Physicochemical Properties and Interactions of *Escherichia coli* Ribonucleic Acid Polymerase Holoenzyme, Core Enzyme, Subunits, and Subassembly $\alpha_2\beta$†

B. Jane Levine,‡ Peter D. Orphanos,§ Betty S. Fischmann,‖ and Sherman Beychok*

ABSTRACT: We have investigated several physicochemical properties of *Escherichia coli* DNA-dependent RNA polymerase, its constituent subunits α, β, β', and σ, and the subassembly $\alpha_2\beta$. These included ultraviolet (UV) absorption, isoelectric points, sulfhydryl content, extinction coefficients, and circular dichroism (CD). Among the most notable results is the observation, based on CD measurements, that the σ subunit, free and combined in holoenzyme, is a highly structured protein with approximately 75% of its residues folded in α-helical conformation and little or no detectable β sheet. No secondary structure changes in either σ or core accompany formation of holoenzyme. In contrast to the conformational independence of the subunits in assembly of holoenzyme, the protein and its components exhibit conformational flexibility as glycerol concentration is varied and in their interaction with DNA. The effect of glycerol on the conformation of σ, core, and holoenzyme was monitored by circular dichroism measurements. In the far-ultraviolet, the residue ellipticity at 220 nm ($[\theta]_{220}$) increased approximately 15% from 0 to 10% glycerol for both core and holoenzyme. In the near-ultraviolet, the residue ellipticity at a peak near 280 nm also varied with glycerol concentration, decreasing in intensity by about 50% with holoenzyme, when glycerol was raised from 5 to 10%, then increasing at still higher glycerol contents. Electrophoretic and molecular sieve analysis showed core and σ to have greater affinity for each other in 50% glycerol than in 10% glycerol. The presence of 10% glycerol in the assay buffer increased the activity of the enzyme. The effect of various DNA templates on the conformations of core, holoenzyme, $\alpha_2\beta$ subassembly, and β' subunit was also monitored by far-ultraviolet circular dichroism. All the protein samples showed between 10 and 20% decrease in secondary structure upon the addition of the DNA.

The DNA-dependent RNA polymerase holoenzyme from *Escherichia coli* (nucleosidetriphosphate:RNA nucleotidyltransferase, EC 2.7.7.6) consists of five subunits, $\alpha_2\beta\beta'\sigma$, with a molecular weight for α of 36 500 (Ovchinnikov et al., 1977), β of 155 000 (Burgess, 1969b), β' of 165 000 (Burgess, 1969b), and σ of 86 000 (Berg et al., 1971). Holoenzyme exists in a dynamic equilibrium with its components, core enzyme ($\alpha_2\beta\beta'$) and σ subunit (Travers, 1975). It is the holoenzyme which recognizes specific promoters on the DNA templates while the core enzyme (after the initiation of transcription and the release of σ at some point) carries out the bulk of the elongation of the RNA product. The released σ combines with another core to restart the well-known "σ cycle" (Travers & Burgess, 1969).

Previous papers from this laboratory (Harding & Beychok, 1973, 1974, 1976) have focused on the sulfhydryl content and reactivity of core enzyme and on a temperature-dependent renaturation of core enzyme. The renatured enzyme occurs in two states, an inactive form at 4 °C, designated RI (renatured inactive), and an active form at 37 °C, designated RA (renatured active). Holoenzyme RI and core RI each exist as an equilibrium mixture represented by $\alpha_2\beta + \beta' + (\sigma) \rightleftarrows \alpha_2\beta\beta'(\sigma)_{\text{inactive}}$ (B. J. Levine, P. D. Orphanos, B. S. Fischman, and S. Beychok, unpublished experiments).

The present paper focuses on physicochemical and analytical properties (UV absorption, isoelectric points, sulfhydryl content, circular dichroism, electrophoresis, and molecular weights) of RNA polymerase subunits, of a subassembly, $\alpha_2\beta$, and of the core and holoenzyme. We also present data on the effect of glycerol on the conformation of σ, core, and holoenzyme and on the σ–core interaction, as well as on enzyme assay systems. The final section deals with changes in the structure of RNA polymerase and its components upon binding to DNA, as monitored by circular dichroism.

Materials and Methods

Ultrogel AcA 22 and ampholines were purchased from LKB. Phosphocellulose (P11) and DEAE-cellulose[1] were purchased from Whatman, Inc. Bio-Gel A-1.5m, Affi-Gel Blue, and most electrophoresis materials were purchased from Bio-Rad. Glycerol (Fisher-certified A.C.S.) was used without further purification for preparative and experimental work.

$NaDodSO_4$–acrylamide gel electrophoresis was performed as described by Weber & Osborn (1969). Analytical gels were run according to Hjerten et al. (1965) as described by Harding & Beychok (1976). All gels were stained for 1 h in a solution of methanol, acetic acid, and water in the ratio 5:1:5 containing 0.25% Coomassie Brilliant Blue R-250 (Eastman Kodak) and destained by diffusion in destaining solution (7.5% acetic acid v/v and 5% methanol v/v in water). The stained bands of the gel were scanned at 570 nm by using a Gilford spectropho-

† From the Departments of Biological Sciences and Chemistry, Columbia University, New York, New York 10027. *Received March 11, 1980.* This investigation was supported by Grant CA-13014, awarded by the National Cancer Institute, Department of Health, Education, and Welfare, Grant PCM 77-08537, awarded by the National Science Foundation, and U.S. Public Health Service Training Grant GM 07216.

‡ Taken in part from a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree, Columbia University, 1980.

§ Taken in part from a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree, Columbia University, 1979. Present address: Procter & Gamble, Winton Hill Technical Center, Cincinnati, OH 45224.

‖ Taken in part from a thesis submitted in partial fulfillment of the requirements for the Ph.D. degree, Columbia University, 1978. Present address: Mount Sinai School of Medicine, Institute of Computer Science, New York, NY 10011.

[1] Abbreviations used: DEAE, diethylaminoethyl; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol.

0006-2960/80/0419-4808$01.00/0

tometer equipped with a linear transport attachment. The relative area under each peak was measured by weighing the piece of paper cut out from each peak.

RNA polymerase was assayed by the method of Burgess (1969a). The templates used were calf thymus DNA (Worthington), poly[d(A-T)] (Miles), and T7 phage DNA. T7 phage DNA was prepared by the method of Thomas & Abelson (1966). Band sedimentation experiments indicated that the T7 DNA was less than 20% nicked (Vinograd et al., 1963). All DNA concentrations are based on an $A_{260} = 20$ for a 1 mg/mL solution.

The Jaenicke method was used for quantitative determinations of nitrogen (Jaenicke, 1974). Phenol crystals (Mallinckrodt) and sodium nitroprussate (Baker) were used without further purification. Ammonium sulfate (Schwarz/Mann Ultra-Pure) and bovine serum albumin (Miles Laboratories, $\epsilon^{1\%}_{280\ nm,\ 1\ cm} = 6.7$) were used as nitrogen standards. The percent nitrogen of RNA polymerase protein was assumed to be 16.5%. Protein samples were dialyzed against a solution containing 0.05 M sodium phosphate, pH 8, 0.45 M NaCl, and 0.1 mM DTT. Five to ten micrograms of protein was used for each assay. The extinction coefficients calculated were an average of four to six determinations.

Isoelectric focusing in 8 M urea (Schwarz/Mann Ultra-Pure) and 2% Nonidet P-40 (Gallard-Schlessinger) was performed by using a combination of two methodologies (Winter et al., 1971; Lowe et al., 1979). The basic setup consisted of an LKB Multiphor 2117 system. The 10 M urea stock solution was deionized with mixed-bed resin [AG 501-X8(D), Bio-Rad] immediately before use. The gel (8 M urea, 2% Nonidet P-40, 5% acrylamide, 9% glycerol, and 2% ampholines, pH 3.5–9.5) was poured the day before the experiment according to Winter et al. (1971). Samples were dialyzed overnight vs. 8 M urea, 2% Nonidet P-40, and 1 mM DTT at 23 °C. The samples were applied to the gel with small Whatman 3MM, Paratex filters, and the focusing was performed at 4 °C, 30 W, for approximately 2 h. The cathode solution contained 1 M NaOH and the anode solution contained 8 M urea, 2% Nonidet P-40 in degassed water, and phosphoric acid to pH 2.5 (Lowe et al., 1979). After the experiment, the pH gradient was determined by incubation of gel slices in degassed deionized water at 4 °C. Staining and destaining of the gel were performed as in Winter et al. (1971).

Ellman's reagent was used for the determination of total thiol residues in 1% $NaDodSO_4$ (Ellman, 1959). Samples were extensively dialyzed overnight against 0.01 M Tris-HCl, pH 8.0, 0.45 M NaCl, and 0.1 mM EDTA at 4 °C. To 9 mL of protein (about 1 mg of core and holoenzyme and 0.15 mg of σ subunit) was added 0.1 mL of Ellman's reagent, and after 20 min 0.1 mL of 10% $NaDodSO_4$ was added. Similar additions were made to 0.9 mL of buffer. An ϵ_M of 13 600 M^{-1} cm^{-1} was used for the 3-carboxy-4-nitrothiophenolate chromophore at 412 nm.

A Cary 14 recording spectrophotometer was used for all ultraviolet and visible absorption work. Circular dichroism spectra were recorded in a Cary 60 spectropolarimeter with a 6001 CD attachment. All $[\Theta]_\lambda$ values reported are expressed as a mean residue ellipticity, assuming a residue weight of 115. Cells were purchased from Pyrocell.

In several experiments, the 2-cm path length tandem cells, consisting of two cells, each of 1-cm path length sealed together, were used. In those cases, individual (protein and DNA) CD spectra were monitored in 1-cm cells from 330 to 210 nm. Exactly 3.0 mL of protein was then pipetted into one compartment of the 2-cm path length tandem cell while the other was filled with the same volume of DNA. This spectrum was recorded. The contents of each cell were then carefully mixed, and the tandem cell was rinsed with the mixture. The cell was refilled and after 20 min the spectrum was recorded. Base lines were obtained before and after each experiment. Control "noninteraction" experiments were performed to check for surface denaturation or adsorption of material to the quartz surfaces. σ subunit was prone to such adsorption, and, accordingly, experiments with σ were not done in tandem cells.

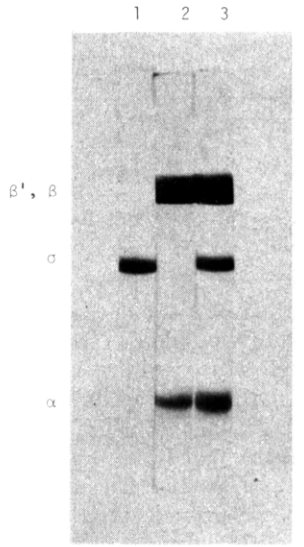

FIGURE 1: 5% $NaDodSO_4$–polyacrylamide gels. (1) 3.1 μg of σ; (2) 14 μg of core; (3) 17 μg of holoenzyme.

All residue ellipticity values reported have been standardized in the far-UV with hemoglobin β^{hCN} ($[\Theta]_{222} = -21.3 \times 10^3$ deg cm^2 $dmol^{-1}$) and in the near-UV with a standard solution of *d*-10-camphorsulfonic acid solution. Far-UV CD experiments in which glycerol concentration was varied were also corrected for the index of refraction (N) of the solvent by using the relation $[\Theta]' = 9N/(N^2 + 2)^2$ (Fasman, 1976). The index of refraction in the far-UV was determined by using the Sellmeir equation (Adler & Fasman, 1968) with $N_{280.4}^{2.4°C} = 1.519$ and $N_{270.0}^{2.4°C} = 1.525$ for glycerol (Guillery, 1930). This yields $N_{220}^{2.4°C} = 1.564$. We assume that the variation in N_{220} for various percent glycerol concentrations is similar to the variation at the sodium D line (Weast, 1972).

Isolation of Holoenzyme, Core, α, β′, β, $\alpha_2\beta$, and σ. The method of Burgess & Jendrisak (1975) was used with certain modifications for the purification of core and holoenzyme.

An Ultrogel AcA 22 column was substituted for the Bio-Gel A-1.5m column. It provided better separation of polymerase from the high molecular weight lipoprotein and from low molecular weight (less than M_r 100 000) contaminants than the Bio-Gel A-1.5m column. The major contaminants after this column (as judged by $NaDodSO_4$ gels) are the M_r 110 000 χ or τ protein and a M_r 68 000 protein, possibly a form of ATPase (Burgess, 1976). The method of Gonzalez et al. (1977) was used to separate core and holoenzyme. This phosphocellulose chromatography, in 50% glycerol, results in the separation of the M_r 110 000 and 68 000 proteins (flow through) from holoenzyme (95–100% σ saturated eluted with buffer containing 0.2 M KCl) and core (eluted with buffer containing 0.5 M KCl). The core and holoenzyme were stored in storage buffer at −20 °C. Densitometry scans of 5% $NaDodSO_4$–acrylamide gels, as shown in Figure 1, revealed that holoenzyme was 95–100% σ saturated. Both core and holoenzyme preparations contained no more than 2% contaminants, judged by densitometry.

Table I: Extinction Coefficients of RNA Polymerase Proteins

protein	$\epsilon^{1\%}_{280\ nm,\ 1\ cm}$ (100 cm²/g)	$\epsilon_M \times 10^{-5}$ (M^{-1} cm^{-1})
α	3.7 ± 0.2	0.14
β	5.2 ± 0.4	0.81
β'	6.2 ± 0.8	1.02
$\alpha_2\beta$	4.7 ± 0.2	1.07
$\alpha_2\beta\beta'$	5.8 ± 0.3	2.28
σ	11.6 ± 0.7	1.00
$\alpha_2\beta\beta'\sigma$	6.7 ± 0.4	3.21

For the preparation of σ subunit, holoenzyme was chromatographed on phosphocellulose (Whatman) in 5% glycerol. The σ flow through was concentrated on DEAE-cellulose (Whatman) and dialyzed vs. storage buffer (0.01 M Tris-HCl, pH 7.8, 0.1 mM EDTA, 0.1 mM DTT, 50% glycerol, and 0.45 M NaCl). No further purification was required, and the subunit was stored at −20 °C. This column also served as another source of core enzyme.

The preparation of $\alpha_2\beta$ subassembly and β' subunit required the denaturation of core enzyme at 30 °C in buffer containing 6.5 M urea. The 10 M urea (Schwarz/Mann Ultra-Pure) stock was deionized with mixed-bed resin AG 501-X8(D) (Bio-Rad) immediately before use. The denatured core was chromatographed on an Affi-Gel Blue column (Wu et al., 1977). $\alpha_2\beta$ appeared in the flow through while β' was eluted with buffer and 0.6 M KCl.

The α, β, and β' subunits were prepared from core enzyme which was denatured in buffer containing 6.5 M urea at 30 °C and then subjected to phosphocellulose chromatography, as in Yarbrough & Hurwitz (1974). α appears in the flow through while β is eluted with buffer and 0.2 M KCl and β' with buffer and 0.5 M KCl. As with holoenzyme, core enzyme, and σ subunit, the α, β, and β' subunits and the $\alpha_2\beta$ subassembly were dialyzed vs. storage buffer (0.01 M Tris-HCl, pH 7.8, 0.1 mM EDTA, 0.1 mM DTT, 50% glycerol, and either 0.2 M NaCl or 0.45 M NaCl) at 4 °C and stored at −20 °C. Densitometry scans of 5% acrylamide–$NaDodSO_4$ gels revealed the following minimum percent purities averaged over several preparations: σ (95%), $\alpha_2\beta$ (95%), β' (90–95%), and α (95%).

Results

Physicochemical Properties. The ultraviolet absorption spectra are unexceptional except for that of σ subunit, which differs from core and holoenzyme spectra in having a shoulder at 290 nm [as Lowe et al. (1979) have also noted] and a low A_{280}/A_{260} ratio between 1.6 and 1.8. The extinction coefficients at 280 nm for the subunits and subassemblies of RNA polymerase are summarized in Table I. For these determinations, concentrations were obtained by using the method of Jaenicke (1974) and all absorption spectra were corrected for light scattering by the method of Leach & Scheraga (1960).

Additional analytical determinations included sulfhydryl content and isoelectric points. In $NaDodSO_4$, the total thiol content (equivalents/mole), determined by Ellman's method, of σ, core, and holoenzyme was 4 ± 0.4, 25 ± 1, and 29 ± 1, respectively. Isoelectric focusing in 8 M urea and 2% Nonidet P-40 was performed. The following p*I* values were calculated for RNA polymerase subunits: β' (6.85); β (5.30); α (4.65); σ (4.35). These p*I* values are subject to an error of ±0.25 pH unit.

Far-UV CD spectra of core, holoenzyme, and σ subunit in buffer containing 10% glycerol are presented in Figure 2. Values of $[\Theta]_{220}$ for core, holoenzyme, and σ are −10 800, −13 100, and −24 500 deg cm² $dmol^{-1}$, respectively. All three showed a second extremum, which varied slightly in position:

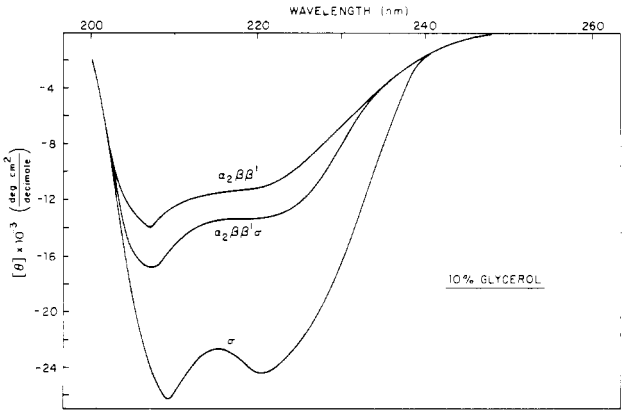

FIGURE 2: Far-UV CD spectra of core, holoenzyme, and σ subunit. All proteins were in buffer containing 0.01 M Tris-HCl, pH 7.9, 0.1 mM DTT, 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.45 M NaCl, and 10% glycerol at 4 °C.

$[\Theta]_{207}$ for core is −14 040 deg cm² $dmol^{-1}$, $[\Theta]_{207.5}$ for holoenzyme is −16 800 deg cm² $dmol^{-1}$, and $[\Theta]_{209}$ for σ is −26 200 deg cm² $dmol^{-1}$. The ratio of magnitudes at the two extrema is 1.30 for core, 1.28 for holoenzyme, and 1.07 for σ. It should be noted that the spectra of σ and core enzyme have different shapes between 220 and 212 nm, probably owing to a contribution by β sheet in core enzyme, and that the experimental holoenzyme spectrum is similar to an average spectrum that would be obtained by weighting according to the mass contributions of σ and core enzyme.

Using the parameters of Chen et al. (1974) and assuming that the average number of peptide units per helical segment is close to the value for their five reference proteins, we have estimated the secondary structure contents of core and holoenzyme. On this basis, core contains 33% of its amino acids in α-helical arrangement and 32% in β sheet, holoenzyme has 43% of its amino acids in α helix form and 27% in β sheet, and σ has 75% of its residues in α-helical segments and less than 10% in β sheet. It may be noted that σ has an exceptionally high α-helix content.

Far-UV CD spectra of α, β, β', and $\alpha_2\beta$ in buffer containing 50% glycerol all showed shapes similar to that of core enzyme. The values of $[\Theta]_{220}$ for α, β, β', and $\alpha_2\beta$ are −4800, −5200, −7600, and −5700 deg cm² $dmol^{-1}$, respectively. These values are anomalously low relative to those of core and holoenzyme. Taken together with the low yield for enzymatic activity following reconstitution of the isolated subunits, these far-UV CD data suggest substantial denaturation of the isolated subunits and $\alpha_2\beta$ fraction.

Effect of Glycerol on σ, Core, and Holoenzyme. The effect of glycerol on the conformation of σ, core, and holoenzyme was measured in the near- and far-UV CD. The far-UV CD spectra are not presented, although it is important to mention that the shape of the spectra and the $[\Theta]_{220}/[\Theta]_{207}$ ratio are unchanged as a function of glycerol concentration. On the other hand, some change was noted in the magnitude of the ellipticities. Table II summarizes values of $[\Theta]_{220}$ as a function of glycerol concentrations. The magnitude of $[\Theta]_{220}$ increases approximately 15% from 0 to 10% glycerol but decreases slightly from 10 to 50% glycerol for both core and holoenzyme. The drop in $[\Theta]_{220}$ for σ from 10 to 50% glycerol is about 20%. $[\Theta]_{220}'$ values (corrected for the index of refraction as under Materials and Methods) are also presented to demonstrate that the phenomenon is not due to differences in solvent indexes of refraction.

Circular dichroism spectra in the near-UV also showed dependence on glycerol concentration, but the signal/noise

Table II: Effect of Glycerol on $[\Theta]_{220}$ Values for Core, Holoenzyme, and σ Subunit

% glycerol (v/v)	$[\Theta]_{220} \times 10^{-3}$[a] core	holoenzyme	σ	$[\Theta]_{220}' \times 10^{-3}$ core	holoenzyme	σ
0	9.30	11.5		7.39	9.14	
5	10.0	12.5		7.91	9.89	
10	10.8	13.1	24.5	8.60	10.3	19.3
25	10.5	12.9		8.15	10.0	
50	10.0	12.0	19.5	7.56	9.1	14.7

[a] Average deviations at 220 nm were ±6% for core and holoenzyme and ±9% for σ.

ratios were low and quantitative estimates were not reliable to better than ±20% in the range 260–300 nm. Qualitatively, the variations at a negative peak at 280 nm can be summarized for both core and holoenzyme as follows. There was no significant change between 0 and 5% glycerol but a decrease of almost one-half in intensity on increasing to 10% glycerol. At higher glycerol concentrations the CD band again increased in magnitude, and at 50% glycerol concentration the spectra were close to those in the absence of glycerol. There were no marked changes in position or shape of bands as glycerol concentration was varied.

If $[\Theta]_{220}$ for holoenzyme is calculated from the sum of core and σ, the resultant value is equal to −13 300 and −11 700 deg cm^2 dmol^{-1} in 10 and 50% glycerol, respectively. The observed values for holoenzyme (from Table II) are −13 100 and −12 000 for deg cm^2 dmol^{-1} in 10 and 50% glycerol. In 50% glycerol, we have found no change in $[\Theta]_{220}$ for reconstituted holoenzyme upon combination of equimolar quantities of core and σ subunit. On the other hand, a 9% decrease in $[\Theta]_{220}$ for reconstituted holoenzyme from −13 300 to −12 000 deg cm^2 dmol^{-1} has been observed in 10% glycerol. However, σ subunit is susceptible to surface denaturation on glass and quartz surfaces at low glycerol concentrations (observed in protein–DNA interactions) and the 9% decrease may not be significant. These results indicate that the secondary structures of core and σ subunit are not demonstrably altered upon combination to form holoenzyme in either 10 or 50% glycerol.

Molecular sieve chromatography of holoenzyme in 10 and 50% glycerol is depicted in Figure 3. The void volume (V_o) and total volume (V_t) of the column were determined by using Blue Dextran and Ni_2SO_4, respectively. Fractions 50–70 from the 10% glycerol column and fractions 45–70 from the 50% glycerol column were subjected to $NaDodSO_4$ gel electrophoresis. The holoenzyme in the 10% glycerol experiment consistently is 40–50% σ saturated while in the 50% glycerol case the σ saturation is between 85 and 95%. The small peak in Figure 3A is assumed to contain σ, although we have not demonstrated this directly. However, analytical gels in 10% glycerol of holoenzyme preparations containing saturating quantities of σ subunit show a band corresponding to free σ subunit. In 50% glycerol, no unbound σ subunit is discerned.

The effect of glycerol on the core and holoenzyme assay system was investigated. Core and holoenzyme samples were in buffers similar to those used in the CD experiments, but containing no glycerol. A 0.1-mL amount of protein was assayed with 0.25 mL of assay buffer containing the various glycerol concentrations. The assay buffer also contained excess concentrations of either poly[d(A-T)], or calf thymus, or T7 DNA. The maximum specific activities for core and holoenzyme with the various DNA templates were obtained when the glycerol concentration in the assay buffer was between 5 and 10%.

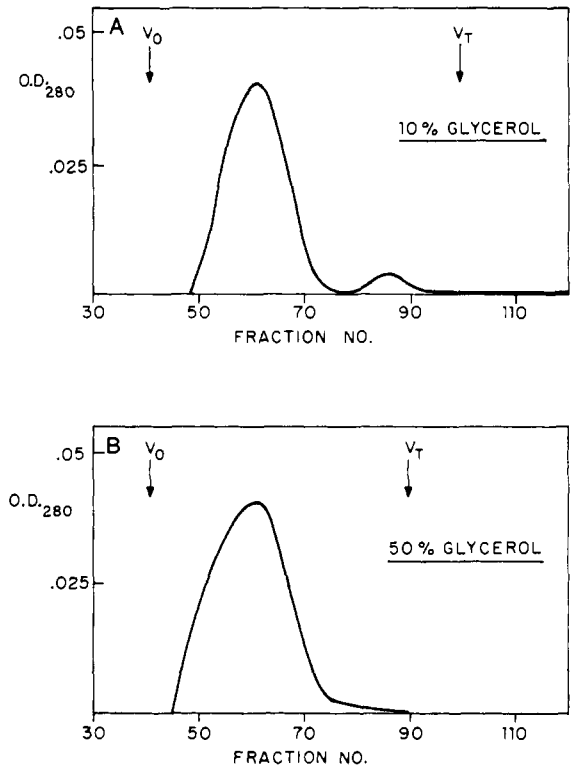

FIGURE 3: Molecular sieve chromatography of holoenzyme in buffer containing 10 and 50% glycerol. Two Bio-Gel A-1.5m columns (1.5 × 45 cm) were packed and equilibrated at 4 °C in buffer containing 0.01 M Tris-HCl, pH 7.9, 1 mM DTT, 0.01 M $MgCl_2$, 0.1 mM EDTA, 0.45 M NaCl, and either (A) 10% glycerol or (B) 50% glycerol. Samples of 0.88 mL of holoenzyme were dialyzed overnight at 4 °C against buffer containing either 10 or 50% glycerol and applied to the columns at a concentration of 9.6×10^{-6} M. The flow rate was 3.3 mL/h, and 0.5-mL fractions were collected. OD_{280} of the fractions was recorded in a 1-cm cell.

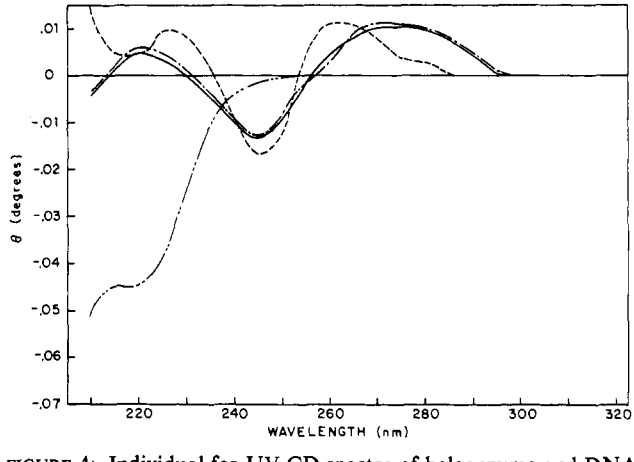

FIGURE 4: Individual far-UV CD spectra of holoenzyme and DNA in a 1-cm cell at 37 °C. Holoenzyme (54 μg/mL) (----); poly[d-(A-T)] (33 μg/mL) (---); calf thymus DNA (43 μg/mL) (—); T7 DNA (46 μg/mL) (---).

Protein–DNA Interactions. The effect of DNA on core and holoenzyme structure was monitored by circular dichroism. Figures 4 and 5 present the experiments performed with holoenzyme and various DNA templates at 37 °C. In these experiments, and all others summarized in Table III, core and holoenzyme appear to lose some of their secondary structure upon binding to DNA. The disorder in structure caused by the DNA is larger at higher temperature. Table III summarizes the percent decrease in magnitude of circular dichroism at 220 nm of $\alpha_2\beta$ subassembly and β′ subunit upon mixing with the various DNA templates at 37 °C. β′ subunit

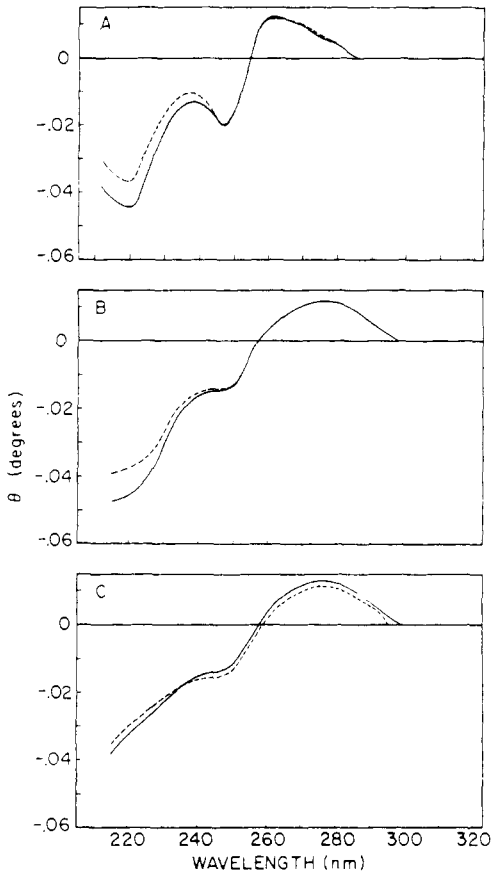

FIGURE 5: Combination far-UV CD spectra of holoenzyme plus DNA in a 2-cm tandem cell at 37 °C. (A) Holoenzyme (54 μg/mL) and poly[d(A-T)] (33 μg/mL): separated (—); mixed (---). (B) Holoenzyme (54 μg/mL) and calf thymus DNA (43 μg/mL): separated (—); mixed (---). (C) Holoenzyme (54 μg/mL) and T7 DNA (46 μg/mL): separated (—); mixed (---).

Table III: Reduction in Magnitude (%) of Circular Dichroism at 220 nm

	4 °C[a]			37 °C[a]		
protein	A-T	CT	T7	A-T	CT	T7
holoenzyme	13	10	3.8	18	19	7.7
core	9.2	11		16	16	
$\alpha_2\beta$				0	10.5	14
β'				0	17	27

[a] CT = calf thymus.

shows a decrease in secondary structure upon the addition of both calf thymus DNA and T7 DNA, the change being larger with T7 DNA. $\alpha_2\beta$ subassembly also shows a decrease in secondary structure in the presence of both calf thymus DNA and T7 DNA, though not as great as that seen with the β' subunit. Neither $\alpha_2\beta$ subassembly nor β' subunit shows a change in secondary structure upon the addition of poly[d-(A-T)].

Discussion

Because of limited quantities that have been available for analytical work and variable degrees of purity, there has been uncertainty about several characteristic physicochemical parameters of RNA polymerase and its subunits. In particular, values of the isoelectric points of the isolated subunits and of certain spectral characteristics of core and holoenzyme, as well as σ subunit, have only recently become available (Lowe et al., 1979). For the core components and the $\alpha_2\beta$ subassembly, extinction coefficients have not been previously published. The $\epsilon^{0.1\%}_{280\ nm,\ 1\ cm}$ values for core and holoenzyme obtained by the nitrogen determination method reported here are in fair agreement with the values reported by others. The ϵ_M for α (1.35×10^4 M^{-1} cm^{-1}) determined by this method agrees reasonably well with the value calculated from the tryptophan and tyrosine contents known from the amino acid sequence (Ovchinnikov et al., 1977). By use of their amino acid sequence and ϵ_M values for tyrosine (1280 M^{-1} cm^{-1}) and tryptophan (5600 M^{-1} cm^{-1}) at 280 nm (Sober, 1970), an ϵ_M for α of 1.20×10^4 M^{-1} cm^{-1} is calculated. The ϵ_M values for β and β' determined by the nitrogen method fit in the range of $\epsilon^{0.1\%}_{280\ nm,\ 1\ cm}$ calculated from the different tyrosine and tryptophan contents reported in the literature (Burgess, 1969b; Fujiki & Zurek, 1975).

An $\epsilon^{0.1\%}_{280\ nm,\ 1\ cm}$ for σ (1.16) determined by the nitrogen method is considerably different from that determined by other methods (Burgess, 1976; Wu et al., 1976; Lowe et al., 1979). Although our ϵ_M value for σ, 1.0×10^5 M^{-1} cm^{-1}, is higher than that in other reports, it is consistent with our values for core (2.28×10^5 M^{-1} cm^{-1}) and holoenzyme (3.21×10^5 M^{-1} cm^{-1}).

The total thiol contents of holoenzyme, core, and σ subunits are consistent with one another. The thiol content of core, 25 equiv/mol, agrees with the value (24 equiv/mol) reported by Nicholson & King (1973) determined by using 2-(chloromercuri)-4-nitrophenol and Ellman's reagent. Harding & Beychok (1973) reported core to have 32 thiol groups reacting with *p*-mercuribenzoate. However, they used an incorrect value for the extinction coefficient; with the value reported in this paper, their sulfhydryl content is in agreement with that given here.

Isoelectric focusing was performed in the presence of the denaturant 8 M urea and the solubilizing nonionic detergent Nonidet P-40, according to the method of O'Farrell (1975). The p*I* values reported here for β', β, α, and σ (6.85, 5.30, 4.65, and 4.40) are close to those reported by Lowe et al. (1979). These values, obtained by using denaturing conditions, are not necessarily those of subunits in their native conformation; both 8 M urea and Nonidet P-40 have been shown to alter the p*I* of native proteins (Righetti et al., 1979). All attempts to focus intact RNA polymerase led to precipitation of the protein.

The $[\Theta]_{220}$ values for core, holoenzyme, and σ were used to estimate the secondary structure characteristics of these proteins in 10% glycerol. σ subunit is a highly structured protein (75% α helix), and its large value of $[\Theta]_{220}$ (-24.5×10^3 deg cm^2 $dmol^{-1}$) may be compared to values for very helical proteins such as human hemoglobin ($[\Theta]_{222} = -21.5 \times 10^3$ deg cm^2 $dmol^{-1}$; Luchins, 1977), sperm whale myoglobin ($[\Theta]_{222} = -24.4 \times 10^3$ deg cm^2 $dmol^{-1}$; Quadrifoglio & Urry, 1968), and rabbit myosin ($[\Theta]_{222} = -23.2 \times 10^3$ deg cm^2 $dmol^{-1}$; Oikawa et al., 1968). The $[\Theta]_{220}$ value for core enzyme in 10% glycerol (-10.8×10^3 deg cm^2 $dmol^{-1}$) differs from that reported by Harding & Beychok (1976) (-8.97×10^3 deg cm^2 $dmol^{-1}$) and Ishihama & Saitoh (1979) (-8.5×10^3 deg cm^2 $dmol^{-1}$). However, the results of the present report would agree with the value reported by Novak & Doty (1970) (-13×10^3 deg cm^2 $dmol^{-1}$) if adjustments are made for the high value of $\epsilon^{0.1\%}_{280\ nm,\ 1\ cm}$ used by those authors. Along with the $[\Theta]_{220}$ for σ, the value for holoenzyme has not been reported elsewhere and comparisons are thus not at present possible.

The $[\Theta]_{220}$ values for α, β, β', and $\alpha_2\beta$ were used to estimate the secondary structure of these proteins in 50% glycerol. The subunits and subassembly $\alpha_2\beta$ all have considerably less secondary structure than the intact core enzyme. In view of the failure to reconstitute these subunits to enzyme activity levels

greater than 30–60%, it is probable that all isolated subunit fractions and the $\alpha_2\beta$ subassembly are irreversibly unfolded to some degree.

Glycerol has been known for some time to stabilize RNA polymerase, and, accordingly, a study of the effect of glycerol on secondary structure as judged by far-UV CD and tertiary structure as judged by near-UV CD of core, holoenzyme, and σ subunit was undertaken. Polyhydric alcohols (ethylene glycerol, sucrose, glycerol, etc.) are not known to cause gross conformational changes in proteins at concentrations less than 50–60% as judged by ORD and CD (Singer, 1962; Tanford, 1962, 1968; Herskovitz et al., 1970). In fact, these alcohols are often added to protein solutions to stabilize activity during purification (Burgess, 1969a; Ballard & Williams-Ashman, 1966), storage (Ostren & Berg, 1974), and renaturation (Jacobson et al., 1970). With RNA polymerase, a small increase in the secondary structure of core and holoenzyme is noted as glycerol concentrations increase from 0 to 10% (Table II). Accompanying this, there are conformational changes in the environment of the aromatic chromophores, as judged by the near-UV CD. The observed changes are not due to significant perturbations of the UV absorption spectra, since quantitative transfers of protein from 0 to 10% glycerol solutions produce less than 5% difference in expected OD_{280}. Nicholson (1971) has shown that by perturbing core enzyme with 20% glycerol, only a 2% change in OD_{280} is noticed. Moreover, the variation in far-UV CD with glycerol content probably does not reflect different degrees of denaturation, since the direction is opposite to that observed in the near-UV. Whereas 10% glycerol yields a maximum magnitude at 220 nm, a minimum magnitude is observed at 280 nm. More likely, the glycerol effect indicates conformational flexibility of the enzyme.

Molecular sieve chromatography (Figure 3) and analytical electrophoresis demonstrate that the σ–core interaction is enhanced at higher glycerol concentrations. This result was implied in the work of Gonzalez et al. (1977), who found that holoenzyme did not dissociate on a 50% glycerol–phosphocellulose column. This phenomenon is not unique for RNA polymerase: sucrose has been found to enhance the polymerization of TMV A protein (Lauffer & Stevens, 1968) and G-actin to F-actin (Kasai et al., 1965); glycerol slows the dissociation of transcarboxylase to its subunits and promotes reassociation (Jacobson et al., 1970); 4 M glycerol (~30%) and 1 M sucrose enhance the assembly of microtubules at 37 °C in the absence of added nucleotides and retard the depolymerization of the tubules at 4 °C (Shelanski et al., 1973).

No reactivation of denatured core enzyme has been reported without glycerol in the reassembly buffer. However, the actual mechanism by which glycerol promotes protein–protein interactions cannot be unequivocally stated, although the suggestion by Lauffer & Stevens (1968) that sucrose and glycerol increase the ordered structure of surface-bound water may have applicability.

The effect of glycerol on in vitro transcription was examined with both core and holoenzyme. Maximal stimulation occurs at glycerol (v/v) concentrations between 5 and 10%. GC-containing DNAs, such as calf thymus and T7, show slightly more dependence on glycerol than does poly[d(A-T)]. Glycerol is known to lower the T_m of DNA (Levine et al., 1963) and probably stabilizes the open configuration, which permits RNA polymerase to bind and initiate transcription (Travers, 1974). Recently, Buss & Stalter (1978) have demonstrated with several polyhydroxylic compounds a marked increase in the transcription activity of rat thymic RNA polymerase II on calf thymus DNA. These increases were also strongly temperature dependent.

The preliminary results of the effect of DNA on the secondary structure of RNA polymerase core and holoenzyme, $\alpha_2\beta$ subassembly, and β' subunit depend on the experimental fact that DNA samples show a very weak signal at 220 nm, whereas the polymerase proteins exhibit a strong signal at that wavelength. Upon mixing, any change is predominantly attributable to a change in the protein structure. In all cases, except for the interactions of poly[d(A-T)] with $\alpha_2\beta$ and β', a loss in secondary structure was observed, the effect being greater, for core and holoenzyme, at higher temperatures. Work is in progress to determine whether the CD changes arise from binding at specific, nonspecific, or both kinds of RNA polymerase binding sites.

References

Adler, A. J., & Fasman, G. D. (1968) *Methods Enzymol. 12B*, 268.

Ballard, P. L., & Williams-Ashman, H. F. (1966) *J. Biol. Chem. 241*, 1602.

Berg, D., Barrett, K., & Chamberlin, M. (1971) *Methods Enzymol. 21D*, 506.

Burgess, R. R. (1969a) *J. Biol. Chem. 244*, 6160.

Burgess, R. R. (1969b) *J. Biol. Chem. 244*, 6168.

Burgess, R. R. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) p 69, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Burgess, R. R., & Jendrisak, J. J. (1975) *Biochemistry 14*, 4634.

Buss, W. C., & Stalter, K. (1978) *Biochemistry 17*, 4825.

Chen, Y.-H., Yang, J. T., & Chau, K. H. (1974) *Biochemistry 13*, 3350.

Ellman, G. L. (1959) *Arch. Biochem. Biophys. 82*, 70.

Fasman, G. D., Ed. (1976) *CRC Handbook of Biochemistry and Molecular Biology 3*, p 3, CRC Press, Cleveland, OH.

Fujiki, H., & Zurek, G. (1975) *FEBS Lett. 55*, 242.

Gonzalez, N., Wiggs, J., & Chamberlin, M. (1977) *Arch. Biochem. Biophys. 182*, 404.

Guillery, V. P. (1930) *Phys. Z. 31*, 700.

Harding, J. D., & Beychok, S. (1973) *Biochem. Biophys. Res. Commun. 51*, 711.

Harding, J. D., & Beychok, S. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3395.

Harding, J. D., & Beychok, S. (1976) in *RNA Polymerase* (Losick, R., & Chamberlin, M., Eds.) p 355, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Herskovitz, T. T., Gradegebka, B., & Jaillet, H. (1970) *J. Biol. Chem. 245*, 2588.

Hjerten, S., Jerstedt, S., & Tiselius, A. (1965) *Anal. Biochem. 11*, 219.

Jacobson, B., Gerwin, B. I., Ahmad, F., Waegeh, P., & Wood, H. G. (1970) *J. Biol. Chem. 245*, 6471.

Jaenicke, L. (1974) *Anal. Biochem. 61*, 623.

Kasai, M., Nakano, E., & Oosawa, F. (1965) *Biochim. Biophys. Acta 94*, 494.

Lauffer, M. A., & Stevens, C. L. (1968) *Adv. Virus Res. 13*, 1.

Leach, S. J., & Scheraga, H. A. (1960) *J. Am. Chem. Soc. 82*, 4790.

Levine, L., Gordon, J. A., & Jencks, W. P. (1963) *Biochemistry 2*, 168.

Lowe, P. A., Hager, D. A., & Burgess, R. R. (1979) *Biochemistry 18*, 1344.

Luchins, J. I. (1977) *Stopped-Flow Circular Dichroism*, Ph.D. Thesis, Columbia University.

Nicholson, B. H. (1971) *Biochem. J. 123*, 117.
Nicholson, B. H., & King, A. M. Q. (1973) *Eur. J. Biochem. 37*, 575.
O'Farrell, P. H. (1975) *J. Biol. Chem. 250*, 4007.
Oikawa, K., Kay, C. M., & McCubbin, W. D. (1968) *Biochim. Biophys. Acta 168*, 164.
Ostren, D. L., & Berg, P. (1974) *Biochemistry 13*, 1338.
Ovchinnikov, Yu. A., Lipkin, V. M., Modyanov, N. N., Chertov, O. Yu., & Smirnov, Yu. V. (1977) *FEBS Lett. 76*, 108.
Quadrifoglio, F., & Urry, D. W. (1968) *J. Am. Chem. Soc. 90*, 2755.
Righetti, P. G., Giannazza, E., Gianni, A. M., Cormi, P., Giglioni, B., Ottolenghi, S., Sechhi, C., & Rossi-Bernardi, L. (1979) *J. Biochem. Biophys. Methods 1*, 45.
Shelanski, M. L., Gaskin, F., & Cantor, C. R. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 765.
Singer, S. J. (1962) *Adv. Protein Chem. 17*, 1.
Sober, H. A., Ed. (1970) *Handbook of Biochemistry*, CRC Press, Cleveland, OH.
Tanford, C. (1962) *J. Biol. Chem. 237*, 1168.
Tanford, C. (1968) *Adv. Protein Chem. 23*, 121.
Thomas, C. A., & Abelson, J. (1966) *Proced. Nucleic Acid Res. 1*, 535.
Travers, A. (1974) *Eur. J. Biochem. 47*, 435.
Travers, A. (1975) *FEBS Lett. 53*, 76.
Travers, A., & Burgess, R. (1969) *Nature (London) 222*, 537.
Vinograd, J., Morris, J., Davidson, N., & Dove, W., Jr. (1963) *Proc. Natl. Acad. Sci. U.S.A. 49*, 12.
Weast, R. C., Ed. (1972) *Handbook of Chemistry and Physics*, CRC Press, Cleveland, OH.
Weber, K., & Osborn, M. (1969) *J. Biol. Chem. 244*, 4406.
Winter, A., Ek, K., & Anderson, V.-B. (1971) *LKB Application Note 250.*
Wu, C. W., Yarbrough, L. W., Wu, F. Y.-H., & Hillel, Z. (1976) *Biochemistry 15*, 2097.
Wu, C. W., Wu, F. Y.-H., & Speckhard, D. C. (1977) *Biochemistry 16*, 5449.
Yarbrough, L. R., & Hurwitz, J. (1974) *J. Biol. Chem. 249*, 5400.

Evidence for Pyrimidine–Pyrimidine Cyclobutane Dimer Formation in the Covalent Cross-Linking between Transfer Ribonucleic Acid and 16S Ribonucleic Acid at the Ribosomal P Site†

James Ofengand* and Richard Liou

ABSTRACT: The covalent cross-linking between the anticodon of tRNA bound at the ribosomal P site and 16S ribosomal RNA which is induced by irradiation at wavelengths greater than 300 nm [Ofengand, J., Liou, R., Kohut, J., III, Schwartz, I., & Zimmermann, R. A. (1979) *Biochemistry 18*, 4322–4332] was shown not to involve any molecule of mRNA as a linker between tRNA and rRNA. After irradiation of a mixture containing oligo([^{3}H]G,U) (G:U = 1.1, average chain length 5), Ac[^{14}C]Val-tRNA, and ribosomes, isolation of the tRNA–ribosome complex by gel filtration in 0.1 mM Mg^{2+} followed by two cycles of dimethyl sulfoxide–sodium dodecyl sulfate denaturation and centrifugation on formamide–sucrose gradients gave a tRNA–rRNA complex with a mole ratio of Ac[^{14}C]valine to [^{3}H]guanosine of 5.6. In a similar experiment using oligo([^{3}H]$U_{1,2}$,G), the mole ratio of ^{3}H to ^{14}C was >50. Thus a minimum of 82% of the tRNA–rRNA complexes were free of any mRNA codons. The ribosome–tRNA covalent complex could be rapidly photolyzed by irradiation at 254 nm. With an incident light flux of 0.45 μEinstein min^{-1} mL^{-1}, the $T_{1/2}$ was 4 min. Both the ribosome and tRNA components which were photolytically split apart could again be cross-linked when reirradiated at 300 nm. Thus the cross-linking reaction was truly *photoreversible*. The kinetics of photolysis for *Escherichia coli* $tRNA_1^{Val}$ and $tRNA_1^{Ser}$ and *Bacillus subtilis* $tRNA^{Val}$ covalent complexes with ribosomes were virtually identical, suggesting a common structure for the cross-link in all three cases. The rate of photolysis at 254 nm was not affected by exposure to 0.1 M HCl at 23 °C for 15 min, ruling out oxetane structures for the cross-link. Photolysis of the *Escherichia coli* $tRNA^{Val}$–16S RNA complex could also be achieved by irradiation at 313 nm in the presence of the sensitizer 5-hydroxytryptamine or 5-hydroxytryptophan. No photolysis occurred when indole-3-carboxaldehyde was the sensitizer and almost none when no sensitizer was present. The sum of these properties is characteristic only of pyrimidine cyclobutane dimers, among the known photoinduced adducts of nucleic acids. Thus, we propose the structure of the cross-link to be such a dimer between the 5′-anticodon base of the tRNA and a pyrimidine in the 16S RNA.

Chemical and photochemical affinity labeling of macromolecules with large or small molecular weight ligands has become a commonly accepted tool in the biochemical armamentarium (Jakoby & Wilchek, 1977). Its utility in investigating the topography of the mRNA–tRNA–ribosome complex, the essential element in protein biosynthesis, is now well established (Kuechler & Ofengand, 1979; Ofengand, 1980). In our laboratory we have used photoactivatable derivatives of tRNA as photoaffinity probes to study the nature of the tRNA binding sites, the A and P sites, on the *E. coli* ribosome (Ofengand et al., 1980) in the belief that once the topography is known, the molecular processes involved in tRNA binding and translocation will become more evident.

As part of this program, we have recently described a unique photochemical cross-linking reaction which joins the anticodon of underivatized tRNA to the ribosome upon irradiation at wavelengths >300 nm (Schwartz & Ofengand, 1978; Ofengand et al., 1979). This cross-linking reaction is highly specific. It requires tRNA occupancy of the ribosomal P but not the

† From the Roche Institute of Molecular Biology, Nutley, New Jersey 07110. *Received March 25, 1980.*

0006-2960/80/0419-4814$01.00/0