Spectroscopic Studies of the Nucleotide Binding Site of Elongation Factor Tu from *Escherichia coli*. An Approach to Characterizing the Elementary Steps of the Elongation Cycle of Protein Biosynthesis†

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ABSTRACT: 9-β-Ribofuranosyl-2-amino-6-mercaptopurine 5′-diphosphate (thioGDP), thioGTP, and 2-azidoinosine 5′-diphosphate have been shown to bind to elongation factor Tu (EF-Tu) from *Escherichia coli*. The binding of thioGDP results in a 4-nm red shift of the absorption band of thioGDP, an increase of approximately 0.7 pH unit of the p*K* of the thiol group, and the formation of a circular dichroism spectrum of the nucleotide distinctly different from that of the free nucleotide. These results are consistent with the purine moiety being bound in a highly hydrophobic site of EF-Tu with specific interactions of the thione group with the protein. When thioGTP binds to EF-Tu, the red shift of the spectrum is only 2 nm, the p*K* of the thiol group is increased by 0.1 pH unit, and the CD spectrum is less intense. This indicates that the nucleotide binding site is less hydrophobic in EF-Tu·thioGTP as compared to EF-Tu·thioGDP. The spectral perturbations have allowed the rate constants for the binding of thioGDP and thioGTP to EF-Tu to be determined by stopped-flow spectrophotometry in the presence of 5 mM $MgCl_2$, at 20 °C and pH 7.6. Values of 1.1×10^5 and 1.6×10^4 M^{-1} s^{-1}, respectively, were obtained. Similarly, values of the dissociation rate constants were 0.01 s^{-1} for thioGDP and 0.21 s^{-1} for thioGTP. The dissociation rate constant of Mg from EF-Tu·Mg·thioGDP was found to be 1.4 s^{-1}, showing that Mg can dissociate independently of the nucleotide. The spectroscopic and kinetic data show that the nucleotide binding site of EF-Tu has some properties similar to that of myosin but also some marked differences. ThioGTP substitutes for GTP in the EF-Tu-dependent binding of aminoacyl-tRNA to ribosomes, and the dissociation of thioGDP from EF-Tu is catalyzed by elongation factor Ts. The potential of thioGTP for investigating the kinetic steps of the elongation cycle of protein biosynthesis by stopped-flow spectrophotometry is discussed.

The prokaryotic elongation factor Tu (EF-Tu)[1] is involved in the elongation cycle of protein biosynthesis by forming a ternary complex with GTP and aminoacyl-tRNA which then binds to the ribosome containing mRNA. GTP is hydrolyzed, the aminoacyl-tRNA is positioned at the A site prior to subsequent peptide bond formation, and a binary complex of EF-Tu·GDP is released [reviewed by Miller & Weissbach (1977)]. In order to understand the role of EF-Tu and GTP in the fast and accurate incorporation of amino acids into the growing peptide chain, we are investigating the elementary steps of the elongation cycle by using rapid reaction kinetic techniques combined with single turnover conditions which have given important information about the mechanism of other energy-transducing nucleoside 5′-triphosphatases (Trentham et al., 1976). Steps involving covalent bond breaking or formation can be followed by chemical quenching, and the rate of GTP hydrolysis and peptide bond formation has been measured when Phe-tRNA·EF-Tu·GTP binds to poly(U)-programmed ribosomes containing acetyl-Phe-tRNA in the P site (Thompson et al., 1980). Steps involving binding, rearrangements, or product release are more difficult to study but can be followed by intrinsic or extrinsic spectral probes. For this reason, I have investigated the binding of potential chromophoric and fluorescent analogues of guanosine nucleotides to EF-Tu.

EF-Tu is a globular protein with a molecular weight of 43 225. Its primary sequence has been determined (Arai et al., 1980), and a high-resolution (2.6-Å) X-ray diffraction analysis has been made on a modified form of the EF-Tu·GDP complex (Morikawa et al., 1978). EF-Tu binds GDP with a K_d of 3×10^{-9} M and GTP with a K_d of 3×10^{-7} M, both measurements being at 20 °C and pH 7.4 in the presence of 5 mM $MgCl_2$ (Miller & Weissbach, 1977). The major functional difference between EF-Tu·GTP and EF-Tu·GDP is that the former complex binds aminoacyl-tRNA ($K_d \sim 10^{-8}$ M) whereas EF-Tu·GDP does not bind aminoacyl-tRNA [or if it does, with a K_d of greater than 10^{-3} M (Shulman et al., 1974)]. Conformational differences between EF-Tu·GTP and EF-Tu·GDP have been studied by a variety of techniques [reviewed by Kaziro (1978)].

Thioguanosine and its corresponding nucleotides (Figure 1) have an absorption maximum at 342 nm enabling accurate spectral measurements to be made in the presence of protein. A weak negative CD peak is associated with this absorption band. The 6-mercapto group ionizes with a p*K* in the region of 8.2–8.6, the resulting anionic form having an absorption maximum at 320 nm. The absorption spectrum, p*K*, and CD spectrum of thioGTP and thioGDP are perturbed when these nucleotides bind to myosin, and the absorption changes have been used to investigate the nucleotide binding site of myosin and determine the kinetic constants of the elementary steps of the myosin ATPase mechanism (Eccleston & Trentham, 1977, 1979; Eccleston & Bayley, 1980). Potential fluorescent analogues of guanosine nucleotides are derived from 2-azidoinosine and 9-β-ribofuranosyl-2-aminopurine. ThioGDP has previously been shown to bind to EF-Tu (Wittinghofer et al., 1977), and 2-azidoinosine 5′-diphosphate has been shown to bind to the eukaryotic elongation factor 1 (Jerome & Heintz, 1980).

The purpose of the work reported here is 2-fold. First, differences in the nucleotide binding site of EF-Tu bound to

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[1] Abbreviations used: EF-Tu, elongation factor Tu; EF-Tu$_f$, nucleotide-free EF-Tu; EF-Ts, elongation factor Ts; thioGTP, 9-β-ribofuranosyl-2-amino-6-mercaptopurine 5′-triphosphate; thioGDP, 9-β-ribofuranosyl-2-amino-6-mercaptopurine 5′-diphosphate; butyl-PBD, 5-(4-biphenylyl)-2-(4-*tert*-butylphenyl)-1,3,4-oxadiazole; HPLC, performance liquid chromatography.

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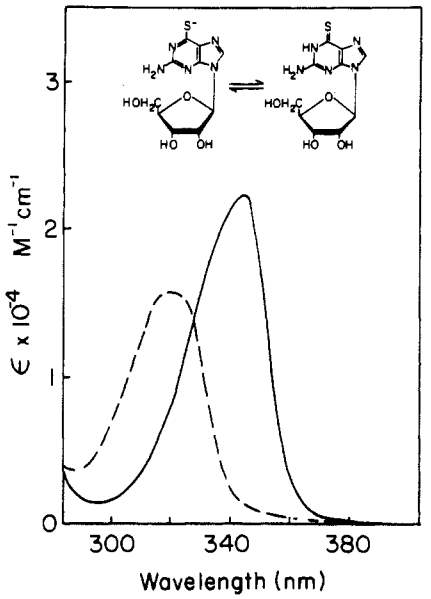

FIGURE 1: Structures of the thione and monoanionic forms of thioguanosine and their absorption spectra. Spectra of thioguanosine were recorded in 0.1 M acetate, pH 4.5 (thione form, λ_{max} = 342 nm), or in 10 mM NaOH (monoanionic form, λ_{max} = 320 nm).

nucleoside 5′-diphosphate and 5′-triphosphate are determined. Second, the potential of the analogues for allowing the measurement of the equilibrium and kinetic constants of the interactions of EF-Tu in the elongation cycle of protein biosynthesis is investigated. These measurements in the past have been made by using filtration techniques and the ability of EF-Tu to bind to cellulose nitrate. While major advances have been made in understanding the role of EF-Tu in protein biosynthesis by using this technique, it is necessarily rather limited in that measurements are made under nonequilibrium conditions, and fast processes cannot be followed. Since the in vivo rate of peptide elongation is on the order of 20 s^{-1} (Gausing, 1972), the elementary processes of this elongation cycle must be at least as fast as this, and therefore, it is necessary to be able to follow reactions on this time scale.

Materials and Methods

Assays of EF-Tu·GDP, EF-Tu_f, and EF-Ts were performed as described by Miller & Weissbach (1974). EF-Tu·GDP was prepared from *E. coli* (Grain Processing Inc., Muscatine, IA) by the method of Leberman et al. (1980) and had a binding capacity of greater than 19 000 pmol of GDP bound per mg of protein. The GTPase activity of the preparations was determined by incubating a solution containing 5 μM EF-Tu·GDP, 100 μM [γ-^{32}P]GTP, 50 mM Tris-HCl, pH 7.2, 50 mM KCl, 50 mM NH_4Cl, and 5 mM $MgCl_2$ for 4 h at 0 °C or 1 h at 20 or 37 °C. [^{32}P]P_i was then measured after extraction as the phosphomolybdate complex into isobutyl alcohol–benzene (1:1) (Martin & Doty, 1948). In most preparations, no detectable hydrolysis (<1 μM [^{32}P]P_i formed) was observed even at 37 °C. Occasional preparations gave a measurable rate of hydrolysis, and these were further purified by back-extraction of an ammonium sulfate precipitate of the EF-Tu·GDP as described by Miller & Weissbach (1974). EF-Tu_f was prepared from EF-Tu·GDP as described by Wittinghofer & Leberman (1976) except that the original incubation mixture contained 30 mg of EF-Tu·GDP and 200 mM ITP in a total volume of 1 mL. This increase in the scale of the procedure resulted in incomplete removal of GDP, and preparations of EF-Tu_f contained approximately 30% EF-Tu·GDP as determined by assays for EF-Tu_f and EF-Tu·GDP and by

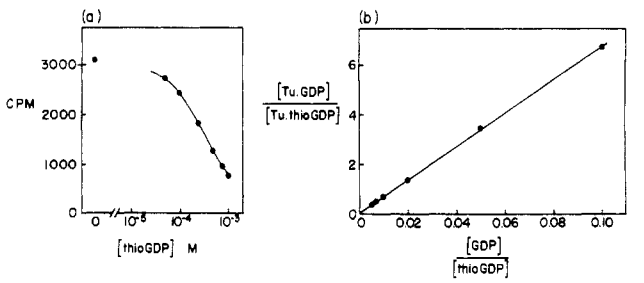

FIGURE 2: Displacement of [^{3}H]GDP from EF-Tu·[^{3}H]GDP by thioGDP. The solutions (total volume 0.26 mL) contained 0.13 μM EF-Tu·GDP, 7.7 μM [^{3}H]GDP (93 cpm/pmol), 50 μM to 1 mM thioGDP, 7.7 mM Tris-HCl, 3.8 mM $MgCl_2$, 6.1 mM KCl, 6.1 mM NH_4Cl, and 0.7 mM DTT, pH 7.6. After incubation at 20 °C for 10 min, the amount of EF-Tu·[^{3}H]GDP was determined by cellulose nitrate filtration as described under Materials and Methods.

HPLC analysis of the nucleotide content of the preparations (see below). The GTPase activity of EF-Tu_f preparations measured as described above for EF-Tu·GDP showed that the rate of formation of P_i was 0.06 and 0.14 μM min^{-1} at 20 and 37 °C, respectively. These rates are comparable to the rates of hydrolysis of EF-Tu·GTP observed by Miller & Weissbach (1970). EF-Tu·thioGDP was prepared by adding a 3-fold excess of thioGDP to a 1 mg/mL solution of EF-Tu_f at pH 8.0 and 5 mM $MgCl_2$. After the solution stood for 5 min at 25 °C, it was made 60% saturated with $(NH_4)_2SO_4$, and the protein was centrifuged down and dissolved in the appropriate buffer. EF-Tu·thioGTP was prepared in the same way, using EF-Tu_f and thioGTP. It was also prepared from EF-Tu·thioGDP by using a regenerating system of phosphoenolpyruvate and pyruvate kinase (see Figure 3 for details). Concentrations of EF-Tu·thioGDP and EF-Tu·thioGTP were determined from the known extinction coefficients of thioguanosine nucleotides (2.48 × 10^4 M^{-1} cm^{-1} at pH 6.5; Fox et al., 1958) after displacement from EF-Tu with GDP. Identity of the bound nucleotide was confirmed by HPLC (see below). Since the EF-Tu_f contained 30% EF-Tu·GDP, this was also present in the preparations of EF-Tu·thioGDP and EF-Tu·thioGTP. This does not interfere with the spectral measurements since these are made between 300 and 400 nm where only thioguanosine nucleotides are observed.

The dissociation constant of guanosine nucleotide analogues was determined under various conditions by comparing them with that of GDP. Varying concentrations of analogues were allowed to equilibrate with EF-Tu and [^{3}H]GDP, and the concentration of EF-Tu·[^{3}H]GDP was measured by using cellulose nitrate filtration. Experimental details are given in the legend in Figure 2.

The relative dissociation constant (K') was calculated by using the following equations (where N = the nucleotide analogue):

$$\text{Tu} + \text{GDP} \underset{}{\overset{K_1}{\rightleftharpoons}} \text{Tu·GDP}$$

$$\text{Tu} + \text{N} \overset{K_2}{\rightleftharpoons} \text{Tu·N}$$

$$K' = \frac{K_2}{K_1} = \frac{[\text{N}][\text{Tu·GDP}]}{[\text{Tu·N}][\text{GDP}]}$$

$$\frac{[\text{Tu·GDP}]}{[\text{Tu·N}]} = \frac{[\text{GDP}]K'}{[\text{N}]}$$

so that a plot of [Tu·GDP]/[Tu·N] against [GDP]/[N] gives a straight line of slope K'.

Since both GDP and N were present in a large excess over EF-Tu, their total concentrations were taken to be equal to their free concentrations. [Tu·N] was calculated on the basis

that no EF-Tu_f existed in the system so that $[Tu]_{total}$ - [Tu·GDP] = [Tu·N].

Cellulose nitrate filters used in the methods described above were dried, and radioactivity was determined in a Beckman LS 7000 scintillation counter after the addition of 0.4% butyl-PBD in toluene (5 mL).

EF-Ts was prepared by the method of Jacobson & Rosenbusch (1977). ThioGDP and thioGTP were prepared as described previously (Eccleston & Trentham, 1977). The 5′-diphosphates of 2-azidoinosine and 9-β-ribofuranosyl-2-aminopurine were prepared by the methods of Weigand & Kaleja (1976) and Ward et al. (1969), respectively.

Absorption spectra were measured with a Cary 118 spectrophotometer, fluorescence spectra with a Farrand Mark 1 spectrofluorometer, and CD spectra with a Jasco J-41A spectropolarimeter, all using 1-cm path length cells. Stopped-flow studies were made on an instrument similar to that described by Gutfreund (1965). Light from a 100-W Osram H 1 tungsten halide lamp with a current-stabilized power supply (Hewlett Packard 6263B) is passed through a grating monochromator (Photochemical Research Associates Inc., Model A200) and monochromatic light with a 10-nm bandwidth passed through a quartz light guide (Scott Mainz, West Germany) to a 1-cm observation cell. Transmitted light was detected by an EMI photomultiplier, and after amplification, the signal was displayed on a type 564 Tektronix storage oscilloscope.

The purity of nucleotides and the identifications of nucleotides of EF-Tu complexes were determined by using HPLC. Separation was achieved by using a Waters system with a Whatman Partisil 10/25 SAX column with a mobile phase of 0.5 M ammonium phosphate, pH 3.0, at a flow rate of 2 mL/min. The eluant was monitored by absorption at the appropriate wavelength.

A ternary complex of Phe-tRNA·EF-Tu·thioGTP was prepared by making a solution containing [^{3}H]Phe-tRNA, EF-Tu_f, and thioGTP in the ratio 1.5:1:1, giving a solution of nominal concentration based on that of EF-Tu_f. Identical procedures to form a ternary complex containing [γ-^{32}P]GTP have shown that the concentration of active ternary complex is approximately 40% of this value (Thompson et al., 1980). Poly(U)-programmed ribosomes containing acetyl-Phe-tRNA in the P site were prepared as described by Thompson et al. (1980).

Results

Binding of Nucleotide Analogues to EF-Tu. The equilibrium binding constants of guanosine nucleotide analogues to EF-Tu were determined by competition with [^{3}H]GDP. A solution of 0.13 μM EF-Tu·GDP and 7.7 μM [^{3}H]GDP was allowed to equilibrate with increasing concentrations of analogue, and then the concentration of EF-Tu·[^{3}H]GDP was determined by cellulose nitrate filtration. Other conditions are given in the legend to Figure 2. Concentrations of analogue were chosen such that between 10% and 70% of GDP was displaced from the EF-Tu. The results were analyzed as described under Materials and Methods and are summarized in Table I.

ThioGDP was found to bind 95-fold more weakly than GDP to EF-Tu at pH 7.6 and 20 °C (Figure 2). Increasing temperature had little effect on the binding although it became weaker at higher values of pH. Analysis of the thioGDP used for these studies by HPLC showed that it contained 98.5% thioGDP, 0.7% thioGTP, and 0.8% thioGMP.

ThioGTP bound 830-fold more weakly than GDP to EF-Tu at pH 7.6 and 20 °C. The binding became slightly tighter at higher pH values. Analysis of the thioGTP showed the presence of 97.9% thioGTP, 0.6% thioGDP, and 1.5% thioGMP. The presence of the small amount of thioGDP, although binding almost 10 times tighter than thioGTP, does not therefore significantly affect the measurement of the binding constant of thioGTP to EF-Tu. Also, no hydrolysis of thioGTP occurred during the time of the experiment.

Table I: Relative Dissociation Constants (K') of Nucleotide Analogues Binding to EF-Tu As Compared to GDP Binding to EF-Tu[a]

analogue	pH	temp (°C)	K'
thioGDP	7.0	20	86
	7.6	20	95
	8.0	20	160
	8.5	20	277
	7.6	37	80
thioGTP	7.0	20	506
	7.6	20	830
	8.0	20	666
	8.5	20	630
	7.6	37	520
2-azido-IDP	7.6	37	100

[a] Experimental conditions are described in the legend to Figure 2.

2-Azido-IDP was found to bind 100-fold more weakly than GDP at pH 7.6 and 37 °C. No binding of 9-β-ribofuranosyl-2-aminopurine 5′-diphosphate could be detected at concentrations up to 1 mM.

Absorption Spectrum of EF-Tu·ThioGDP. The absorption of EF-Tu·thioGDP at pH 7.0 was recorded between 300 and 400 nm. Sufficient GDP was then added to the cuvette so that all of the thioGDP was displaced from EF-Tu, giving a solution containing EF-Tu·GDP and thioGDP. Differences between free thioGDP and the same concentration of thioGDP bound to EF-Tu could therefore be measured.

The absorption maximum of thioGDP is shifted from 342 to 346 nm on binding to EF-Tu. ϵ_{342} decreases from 24.8×10^3 to 21.7×10^3 M^{-1} cm^{-1} and ϵ_{320} from 12.9×10^3 to 8.8×10^3 M^{-1} cm^{-1}. This spectral perturbation is recorded as a difference spectrum in Figure 3a. It shows a minimum at 328 nm ($\Delta\epsilon = -4.7 \times 10^3$ M^{-1} cm^{-1}) and a maximum at 352 nm ($\Delta\epsilon = +5.2 \times 10^3$ M^{-1} cm^{-1}) with an isosbestic point at 346 nm. Since these spectra were recorded at pH 7.0, and the pK of the thiol group of thioGDP is 8.35 in 5 mM $MgCl_2$, these spectra are essentially those of the thione form of thioGDP.

The spectra of thioGDP and EF-Tu·thioGDP were also recorded at pH values in the region of the pK of the thiol group of thioGDP in order to measure perturbations of this pK on binding to EF-Tu. The spectrum of EF-Tu·thioGDP, and thioGDP after displacement with GDP, at pH 8.28 is shown in Figure 4a. The absorbance at 320 and 342 nm was determined for each solution.

Since

$$A_{342} = [SH]\epsilon_{SH,342} + [S^-]\epsilon_{S^-,342}$$

$$A_{320} = [SH]\epsilon_{SH,320} + [S^-]\epsilon_{S^-,320}$$

(where SH represents the thione form of thioGDP and S^- its conjugate base), the pK of the thiol group could be calculated with

$$pK = pH + \log\left(\frac{A_{342}\epsilon_{S^-,320} - A_{320}\epsilon_{S^-,342}}{A_{320}\epsilon_{SH,342} - A_{342}\epsilon_{SH,320}}\right)$$

The molar extinction coefficients used for free thioGDP are

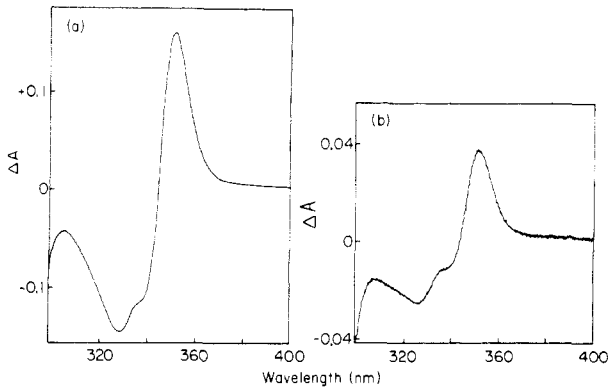

FIGURE 3: Absorption difference spectra between free and EF-Tu-bound thioguanosine nucleotides at pH 7.0. (a) Difference spectrum between EF-Tu·thioGDP and thioGDP. The 1-cm reference cuvette contained 30.6 μM EF-Tu·thioGDP, 5 mM $MgCl_2$, 50 mM Tris-HCl, 0.5 mM DTT, and 0.1 mM GDP. The sample cuvette contained an identical solution except that the GDP was omitted. (b) Difference spectrum between EF-Tu·thioGTP and thioGTP. The 1-cm reference cuvette contained 11.7 μM EF-Tu·thioGDP, 0.1 M Tris-HCl, 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM phosphoenolpyruvate, 0.25 mg/mL pyruvate kinase, and 0.1 mM GDP. The sample cuvette contained an identical solution except that GDP was omitted. The spectrum was recorded 15 min after the addition of pyruvate kinase, when HPLC analysis showed that all of the thioGDP had been converted to thioGTP.

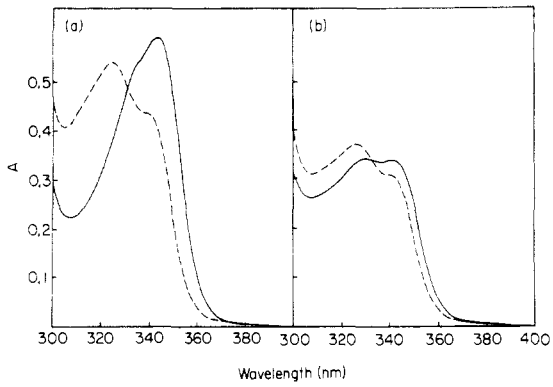

FIGURE 4: Absorption spectra of EF-Tu·thioGDP and EF-Tu·thioGTP at pH 8.28 and 8.38, respectively. (a) The 1-cm path-length cuvette contained 30.2 μM EF-Tu·thioGDP, 0.1 M Tris-HCl, pH 8.28, 5 mM $MgCl_2$, and 0.5 mM DTT (solid line). GDP was then added to a final concentration of 0.1 mM, and the spectrum was recorded after 5 min (dashed line). (b) The 1-cm path-length cuvette contained 15.0 μM EF-Tu·thioGDP, 0.1 M Tris-HCl, pH 8.38, 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM phosphoenolpyruvate, and 0.25 mg/mL pyruvate kinase. The spectrum was recorded 15 min after the addition of pyruvate kinase (solid line). GDP was then added to a final concentration of 0.1 mM and the spectrum recorded after 5 min (dashed line).

as follows: $\epsilon_{SH,342} = 24.8 \times 10^3$ M^{-1} cm^{-1}; $\epsilon_{SH,320} = 12.9 \times 10^3$ M^{-1} cm^{-1}; $\epsilon_{S^-,342} = 1.0 \times 10^3$ M^{-1} cm^{-1}; and $\epsilon_{S^-,320} = 21.0 \times 10^3$ M^{-1} cm^{-1} (Fox et al., 1958). Molar extinction coefficients for the thione form of thioGDP bound to EF-Tu are those calculated from the absorption spectrum of EF-Tu·thioGDP at pH 7.0. The values for the S^- form of thioGDP bound to EF-Tu could not be determined since EF-Tu is unstable above pH 9.0, so the value for free thioGDP was used. Any perturbations of the value on binding to EF-Tu are likely to be small on the basis of the good agreement (<3%) obtained by using these molar extinction coefficients to calculate the sum of thione and anionic forms in the solutions of EF-Tu·thioGDP and the same concentration of free thioGDP.

The p*K* of the thiol group of thioGDP bound to EF-Tu was calculated to be 9.13 compared to 8.35 for free thioGDP. This is probably a lower limit since the concentration of the anionic form is difficult to determine accurately in the presence of a large excess of the thione form, which results from the necessity of making measurements well below the p*K*.

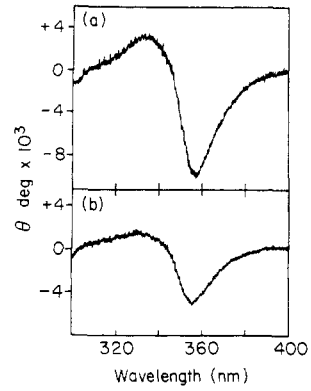

FIGURE 5: CD spectrum of EF-Tu·thioGDP and EF-Tu·thioGTP at pH 7.0, 20 °C. (a) The solution contained 26.7 μM EF-Tu·thioGDP, 0.1 M Tris-HCl, pH 7.0, 50 mM KCl, 5 mM $MgCl_2$, and 1 mM dithiothreitol. (b) The solution contained 25.3 μM EF-Tu·thioGDP and a regenerating system of 0.5 mM phosphoenolpyruvate and 0.1 mg/mL pyruvate kinase to produce EF-Tu·thioGTP. Other solvent conditions were as in (a). Each spectrum is the average of four spectra scanned at 10 nm/min after the subtraction of a blank containing buffer solution only.

Absorption Spectrum of EF-Tu·ThioGTP. The perturbations of the spectrum of the thione form of thioGTP and of the p*K* of the thiol group when thioGTP binds to EF-Tu were measured in the same way as for thioGDP. The spectra were recorded in the presence of a thioGTP-regenerating system (phosphoenolpyruvate and pyruvate kinase). No changes in the spectra of thioGTP were observed on adding it to the regenerating system without EF-Tu. Measurements of the EF-Tu·thioGTP complex made by adding thioGTP to $EF\text{-}Tu_f$ gave the same results as shown here. The absorption maximum of thioGTP is shifted from 342 to 344 nm on binding to EF-Tu at pH 7.0. ϵ_{342} decreases from 24.8×10^3 to 23.8×10^3 M^{-1} cm^{-1}, and ϵ_{320} decreases from 12.9×10^3 to 11.5×10^3 M^{-1} cm^{-1}. The difference spectrum between EF-Tu·thioGTP and free thioGTP (Figure 3b) shows a minimum at 328 nm ($\Delta\epsilon = -2.2 \times 10^3$ M^{-1} cm^{-1}) and a maximum at 352 nm ($\Delta\epsilon = +3.1 \times 10^3$ M^{-1} cm^{-1}) with an isosbestic point at 346 nm.

The perturbation in the value of the p*K* of the thiol group of thioGTP was calculated from spectral measurements at pH values near the p*K* of the group. Figure 4b shows the spectrum of EF-Tu·thioGTP and free thioGTP after displacement with GDP, at pH 8.38. The p*K* values were calculated as described for thioGDP and were found to be 8.36 for free thioGTP and 8.45 for EF-Tu·thioGTP.

CD Spectra of EF-Tu·ThioGDP and EF-Tu·ThioGTP. Solutions of the two complexes, EF-Tu·thioGDP and EF-Tu·thioGTP, were prepared at pH 7.0 as described for the absorption spectra measurements. The CD spectra between 300 and 400 nm were recorded at 20 °C. The free thioguanosine nucleotides have a small negative CD peak at 342 nm ($[\theta] = 1.5 \times 10^3$ deg cm^2 $dmol^{-1}$, $\Delta\epsilon = 0.45$ M^{-1} cm^{-1}; Eccleston & Bayley, 1980). The small negative peak of thioGDP is inverted and intensified and a new long-wavelength negative peak appears on binding to EF-Tu (Figure 5a). By use of the concentration of EF-Tu·thioGDP, the molar ellipticity, $[\theta]$, of the bound nucleotide was calculated to be 3.7×10^4 deg cm^2 $dmol^{-1}$ ($\Delta\epsilon = 11.2$ M^{-1} cm^{-1}) for the negative peak at 357 nm and 1.2×10^4 deg cm^2 $dmol^{-1}$ ($\Delta\epsilon = 3.6$ M^{-1} cm^{-1}) for the positive peak at 333 nm. A qualitatively similar effect is seen with thioGTP, but the intensities of both peaks are less. $[\theta]$ for the negative peak at 357 nm was 2.0×10^4

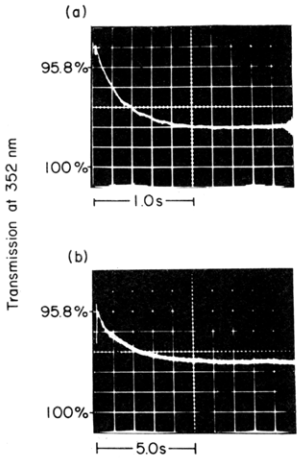

FIGURE 6: Stopped-flow spectrophotometric record of the binding of thioGDP and thioGTP to EF-Tu at 20 °C, pH 7.6. (a) One syringe contained 10 μM EF-Tu$_f$ and the other 30 μM thioGDP. (b) One syringe contained 10 μM EF-Tu$_f$ and the other 30 μM thioGTP. (Concentrations after mixing.) All syringes also contained 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, and 0.5 mM DTT.

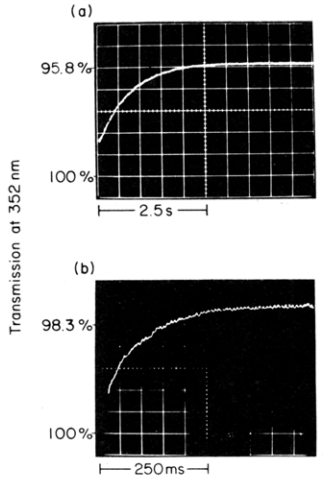

FIGURE 7: Stopped-flow spectrophotometric records of (a) the displacement of thioGDP from EF-Tu·thioGDP by EDTA and (b) the displacement of thioGDP from EF-Tu·thioGDP by GDP and EF-Ts, at pH 7.6 and 20 °C. (a) One syringe contained 5 μM EF-Tu·thioGDP and 5 mM $MgCl_2$. The other contained 10 mM EDTA. Both syringes also contained 50 mM Tris-HCl, pH 7.6, and 0.5 mM DTT. (b) One syringe contained 1.25 μM EF-Tu·thioGDP, and the other contained 100 μM GDP and 0.5 μM EF-Ts. Both syringes also contained 50 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, and 0.5 mM DTT.

deg cm^2 dmol^{-1} ($\Delta\epsilon$ = 6.1 M^{-1} cm^{-1}) and for the positive peak at 333 nm was 0.6 × 10^4 deg cm^2 dmol^{-1} ($\Delta\epsilon$ = 1.8 M^{-1} cm^{-1}).

Rate of Association and Dissociation of Thioguanosine Nucleotides and EF-Tu. The spectral changes associated with the binding of thioguanosine nucleotides to EF-Tu described above enabled the second-order association rate constants and first-order dissociation rate constants for these interactions to be measured by using stopped-flow spectrophotometry. Measurements were made at 352 nm at pH 7.6 at 20 °C in the presence of 5 mM $MgCl_2$. Figure 6a shows the reaction between 10 μM EF-Tu$_f$ and 30 μM thioGDP (final reaction chamber concentrations). Analysis of this reaction trace showed it to be exponential, giving a pseudo-first-order rate constant of 3.35 s^{-1}. A similar experiment at 60 μM thioGDP gave a pseudo-first-order rate constant of 7.0 s^{-1}. Since EF-Tu$_f$ is unstable at 20 °C (half-life is 5 min at 37 °C; Arai et al., 1974), the actual concentration of EF-Tu$_f$ was less than 10 μM. It also contained approximately 30% EF-Tu·GDP (see Materials and Methods). However, since the experiment was carried out under pseudo-first-order conditions, the lower concentration of EF-Tu$_f$ will not affect the measured rate constant through the amplitude of the absorption change will be decreased.

The dissociation rate constant of thioGDP from EF-Tu in 5 mM $MgCl_2$ was measured by mixing 10 μM EF-Tu·thioGDP with 100 μM GDP so that once dissociated the free thioGDP could not effectively compete with GDP for EF-Tu. An exponential process was observed, giving a first-order rate constant of 0.01 s^{-1}. Increasing the concentration of GDP to 500 μM did not change this rate constant.

If the binding of thioGDP to EF-Tu is considered as a single-step process

$$\text{EF-Tu} + \text{thioGDP} \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} \text{EF-Tu·thioGDP}$$

then the observed rate of binding is given by

$$k_{obsd} = k_{+1}[\text{EF-Tu}][\text{thioGDP}] + k_{-1} \qquad (1)$$

Since k_{-1} is relatively small (0.01 s^{-1}) compared to the observed binding rates, it can be ignored, and the values of k_{obsd} obtained at 30 and 60 μM thioGDP give second-order binding rate constants of 1.12 × 10^5 and 1.17 × 10^5 M^{-1} s^{-1}, respectively. Combining the average value with the dissociation rate constant gives a value for the apparent dissociation constant (K_d^{app} = k_{-1}/k_{+1}) of 8.7 × 10^{-8} M.

Kinetic constants for the interaction of EF-Tu and thioGTP were measured in the same way. Mixing 10 μM EF-Tu$_f$ with 30 μM thioGTP (Figure 6b) and with 60 μM thioGTP gave observed rate constants of 0.55 and 1.27 s^{-1}, respectively. The dissociation rate constant obtained by displacing thioGTP from EF-Tu with an excess of GDP was 0.21 s^{-1}. Analysis by eq 1 gives a second-order binding rate constant of 1.6 × 10^4 M^{-1} s^{-1}, which together with the dissociation rate constant gives a value for the apparent dissociation constant of 1.3 × 10^{-5} M.

The dissociation rate constants of thioGDP and thioGTP from EF-Tu described above were measured in the presence of 5 mM $MgCl_2$. It is of interest to determine whether Mg is released from EF-Tu as its complex with GDP or whether it can be released independently of the nucleotide. For example, if EF-Tu·Mg·thioGDP is mixed with EDTA, two distinct mechanisms could occur for the release of thioGDP and Mg (see Schemes I and II). A mechanism analogous to Scheme I

$$\text{EF-Tu·Mg·thioGDP} + \text{EDTA} \rightleftharpoons \text{EF-Tu·thioGDP} + \text{Mg·EDTA}$$

$$\text{EF-Tu·thioGDP} \rightleftharpoons \text{EF-Tu} + \text{thioGDP}$$

Scheme II

$$\text{EF-Tu·Mg·thioGDP} \rightleftharpoons \text{EF-Tu} + \text{Mg·thioGDP}$$

$$\text{Mg·thioGDP} + \text{EDTA} \rightleftharpoons \text{thioGDP} + \text{Mg·EDTA}$$

Scheme II occurs in the release of Mg·ADP from myosin (Bagshaw & Trentham, 1974). In order to distinguish between these two possibilities for EF-Tu, a solution of EF-Tu·thioGDP in $MgCl_2$ was mixed with excess EDTA and the absorption monitored at 352 nm. Figure 7a shows that an exponential process is observed, giving a first-order rate constant of 1.4 s^{-1}. Varying the EDTA excess concentration over that of $MgCl_2$ between 7.5 and 97.5 mM did not affect the rate constant of this process. This result is consistent with Scheme I but not Scheme II. The addition of 100 μM GDP to the EDTA also did not affect the rate constant although the amplitude of the process was increased by approximately 20%. This is the expected result since the equilibrium of the second step of Scheme I will be displaced to the right in the presence

of GDP by the formation of EF-Tu·GDP. The rate constant of 1.4 s^{-1} probably represents the dissociation of Mg from EF-Tu·Mg·thioGDP although the results do not rule out Mg release being faster than this and the release of thioGDP from EF-Tu·thioGDP occurring at 1.4 s^{-1}.

EF-Ts catalyzes the exchange of GTP for GDP bound to EF-Tu (Miller & Weissbach, 1977). This process is much faster than the dissociation rate of GDP from EF-Tu, and so the effect of EF-Ts is to increase this dissociation rate (Chau et al., 1981). Figure 7b shows that EF-Ts also catalyzes the dissociation of thioGDP from EF-Tu. When 1.25 μM EF-Tu·thioGDP is mixed with 100 μM GDP containing 0.5 μM EF-Ts at pH 7.6, 20 °C, in 5 mM $MgCl_2$, an exponential process is observed giving a first-order rate constant of 11 s^{-1}. This compares to the value of 0.01 s^{-1} obtained in the absence of EF-Ts and is similar to the in $MgCl_2$, protein biosynthesis.

Effect of ThioGTP on the EF-Tu-Dependent Binding of Aminoacyl-tRNA to Ribosomes. In order to assess the feasibility of using the absorption changes of thioguanosine nucleotides described above in kinetic studies of the elongation cycle of protein biosynthesis, the ability of thioguanosine nucleotide to substitute for guanosine nucleotides in the EF-Tu-dependent binding of aminoacyl-tRNA to ribosomes was investigated.

A ternary complex of [^{3}H]Phe-tRNA·EF-Tu·thioGTP (final concentration 0.5 μM) was mixed with poly(U)-programmed ribosomes (final concentration 0.125 μM) containing acetyl-Phe-tRNA in the P site at 0 °C in 20 μL of a solution containing 50 mM Tris-HCl, pH 7.2, 50 mM KCl, 1 mM NH_4Cl, 5 mM $MgCl_2$, and 1 mM dithiothreitol. After 30 s, analysis of the reaction mixture as described under Materials and Methods showed that 1.5 pmol of [^{3}H]acetyl-Phe-tRNA bound to the ribosomes, 0.4 pmol of which was extractable as [^{3}H]acetyl-Phe-Phe.

Fluorescence Measurements of EF-Tu·2-Azido-IDP. EF-Tu·2-azido-IDP was prepared as described for EF-Tu·thioGDP except that 2-azido-IDP replaced thioGDP. The fluorescence emission spectrum of 5 μM solution of the complex was recorded in solvent conditions described in Figure 3 after excitation at 305 nm and showed an emission maximum at 400 nm. The addition of GDP to a final concentration of 100 μM increased the intensity of this emission by 15%, showing that 2-azido-IDP fluorescence is quenched on binding to EF-Tu.

Discussion

EF-Tu shows a high specificity for guanosine nucleotides (Miller & Weissbach, 1977) although the results presented here show that some modifications of the 6-oxy and 2-amino groups of GDP can be made and significant binding still occurs. Replacement of the 6-oxy by 6-thio (i.e., thioGDP) or replacement of 2-amino by 2-azido (i.e., 2-azido-IDP) results in an approximately 100-fold weaker binding constant. Omission of the 6-oxy group (i.e., 9-β-ribofuranosyl-2-aminopurine 5′-diphosphate) results in no detectable binding.

The interaction of thioGDP and thioGTP with EF-Tu was studied in detail to gain structural information about the nucleotide binding site of EF-Tu and to enable measurements of the rate constants of the interactions of EF-Tu to be made. Extensive studies have previously been made on the absorption spectra of thioguanosine nucleotides and their interaction with other proteins and on the structure of thioguanosine. Thewatt & Bugg (1972) have shown that in thioguanosine crystals the replacement of an oxygen atom with a sulfur atom has little effect on the bond length or bond angles within the purine ring. The stacking patterns of thioguanosine in the crystal were very similar to those of guanosine, showing that the sulfur is a good hydrogen bond acceptor although the hydrogen bonds formed were of a longer length than those of guanosine.

When thioGDP binds to EF-Tu, the absorption spectrum of the thione form is shifted 4 nm to the red and decreased in amplitude, a major change in its CD spectrum occurs, and the pK of the thiol group is increased by at least 0.7 unit. Several factors influence absorption and CD spectra of compounds which often cannot be separated into single effects. Cheong et al. (1969) have proposed that the absorption band of thioguanosine derives from at least two transitions, a π–π^* charge-transfer transition together with a n–π^* transition of weaker intensity. Any factors affecting the spectrum of thioguanosine nucleotides must take into account these two transitions. These factors may include polarizability of the environment, conformation of the glycosidic bond of the nucleotide, specific interactions between the nucleotide and amino acid residues of the protein, and other tautomeric structures of the purine ring which could be stabilized within the protein–nucleotide complex. Comparison of the spectral perturbation of thioguanosine nucleotides bound to EF-Tu with spectra in model solvents and when bound to other proteins (Eccleston & Trentham, 1977; Eccleston & Bayley, 1980) allows some of these factors to be studied and hence gives information on the nucleotide binding site of EF-Tu.

When thioguanosine nucleotides are dissolved in 70% 2-propanol, the difference spectrum obtained compared to that in aqueous solvent is similar in shape and intensity to that obtained when these nucleotides bind to myosin and that when thioGDP binds to EF-Tu (Figure 3). Although the intensities of the difference spectrum peaks are not linear with 2-propanol concentration, the simplest interpretation of the spectral perturbation occurring when thioGDP binds to myosin or EF-Tu is that the purine moiety is located in a hydrophobic environment in the protein. The large increase in the pK of the thiol group of thioGDP on binding to EF-Tu is consistent with this interpretation. The pK of the thiol group of thioguanosine is increased by 0.7 pH unit between 0 and 68% methanol. It should be noted that although the spectral perturbation of thioGDP bound to EF-Tu is similar to that when thioGMP·P(NH)P binds to myosin, there is a smaller increase in the pK in the latter case. Eccleston & Trentham (1977) attributed this to the presence of a cationic group in the active site of myosin which suppresses this expected increase.

The weak negative CD peak of thioguanosine nucleotides is not affected by concentrations of 2-propanol up to 75%. (Above this concentration, base stacking occurs, giving rise to a characteristic "conservative" band system in which there are equal bands of positive and negative CD.) The CD spectrum of thioguanosine nucleotides bound to proteins can arise from immobilization of the purine with respect to the ribose moiety of the nucleotide (Teng et al., 1971) or from interactions with the binding site. These two mechanisms will both contribute to the observed CD spectrum although Eccleston & Bayley (1980) concluded that the CD spectra of myosin·thioguanosine nucleotide complexes derive mainly from a specific asymmetric interaction involving the thione group. The CD spectrum of the EF-Tu·thioGDP complex shows peaks at the same positions as those in the myosin complexes except that the signs are reversed. This suggests that similar electronic factors are involved in both complexes but with different symmetry.

The spectral perturbations occurring when thioGTP binds to EF-Tu show marked differences as compared to those of

thioGDP binding to EF-Tu. The red shift in the spectrum is less, and the pK of the thiol group increases by only 0.1 pH unit. Although the peaks of the CD spectrum are at the same wavelength and sign, the intensity of EF-Tu·thioGTP is much smaller than that of EF-Tu·thioGDP. The dissociation constant of thioGTP and EF-Tu has been determined to be 2.5×10^{-6} M by competitive binding studies (based on thioGTP binding 830-fold more weakly than GDP, which has a binding constant of 3×10^9 M) and 1.3×10^{-5} M from kinetic measurements. If this second (higher) value is the correct binding constant, then some free thioGTP is present in the solutions of EF-Tu·thioGTP. This would result in measurements of absorption, pK, and CD perturbations lower than the true values, and so these results should be interpreted with caution. However, the observed differences are consistent with a more open (and less hydrophobic) structure surrounding the purine ring in EF-Tu·thioGTP compared to EF-Tu·thioGDP. Wilson & Cohn (1977) have previously shown small but reproducible differences in the EPR spectra and proton relaxation rate of water between the Mn complexes of EF-Tu·GTP and EF-Tu·GDP. It appears that these differences of structure are not confined to the phosphate moiety but extend to the whole nucleotide binding site. The mechanism by which this change in structure at the nucleotide binding site creates a site for the binding of aminoacyl-tRNA is not known.

The rates of association of GTP and GDP with EF-Tu have been measured previously by Arai et al. (1974) and by Fasano et al. (1978), using very low concentrations of reactants to slow the second-order reaction down to times long enough for the cellulose nitrate filtration technique to be used. The spectral perturbations described above have allowed the rates of association of thioGTP and thioGDP to be measured directly by using stopped-flow spectrophotometry at concentrations of EF-Tu closer to those existing in vivo. The important feature of these results (Figure 6) is that thioGTP binds to EF-Tu an order of magnitude slower than the binding of thioGDP. This agrees with the results of Fasano et al. (1978), who showed the same effect with GTP and GDP, and contrasts with the results of Arai et al. (1974), who obtained almost equal second-order rate constants for the binding of both GTP and GDP to EF-Tu. Although the measured rate constants are proportional to thioGTP or thioGDP concentration, it is unlikely from the values obtained that they represent a simple second-order process since such processes are typically in the range of 10^7–10^8 M^{-1} s^{-1} for protein–nucleotide interactions (Gutfreund, 1975). A more likely mechanism is that an initial rapid equilibrium is established between EF-Tu and nucleotide (equilibrium association constant K_1) followed by a first-order isomerization step (first-order rate constant k_2) as occurs with myosin and ATP or ADP (Trentham et al., 1976):

$$\text{EF-Tu} + \text{nucleotide} \overset{K_1}{\rightleftharpoons} \text{EF-Tu·nucleotide} \overset{k_2}{\rightleftharpoons} \text{EF-Tu*·nucleotide}$$

where the measured rate constant is $K_1 k_2$. (Resolution of K_1 from k_2 could theoretically be obtained by increasing the concentration of nucleotide until k_2 becomes rate limiting in the reaction. However, the high absorption of thioguanosine nucleotides in such experiments makes this approach impractical.) Although thioGDP dissociated from EF-Tu·thioGDP as a complex with Mg in 5 mM $MgCl_2$, Figure 7a shows that it is possible for Mg to dissociate from the protein with a faster rate constant than the rate constant for Mg·thioGDP dissociation. This fast dissociation of Mg^{2+} from EF-Tu·Mg·thioGDP with the concomitant dissociation of thioGDP is consistent with the preparation of EF-Tu_f from EF-Tu·GDP in 1 min at 0 °C in the presence of EDTA (Thompson et al., 1981) when the dissociation of GDP from EF-Tu·GDP in 5 mM $MgCl_2$ at 0 °C has a half-time of 50 min (Fasano et al., 1978). Wittinghofer & Leberman (1979) have presented evidence that Mg^{2+} can bind to EF-Tu from *Bacillus stereothermophilus* in the absence of nucleotide. It appears, therefore, that divalent metals can bind to and dissociate from EF-Tu independently of guanosine nucleotides.

The ability of thioguanosine nucleotides to substitute for GTP and GDP in the elementary steps of the elongation cycle of protein biosynthesis suggests a spectroscopic approach for analysis of these processes to complement the transient kinetic approaches using radioisotopes (Thompson et al., 1980) and spectroscopic studies of the effect of antibiotics in the system (Eccleston, 1981). The availability of thioGTP as an alternate nucleotide to GTP may also be advantageous in future studies directed at the fidelity of the translation process.

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Reconstitution of *Escherichia coli* Membrane Vesicles with D-Amino Acid Dehydrogenase†

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ABSTRACT: When purified D-amino acid dehydrogenase [Olsiewski, P. J., Kaczorowski, G. J., & Walsh, C. T. (1980) *J. Biol. Chem. 255*, 4487] is incubated with right-side-out membrane vesicles from *Escherichia coli*, the enzyme binds to the membrane in a time- and concentration-dependent manner. As a result, the vesicles acquire the ability to oxidize D-alanine and catalyze D-alanine-dependent active transport. Similarly, incubation of D-amino acid dehydrogenase with inside-out vesicles results in binding of enzyme and D-alanine oxidase activity. Antibody inhibition studies indicate that the enzyme is bound exclusively to the inner cytoplasmic surface of the membrane in native vesicles (i.e., membrane vesicles prepared from cells induced for D-amino acid dehydrogenase). In contrast, similar studies with reconstituted vesicles demonstrate that enzyme binds to the surface exposed to the medium regardless of the orientation of the membrane. Thus, enzyme bound to right-side-out vesicles is located on the opposite side of the membrane from where it is normally found. Remarkably, in the presence of D-alanine, reconstituted right-side-out and inside-out vesicles generate electrochemical proton gradients of similar magnitude but opposite polarity, indicating that enzyme bound to either surface of the membrane is physiologically functional. The results suggest that vectorial proton translocation via the respiratory chain occurs at a point distal to the site where electrons enter the respiratory chain from the primary dehydrogenase, a conclusion that is inconsistent with the notion that the dehydrogenase forms part of a proton-translocating loop.

Cytoplasmic membrane vesicles prepared from *Escherichia coli* by osmotic lysis have become increasingly useful for studying active transport (Kaback, 1970, 1974a, 1980). These vesicles exhibit the same polarity and configuration as the membrane in the intact cell, as shown by many criteria (Kaback, 1971, 1974a; Owen & Kaback, 1978, 1979a,b). Furthermore, the vesicles retain the ability to couple respiration and, as demonstrated recently (Hugenholtz et al., 1981), ATP hydrolysis to the active transport of many different solutes by mechanisms in which chemiosmotic forces play a central, obligatory role (Kaback, 1976; Harold, 1976; Ramos & Kaback, 1977a–c; Tokuda & Kaback, 1977; Kaczorowski et al., 1980). Thus, as postulated by Mitchell (1961, 1968, 1979a,b), the immediate driving force for active transport in this system is a transmembrane electrochemical proton gradient ($\Delta\bar{\mu}_{H^+}$)[1] composed of an electrical potential ($\Delta\psi$, interior negative) and a chemical gradient of hydrogen ions (ΔpH, interior alkaline).

An important achievement in these studies was the development of techniques that allow quantitation of the electrical and chemical components of $\Delta\bar{\mu}_{H^+}$ (Schuldiner & Kaback, 1975; Ramos et al., 1976, 1979; Ramos & Kaback, 1977a–c; Felle et al., 1980). Recently, these techniques were applied to vesicles in which the membrane is inverted relative to the intact cell (Reenstra et al., 1980). In this case, substrate oxidation or ATP hydrolysis leads to the generation of a $\Delta\bar{\mu}_{H^+}$

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[1] Abbreviations used: $\Delta\bar{\mu}_{H^+}$, electrochemical proton gradient; $\Delta\psi$, electrical potential; ΔpH, chemical gradient of hydrogen ions; NEM, *N*-ethylmaleimide; D-ADH, D-amino acid dehydrogenase; D-LDH, D-lactate dehydrogenase; PMS, phenazine methosulfate; DCIP, dichloroindophenol; Q_1, ubiquinone 1; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

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