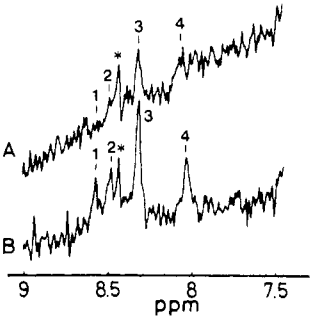

FIGURE 3: ^1H NMR spectra, in the histidine C_2 proton region, of EF-Tu·GDP which show the effect of hydrogen–deuterium exchange on the state of free EF-Tu. NMR samples (pH 5.8) were prepared by adding 1 mM GDP and 10 mM $MgCl_2$ to a ^2H_2O solution of 1 mM free EF-Tu. (A) Solution of free EF-Tu was incubated at 50 °C for 64 h (Figure 2D). (B) Solution of free EF-Tu was kept at 4 °C for a week. Signal H3 overlaps other signals at this pH (see Figure 1B).

incubation for as long as 64 h. Figure 3A shows the spectrum of EF-Tu·GDP, which was prepared by adding GDP to a ^2H_2O solution of free EF-Tu after incubation for 64 h (Figure 2D). In comparison, the control spectrum is shown in Figure 3B, which was obtained by adding GDP to a ^2H_2O solution of free EF-Tu kept at 4 °C.

Signal H5f of free EF-Tu remained with as much as 50% of the original intensity even after 64 h of incubation (Figure 2D), but signal H1d in the spectrum of EF-Tu·GDP (Figure 3A), as prepared by adding GDP to 64-h incubated free EF-Tu, was completely extinguished. The hydrogen–deuterium exchange of signal H1d must have occurred mostly during the incubation of free EF-Tu at 50 °C, since signal H1d is clearly observed in the control spectrum (Figure 3B). Therefore, the above observations demonstrate that signals H5f and H1d arise from *different* histidine residues. These signals are thus to be denoted with different numerals such as H5f and H*1*d, but not with the same symbol such as a filled square in Figure 2 of the previous paper (Nakano et al., 1979). The signal (H1f) from histidine residue H1 in free EF-Tu and the other signal (H5d) from histidine residue H5 in EF-Tu·GDP do not show up either, probably because of significant signal overlapping.

Binding of Hexacyanochromate(III) Anion. The hexacyanochromate(III) anion, $Cr(CN)_6^{3-}$, has a long electron spin relaxation time and accordingly may be used as a paramag-

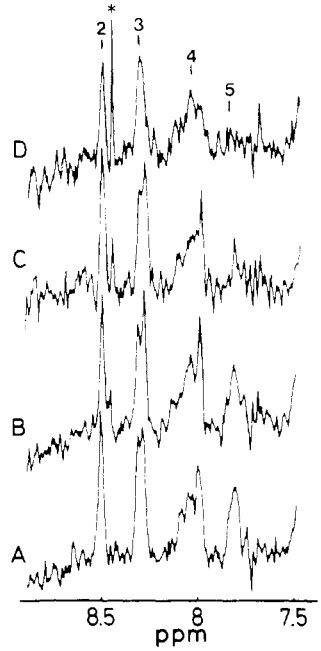

FIGURE 4: Effect of the addition of $Cr(CN)_6^{3-}$ ion on the ^1H NMR spectra of free EF-Tu (pH 6.1) in the histidine region. To a ^2H_2O solution of 1.5 mM free EF-Tu containing 0.1 M NaCl and 0.1 mM dithiothreitol was added $K_3Cr(CN)_6$ to a final concentration of 0 (A), 0.14 (B), 0.44 (C), and 1.44 mM (D).

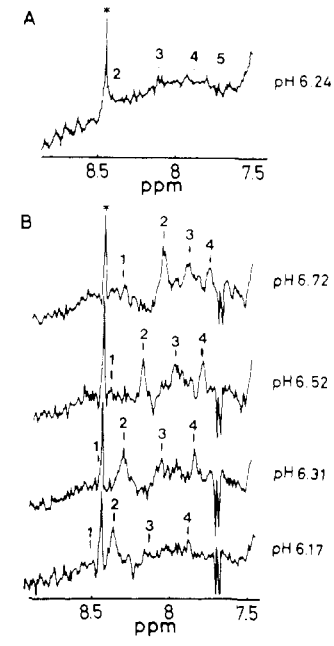

FIGURE 5: Histidine region of the ^1H NMR spectra of photooxidized EF-Tu. (A) Spectrum of photooxidized free EF-Tu (1.2 mM) in ^2H_2O solution at pH 6.24 containing 0.1 M NaCl and 0.1 mM dithiothreitol. (B) Spectra of photooxidized EF-Tu·GDP (1.2 mM) in ^2H_2O solution at various pH values containing 10 mM $MgCl_2$, 0.1 M NaCl, and 0.1 mM dithiothreitol. Photooxidation was carried out on free EF-Tu and EF-Tu·GDP, respectively, as described under Experimental Procedures. The asterisked peak arises from impurity, and the negative doublet peak at 7.7 ppm is due to quadrature fold-over of the intense peak at 1.3 ppm.

netic relaxation probe in ^1H NMR studies of enzymes (Campbell et al., 1974, 1975). This anion is especially useful for mapping the active sites in cationic environments such as the nucleotide binding site of ribonuclease A (Inagaki et al., 1979).

In fact, the spectrum of free EF-Tu at pH 6.1 was affected by the addition of $Cr(CN)_6^{3-}$ ion (parts A–D of Figure 4); signal H5f was selectively broadened, while the other signals were little affected. On the contrary, no spectral change was observed upon the addition of $Cr(CN)_6^{3-}$ ion to a solution of EF-Tu·GDP or EF-Tu·GTP at pH 5.8 (data not shown). These observations suggest that $Cr(CN)_6^{3-}$ ion is bound to the nucleotide binding site of *T. thermophilus* EF-Tu, as is the case for ribonuclease A (Inagaki et al., 1979), and that histidine residue H5 lies near the guanine nucleotide binding site of EF-Tu. There may be another possibility, that selective broadening of signal H5d (H5t) in the spectrum of EF-Tu·GDP (·GTP) is just not observed because of the significant signal overlapping. Nevertheless, the environment around histidine residue H5 is certainly altered upon the binding of GDP or GTP, since the pH titration curve of histidine residue H5 shows an appreciable shift into the signal overlapping region by the binding of GDP or GTP (Figure 1). Signal H5f exhibits a relatively slow hydrogen–deuterium exchange rate (Figure 2) and is broader than other numbered signals, indicating that this histidine residue H5 is buried and immobilized to some extent. Probably, histidine residue H5 is not directly involved in the binding with guanine nucleotides, although it lies near the guanine nucleotide binding site.

^1H NMR Spectra of Photooxidized EF-Tu. As an aid in the assignment of histidine C_2 proton signals to functionally important histidine residues, ^1H NMR spectra of EF-Tu photooxidized under various states were analyzed. Figure 5A shows the spectrum of photooxidized free EF-Tu (at pH 6.24), where the histidine C_2 proton signals H2f–H5f are hardly observed. On the other hand, in the spectra of photooxidized EF-Tu·GDP (at various pH values), several C_2 proton signals can be observed as shown in Figure 5B. Signals H2d and H4d are clearly observed, whereas the intensities of signals H1d and H3d are markedly diminished as compared with those of intact EF-Tu·GDP (Figure 3B). Signal H1d is almost completely extinguished out. These observations indicate that the histidine residues responsible for signals H1d and H3d are not protected from photooxidation by the binding of GDP.

Next, the role of these histidine residues in the binding with aa-tRNA was investigated by analyzing the ^1H NMR spectra of EF-Tu photooxidized in the form of ternary aa-tRNA·EF-Tu·GTP complex. Because a large number of base proton signals of aa-tRNA appear in the histidine C_2 proton region (9.0–7.5 ppm), the spectra of EF-Tu·GDP photooxidized in the form of the ternary complex are analyzed after isolation of EF-Tu·GDP from the ternary complex. They are shown in Figures 6A (pH 5.9), 6B (pH 6.7), 6C (pH 6.0), and 6D (pH 6.8). In each pair of spectra, the lower trace was obtained from the photooxidation of the ternary complex, while the upper trace was obtained from the control experiment on tRNA + EF-Tu·GDP (parts A and B of Figure 6) or on aa-tRNA + EF-Tu·GDP (parts C and D of Figure 6). As clearly shown in the lower trace of Figure 6A, signals H1d and H3d are now observed even after the photooxidation in the form of the ternary complex, indicating that the histidine residues H1 and H3 are protected by the binding with aa-tRNA. In contrast, the intensities of signals H1d and H3d are reduced in the control experiment (the upper trace of Figure 6A), where EF-Tu·GDP does not bind deacylated tRNA at all and accordingly suffers photooxidation to the same extent as in the photooxidation of EF-Tu·GDP alone. Figure 6C shows that signal H1d appears to be protected even in the control experiment where aa-tRNA + EF-Tu·GDP was photooxidized, while the signal intensity of H3d is still decreased in this control experiment. At higher pH (parts B and D of Figure 6), signals H2d–H4d are well resolved, while signal

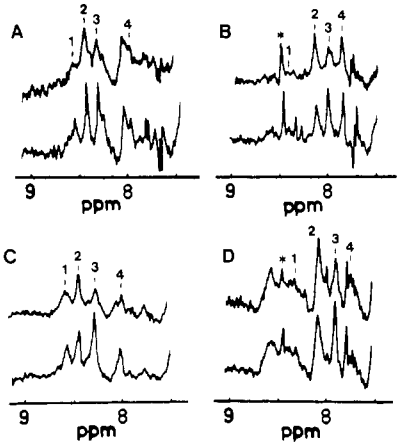

FIGURE 6: ^{1}H NMR spectra, in the histidine C_2 proton region, of EF-Tu·GDP which show the effect of aa-tRNA binding on photooxidation. In each pair of spectra at pH 5.9 (A), 6.7 (B), 6.0 (C), and 6.8 (D), the lower trace was obtained from the photooxidation of the ternary complex aa-tRNA·EF-Tu·GTP. The upper traces were obtained from the control experiments: photooxidation on tRNA + EF-Tu·GDP (A and B) or photooxidation on aa-tRNA + EF-Tu·GDP (C and D). The sample solutions commonly contained about 1 mM EF-Tu·GDP with 10 mM $MgCl_2$, 0.1 M NaCl, and 0.1 mM dithiothreitol. For details, see Experimental Procedures.

H1d is not strong probably because of its faster hydrogen–deuterium exchange rate.

As will be discussed later, histidine residue H3 does not appear to be important in the function of EF-Tu, and therefore histidine residue H1 is concluded to lie in the aa-tRNA binding site and to be essential for the binding with aa-tRNA.

Discussion

Assignment of Histidine C_2 Proton Signals. Histidine residues have been found to be important in *T. thermophilus* EF-Tu for binding both with guanine nucleotides and with aa-tRNA (Nakano et al., 1979). Although the tertiary structure of this EF-Tu is not yet known, it is possible to assign the histidine C_2 proton signals to the functionally important histidine residues in the ^{1}H NMR spectra of EF-Tu. In fact, some histidine C_2 proton signals are affected either by the binding of GDP or by the ligand substitution from GDP to GTP (Figure 1). However, because the exchange rate of bound nucleotides is extremely slow as compared with the NMR chemical shift time scale, the correspondence of signals between different states of EF-Tu, such as between free EF-Tu and EF-Tu·GDP, has not been determined uniquely by a simple comparison of pH titration curves or by the observation of spectral change upon the gradual addition of GDP (Nakano et al., 1979). In the present study, however, the hydrogen–deuterium exchange experiment was found to be of great use for the identification of such signals between different states. That is, signals H5f and H1d (Figure 1) were proved to arise from different histidine residues. This hydrogen–deuterium exchange method will be promising even for a larger protein, with well resolved histidine signals, if the protein is resistant to incubation at relatively high temperature.

Binding of Paramagnetic Anion. The paramagnetic probe effect by $Cr(CN)_6^{3-}$ ion suggests that this anion is bound to the guanine nucleotide binding site of EF-Tu and that histidine residue H5 lies near that binding site. However, the structure of free EF-Tu appears to be rather mobile, so that there is still an alternative possibility that histidine residue H5 lies in another region, e.g., near the hinge region of two domains of EF-Tu (Morikawa et al., 1978), which may be open to $Cr(CN)_6^{3-}$ ion only in the absence of guanine nucleotides. In such a case, the observation that the pH titration curve of histidine residue H5 shifts into the signal overlapping region by the binding of GDP or GTP (Figure 1) may also be explained by the local conformational transition induced by the binding with guanine nucleotides.

Effect of GDP Binding on Photooxidation. Photooxidation of *T. thermophilus* EF-Tu exclusively modifies histidine residues (Nakano et al., 1979). Therefore, an analysis of ^{1}H NMR spectra of EF-Tu photooxidized under various conditions is expected to provide important information for studying the microenvironments of histidine residues. From a comparison of the spectra of photooxidized free EF-Tu (Figure 5A) and photooxidized EF-Tu·GDP (Figure 5B), almost all the histidine signals are found to disappear unless GDP is bound to EF-Tu during photooxidation. Here, the histidine residues responsible for the remaining signals (H2d and H4d) in the spectrum of photooxidized EF-Tu·GDP (Figure 5B) are not necessarily protected from photooxidation directly by the binding with GDP, since the apparent pK_a values of these histidine signals are little affected by the GDP binding. These histidine residues may also be protected indirectly through the structural stabilization of EF-Tu brought about by the binding of GDP.

In contrast to the signals H2d and H4d, the intensities of signals H1d and H3d are apppreciably diminished by the photooxidation of EF-Tu·GDP (Figure 5B). This observation is consistent with the result of amino acid analysis that *two* histidine residues are modified by the photooxidation of *T. thermophilus* EF-Tu·GDP (Nakamura et al., unpublished experiments). It may be noted here that the titration curve of histidine residue H1 is most significantly affected by the ligand substitution from GDP to GTP (parts B and C of Figure 1) and the apparent pK_a of this histidine residue is decreased by 0.3–0.4 pH unit (Nakano et al., 1979). There are two possible interpretations for this observation: (1) histidine residue H1 is directly involved in the binding with guanine nucleotides and the electrostatic interaction between the imidazolium group and the phosphate group affects the pK_a of histidine residue H1 or (2) the conformational transition induced by the ligand substitution from GDP to GTP alters the microenvironment around histidine residue H1. In the latter case, histidine residue H1 is possibly located in the aa-tRNA binding site, as is the case for a cysteine residue of *E. coli* EF-Tu (Arai et al., 1974a, 1975, 1976; Crane & Miller, 1974; Wilson et al., 1978). The photooxidation experiment on EF-Tu·GDP in the present study clearly rules out the former interpretation. That is, signal H1 disappeared even by the photooxidation of EF-Tu in the presence of GDP, indicating that this histidine residue H1 was not protected by the binding with GDP. Since the GDP binding activity of free EF-Tu is protected from photooxidation by the binding with GDP (Nakano et al., 1979), histidine residue H1 is not directly involved in the GDP binding.

Binding Site for aa-tRNA. The activity of EF-Tu for promoting the aa-tRNA binding to ribosomes is lost if EF-Tu is photooxidized in the absence of aa-tRNA, whereas this activity is protected from photooxidation by the binding of GTP and aa-tRNA (Nakano et al., 1979). Therefore, an analysis of the ^{1}H NMR spectra of EF-Tu, photooxidized with and without protection by aa-tRNA binding, is expected to be important for identifying the C_2 proton signal of the histidine residue(s) essential for aa-tRNA binding. The intensities of signals H1d and H3d are reduced by the photooxidation of EF-Tu·GDP (Figure 5B) but are not decreased by the photooxiddation in the form of ternary complex (see Figure

6A), indicating that histidine residues H1 and H3 of EF-Tu are protected finally by the binding with aa-tRNA. However, histidine residue H3 does not appear to be important for the binding with aa-tRNA since the titration curve of H3 is not affected by the binding of GDP nor the ligand substitution from GDP to GTP (Figure 1). Furthermore, this signal H3d is significantly sharp, suggesting that histidine residue H3 is located in a mobile environment such as the molecular surface of EF-Tu. Therefore, it may be concluded that the histidine residue H1 *is* located in the aa-tRNA binding site and *is* essential for the binding with aa-tRNA. In fact, the appparent pK_a of histidine residue H1 is most markedly affected by the ligand change from GDP to GTP (parts B and C of Figure 1). The conclusion that histidine residue H1 is located in the aa-tRNA binding site therefore means that the microenvironment around H1 in the aa-tRNA binding site is allosterically altered on the ligand substitution from GDP to GTP. It may be recalled, for the case of *E. coli* EF-Tu, that the conformational transition around the aa-tRNA binding site is controlled by the ligand substitution in the guanine nucleotide binding site [see Kaziro (1978)]. Such a scheme is now confirmed more clearly on *T. thermophilus* EF-Tu. The change in the apparent pK_a of histidine residue H1 upon the ligand change will be understood in more detail if the primary or higher order structure of this protein is determined in the future.

Signal H1d appears to be protected also in the control experiment where aa-tRNA + EF-Tu·GDP is photooxidized (parts C and D of Figure 6). In fact, the decrease in the activity of EF-Tu for promoting aa-tRNA binding to ribosomes is appreciably slower in the photooxidation of aa-tRNA + EF-Tu·GDP than in that of tRNA + EF-Tu·GDP (data not shown), indicating that the activity of EF-Tu·GDP is protected from photooxidation to some extent in the presence of aa-tRNA. Although the complex formation between aa-tRNA and EF-Tu·GDP cannot be detected by the ususal assay method (Gordon, 1968; Arai et al., 1974c), there is possibly a weak interaction between aa-tRNA and EF-Tu·GDP at a high concentration of ~ 100 μM and this interaction may protect histidine residue H1 in the aa-tRNA binding site against the photooxidation. In this connection, Ringer & Chládek (1975) have previously reported that there is certainly an interaction between *E. coli* EF-Tu·GDP and the aminoacyl dinucleoside phosphates with a nucleotide sequence at the 3′ terminus of aa-tRNA.

The intensity of signal H3d is still decreased by the photooxidation of aa-tRNA + EF-Tu·GDP (parts C and D of Figure 6), indicating that a weak interaction between aa-tRNA and EF-Tu·GDP does not protect histidine residue H3. This is reasonable if histidine residue H3 is exposed to solvent on the molecular surface around the aa-tRNA binding site. Thus, signal H3 is protected from photooxidation only in the tight ternary complex aa-tRNA·EF-Tu·GTP.

Conclusion. The microenvironments of five histidine residues in *T. thermophilus* EF-Tu were analyzed in the present study. The binding experiments of hexacyanochromate(III) ion suggested that signal H5 arises from a histidine residue near the guanine nucleotide binding site but is not directly involved in the binding with guanine nucleotides. From the pH titration of histidine C_2 protons and the NMR analyses of photooxidized EF-Tu, signal H1 was assigned to a histidine residue which is located in the aa-tRNA binding site and is probably essential for the binding with aa-tRNA. The histidine residue corresponding to signal H3 is exposed to solvent near the aa-tRNA binding site.

Recently, a preliminary experiment on the photooxidative inactivation of *E. coli* EF-Tu has shown that histidine residues are also important in the function of *E. coli* EF-Tu in a similar manner as those of *T. thermophilus* EF-Tu. The activity of *E. coli* EF-Tu for binding with GDP is protected from photooxidation by the GDP binding, while the activity for promoting aa-tRNA binding to ribosomes is protected only by the binding with both GTP and aa-tRNA (Nakamura et al., unpublished experiments). These observations suggest that the structures of the active sites are similar for *E. coli* EF-Tu and *T. thermophilus* EF-Tu, with respect to the microenvironments around histidine residues. Therefore, the identification of functionally essential histidine residues in the amino acid sequence of *E. coli* EF-Tu is expected to provide important information for the architecture of active sites not only in *E. coli* EF-Tu but also in *T. thermophilus* EF-Tu.

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Thermodynamic and Conformational Studies on *sn*-2-Phosphatidylcholines in Monolayers and Bilayers†

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ABSTRACT: 1,3-Dipalmitoyl-*sn*-glycero-2-phosphocholine has been spread at an air–water interface. The area–pressure isotherms of the *monolayer* have been recorded by using the Wilhelmy plate method and are compared with those of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine. At a given surface pressure the *sn*-2-phosphatidylcholine is always found to be more expanded than the *sn*-3-phosphatidylcholine. The thermodynamic behavior of *bilayers* composed of 1,3-dipalmitoyl-*sn*-glycero-2-phosphocholine was investigated by means of differential scanning calorimetry. The lipid undergoes an endothermic pretransition at 17–23 °C with a transition enthalpy of ΔH = 1.7–4.2 kcal/mol and an endothermic gel to liquid-crystal transition at 37 to 38 °C with ΔH = 9.4 kcal/mol. The pretransition is strongly dependent on the prehistory of the sample. The conformational properties of 1,3-dipalmitoyl-*sn*-glycero-2-phosphocholine in liquid-crystalline bilayer membranes were studied by deuterating the lipid at various chain and head group segments and measuring the deuterium and phosphorus-31 nuclear magnetic resonance spectra of the corresponding bilayer phases. The ^2H and ^{31}P NMR parameters of the phosphocholine head group are very similar for *sn*-2- and *sn*-3-phosphatidylcholines, suggesting almost identical head group structures for the two lipids. On the other hand, distinct conformational differences are observed for the hydrocarbon region. In bilayers of *sn*-2-phosphatidylcholine both fatty acyl chains begin parallel to the bilayer surface and are bent perpendicular to it after the C-2′ segment. In corresponding bilayers of *sn*-3-phosphatidylcholines only the *sn*-2 chain is bent, whereas the *sn*-1 chain is perpendicular to the membrane surface at all segments. As a consequence of the unusual conformation at the chain beginnings, the hydrocarbon interior of bilayers of *sn*-2-phosphatidylcholines appears to be more disordered than that of the *sn*-3 analogue.

sn-2-Phosphatidylcholines (β-lecithins), though not naturally occurring, have been shown to be substrates for phospholipase A_2, and the enzyme specifically cleaves the ester bond at the C-1 position (de Haas & van Deenen, 1963, 1964). In the case of naturally occurring *sn*-3-phosphoglycerides phospholipase A_2 catalyzes the hydrolysis of the C-2 ester bond. The enzyme acts highly stereospecifically, and only *sn*-3-phospholipids are degraded whereas the *sn*-1-phospholipids are competitive inhibitors characterized by the same binding constant as the *sn*-3 stereoisomers (Bonsen et al., 1972a,b). The stereospecificity of phospholipase A_2 action originates from the size, the shape, and the chemical nature of the active site, and the question can be raised of how the conformation of *sn*-2-phosphatidylcholines compares with that of the naturally occurring *sn*-3 analogues.

In the present study we have investigated this problem with deuterium and phosphorus-31 nuclear magnetic resonance (Seelig, 1977, 1978). For this purpose 1,3-dipalmitoyl-*sn*-glycero-2-phosphocholine was selectively deuterated at the C-2′ segment of the *sn*-3 chain and at segments 2′, 3′, 4′, and 8′ of the *sn*-1 chain. The hydrocarbon chain ordering in the presence and absence of cholesterol was deduced from the residual deuterium quadrupole splittings. Analogously, the conformational properties of the head group were studied by selective deuteration of the two choline methylene segments, whereas the motion of the phosphate segments was studied with phosphorus-31 nuclear magnetic resonance. In addition, the thermodynamic properties of 1,3- and 1,2-dipalmitoyl-*sn*-glycerophosphocholine were compared by means of differential scanning calorimetry and monolayer experiments.

Materials and Methods

Chemical Synthesis. Selectively deuterated fatty acids (labeled at carbon atoms no. 2, 3, 4, and 8) were synthesized as described previously (Seelig & Seelig, 1974). 1,3-Bis([2′,2′-$^2H_2$]palmitoyl)-*sn*-glycero-2-phosphocholine was prepared from the corresponding deuterated 1,3-dipalmitin according to Hirt & Berchtold (1958). Phospholipase A_2 degradation led to the lyso compound 3-[2′,2′-$^2H_2$]palmitoyl-*sn*-glycero-2-phosphocholine which was reacylated with C-3-, C-4-, and C-8-deuterated palmitic acid (de Haas & van Deenen, 1963).

[1-$^2H_2$]Choline iodide, $(CH_3)_3NCD_2CH_2OH^+I^-$, and [2-$^2H_2$]choline iodide, $(CH_3)_3NCH_2CD_2OH^+I^-$, were synthesized as described by Gally et al. (1975). 1,3-Dipalmitin was made from *rac*-2-benzylglycerol as described by Bonsen et al. (1972a,b) and was reacted with $POCl_3$ and deuterated cholines following the method of Eibl (1978). The head group deuterated 1,3-dipalmitoyl-*sn*-glycero-2-phosphocholines are abbreviated as $^+NCD_2CH_2O$-DPPC and $^+NCH_2CD_2O$-DPPC.

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