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Preparation and Characterization of *N*-(1-Pyrenyl)iodoacetamide-Labeled *Escherichia coli* RNA Polymerase

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ABSTRACT: *N*-(1-Pyrenyl)iodoacetamide has been used to introduce fluorescent probes into *Escherichia coli* RNA polymerase. After an incubation time of 15 min, approximately 2 pyrene equiv was introduced per enzyme molecule. There was no further increase in modification after more extended periods of incubation. Neither calf thymus DNA nor nucleotides protected the holoenzyme from modification. Thus, the sites of modification do not appear to involve the binding sites for polynucleotides or the ribonucleoside triphosphates. From the isolation and analysis of the individual subunits, it was found that σ contained approximately 1 pyrene equiv, β contained 0.6, β' contained 0.6, and α less than 0.1. Spectral and Stern–Volmer analyses indicate that the covalently attached pyrene molecules are in comparable apolar microenvironments. On the basis of CD analyses, the introduction of pyrene molecules into RNA polymerase alters its secondary structure. This alteration in secondary structure manifests itself by a reduction in overall enzymatic activity. Transcript analysis of the products obtained by using a linearized plasmid containing the A1 promoter and the T_e terminator of bacteriophage T7 indicates that the pyrenyl derivative is capable of producing full-length transcripts and that it has an efficiency of chain termination comparable to the native enzyme. Analysis of τ plots for the interaction of the pyrenyl derivative and the native enzyme, respectively, with the A1 promoter yielded comparable values for the isomerization constant in the conversion of the closed complex to an open one. Comparable values were also obtained for the association constant. The rate of chain elongation for the pyrenyl derivative, however, is approximately 54% of that observed for the native enzyme. Thus, the decrease in overall transcriptional activity observed with the pyrenyl derivative is not due to a decrease in the efficiency of initiation or premature termination, but rather to a decrease in the rate of chain elongation.

The reaction catalyzed by *Escherichia coli* RNA polymerase has been divided formally into four major steps, each of which consists of several substeps (Chamberlin, 1970; McClure, 1985). RNA polymerase recognizes and binds to promoter sites, initiates transcription at these sites, catalyzes the process of chain elongation, and terminates transcription at discrete sites. It has been postulated that many of these steps are accompanied by conformational changes in the enzyme. However, precise experimental documentation of these structural alterations is limited. A biphasic change in the

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fluorescence intensity of fluorophores [*N*-(1-pyrenyl)maleimide] located in the σ subunit, upon the rapid mixing of the labeled σ subunit with an excess of core polymerase, has been interpreted to indicate a fast bimolecular binding of σ to the core polymerase followed by a relatively slow isomerization of the holoenzyme which is formed (Wu et al., 1976). These results have been used to account for the fact that the σ subunit confers selective asymmetric transcription to the core polymerase. The resulting changes in fluorescence upon the binding of DNA or nucleoside triphosphates to an inactive derivative of RNA polymerase containing a fluorescent analogue of 6-methylthioinosine dicarboxaldehyde covalently attached to the β subunit have been interpreted to indicate structural alterations in RNA polymerase upon the binding of ligands (Wu & Wu, 1974). In a neutron small-angle scattering study, it has been shown that RNA polymerase undergoes a conformational change upon the formation of a specific complex with the A1 promoter of bacteriophage T7 (Heumann et al., 1988). The interaction of ppGpp with RNA polymerase has been investigated by circular dichroism (Woody et al., 1987). Using this approach, it was demonstrated that the binding of ppGpp to RNA polymerase results in a conformational change in the enzyme. It was inferred from these studies that the effect of ppGpp on transcription is mediated by this conformational change.

To gain further insight into the mechanism of transcription at the molecular level, the fluorescent probe *N*-(1-pyrenyl)-iodoacetamide has been coupled to *E. coli* RNA polymerase. The usefulness of fluorescence labels as reporter groups is based on the low concentrations (10–100 nM) of fluorophore required to obtain a detectable signal. Furthermore, the fluorescent probe is sensitive to a variety of molecular and environmental conditions. As a result of these properties, it is possible to analyze the microenvironment of the fluorophore and thus the microenvironment of the group to which it is attached. Not only can this approach be used to demonstrate perturbations on ligand binding, but it also can be used to quantify protein–ligand interactions. This paper details the preparation and characterization of *N*-(1-pyrenyl)carboxamidomethyl RNA polymerase (pyrenyl RNA polymerase; P-RPase).[1] In the following paper (Johnson, 1991), the results obtained from an investigation of the interaction of this enzymatically active derivative with synthetic polynucleotides are presented.

Materials and Methods

Materials. E. coli K12 cell paste (three-fourths log phase, enriched medium) was purchased from Grain Processing Corp. *N*-(1-Pyrenyl)iodoacetamide was obtained from Molecular Probes. [2,8-^{3}H]ATP and [8,5-^{3}H]GTP were obtained from New England Nuclear, and [α-^{32}P]UTP was obtained from either New England Nuclear or ICN. Calf thymus DNA was purchased from Worthington, and poly[d(A-T)][1] and poly-[d(G-C)] were from P-L Biochemicals. Bacteriophage T7 DNA was purchased from Sigma. pBR322, blue agarose, and red agarose were obtained from Bethesda Research Laboratories. DNA–cellulose was prepared according to the procedure of Alberts and Herrick (1971). All other reagents were of the highest purity available from commercial sources.

Purification of RNA Polymerase and Isolation of the Individual Subunits. For the purification of RNA polymerase, the method of Burgess and Jendrisak (1975) as modified by Lowder and Johnson (1987) was used. Separation of the core polymerase and holoenzyme was accomplished by using the method of Gonzalez et al. (1977). As the final step in the purification of the holoenzyme, DNA–cellulose column chromatography was used according to the protocol of Burgess and Jendrisak (1975). Either a linear salt gradient from 0.15 to 0.9 M NaCl or a step gradient at 0.5 M NaCl was used to elute the column. Densitometric scans of Coomassie Blue stained sodium dodecyl sulfate–8.75% polyacrylamide gels, which were run according to the procedure of Laemmli (1970), indicated in all cases that the holoenzyme was at least 90% saturated with σ. The σ subunit was isolated from the holoenzyme according to the method of Lowe et al. (1979). The individual subunits in the core polymerase were isolated by chromatography on a blue agarose column as described by Lowder and Johnson (1987). The concentration of the different species in solution was determined from the respective molar extinction coefficients (Levine et al., 1980). When necessary, the contribution to the absorbance due to light scattering was determined by using the method of Leach and Scheraga (1960).

Purification of Plasmids. E. coli strain HMS174 containing either plasmid pAR1435 or plasmid pAR1707 was generously provided by Drs. W. Studier and A. H. Rosenberg (Biology Department, Brookhaven National Laboratory, Upton, NY). Bacteria were grown in LB medium [1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto yeast extract, and 1% (w/v) NaCl] containing 40 μg/mL ampicillin. LB medium (100 mL) containing ampicillin was inoculated with a single bacterial colony. This solution was incubated overnight at 37 °C with vigorous shaking. The following morning, two 1-L volumes of LB medium containing ampicillin were inoculated with 5 mL of the overnight culture and were incubated overnight at 37 °C with vigorous shaking. They served as the inoculum for 50 L of LB medium containing ampicillin in the Lab-Line 500-L fermentor. After the OD_{600} reached 0.4 (approximately 3 h after inoculation), chloramphenicol was added such that its final concentration was 0.17 mg/mL. Incubation at 37 °C was continued for an additional 4–5 h, at which time the bacterial cells were harvested. The bacterial cells were stored frozen at −20 °C until later use.

Plasmid DNA (pAR1435 or pAR1707) was isolated from the cells using lysis by SDS according to the procedure of Maniatis et al. (1982) with the following modifications. After the addition of NaCl and centrifugation to remove high molecular weight DNA and bacterial debris, a sufficient amount of a 30% solution of poly(ethylene glycol) (PEG) containing 1.5 M NaCl was added to the supernatant to yield a 10% final concentration of PEG. This solution was allowed to stand overnight at 4 °C. The solution was subjected to centrifugation at 6000 rpm in a GSA rotor for 5 min. The pellet was resuspended in 10 mL of buffer containing 50 mM Tris-HCl (pH 7.8), 0.1 M NaCl, and 0.1 mM EDTA. The plasmid DNA was subjected twice to purification by CsCl sedimentation in the presence of ethidium bromide as described by Maniatis et al. (1982).

Linearized plasmid was obtained by digestion of the isolated plasmid with the restriction endonuclease *Sal*I (Bethesda Research Laboratories) according to the protocol given by the manufacturer. After the reaction was complete, as judged by the results of ethidium bromide stained agarose gels, the sample was extracted extensively with a 1:1 mixture of phenol and chloroform/isoamyl alcohol (24:1, v/v), followed by extraction with ether. The extracted DNA was precipitated with

[1] Abbreviations: poly[d(A-T)], poly[d(A-T)]·poly[d(A-T)]; poly[d-(G-C)], poly[d(G-C)]·poly[d(G-C)]; CD, circular dichroism; GdnHCl, guanidine hydrochloride; RPase, RNA polymerase; P-RPase, pyrenyl RNA polymerase; DMF, dimethylformamide.

ethanol and dissolved in 10 mM Tris-HCl (pH 8.0)/1 mM EDTA. DNA concentrations were determined by measuring the absorbance at 260 nm.

Coupling of N-(1-Pyrenyl)iodoacetamide to RNA Polymerase. The reaction between RNA polymerase holoenzyme and *N*-(1-pyrenyl)iodoacetamide was performed at ambient temperature in a buffer containing 50 mM Tris-HCl (pH 8.0), 0.1 M KCl, 10 mM $MgCl_2$, 0.1 mM EDTA, and 10% glycerol (buffer A). Prior to modification, the enzyme was extensively dialyzed against buffer A to remove dithiothreitol. The appropriate amount of *N*-(1-pyrenyl)iodoacetamide was added in DMF to the protein solution, and the reaction was gently stirred in the dark. The reaction was stopped at the desired time by the addition of excess 2-mercaptoethanol (approximately 10 mM). Excess unreacted insoluble reagent was removed by centrifugation at 2500*g* for 15 min. The modified enzyme was dialyzed extensively against buffer A containing 0.1 mM dithiothreitol.

The extent of protein modification was determined in a solution which contained 4 M GdnHCl using an extinction coefficient of 2.2×10^4 M^{-1} cm^{-1} at 344 nm for the pyrene adduct (Kouyama & Mihashi, 1981). It was found that the concentration of pyrene determined under these conditions was comparable to that observed for the native species. Therefore, the extent of modification was routinely determined in the absence of GdnHCl. The protein concentration was determined by the method of Lowry et al. (1951). The sulfhydryl content was determined by using Ellman's reagent according to the method of Habeeb (1972).

RNA Polymerase Assay. Enzyme activity was assayed according to the method of Johnson (1985). The activities of the holoenzyme and core enzyme were determined under conditions similar to those used for the spectroscopic studies which are described in the following paper (Johnson, 1991). The transcriptional activities with calf thymus DNA and poly[d(G-C)] were determined in 10-min assays, whereas 5-min assays were used in the case of poly[d(A-T)]. One unit of enzyme activity is defined as the nanomoles of ATP or GTP incorporated into acid-precipitable RNA per milligram of enzyme in 10 min. The assay solutions (0.25 mL) contained 0.1 mM EDTA, 0.4 mM each of the required nucleoside triphosphates with either [^{3}H]ATP or [^{3}H]GTP at a specific activity of 15000–20000 cpm/nmol, 5 μg of enzyme, 0.1 mM DNA, 0.1 mM DTT, and one of the buffer systems listed in Table II. The concentrations of the DNA templates were determined by using the following extinction coefficients (per millimole of phosphate): poly[d(A-T)], 6.65 mM^{-1} cm^{-1} at 262 nm (Inman & Baldwin, 1962); poly[d(G-C)], 7.10 mM^{-1} cm^{-1} at 255 nm (Pohl et al., 1973); calf thymus DNA, 6.5 mM^{-1} cm^{-1} at 260 nm (McConnell & von Hippel, 1970).

Abortive Initiation Assays. The procedure of Oen et al. (1979), as modified by Solaiman and Wu (1984), was used to estimate the number of active enzyme molecules present in the samples. Reaction mixtures contained, in a final volume of 30 μL, 40 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, 2 mM AMP, 5 nM plasmid pAR1435, pAR1707, or pBR322 DNA, 7.8 μM [α-^{32}P]UTP, and various concentrations of enzyme. Aliquots (15 μL) of cocktail A [80 mM Tris-HCl (pH 8.0), 5 mM $MgCl_2$, 50 mM KCl, 2 mM DTT, 10 nM plasmid DNA, and various concentrations of RNA polymerase] were incubated at 37 °C for 10 min. An equal volume of cocktail B [15.6 μM [α-^{32}P]UTP (360 cpm/pmol), 4.0 mM AMP, 5 mM $MgCl_2$, and 50 mM KCl] was added to each aliquot, and the incubation was allowed to proceed for an additional 30 min at 37 °C. The reactions were terminated by the addition of 10 μL of 0.2 M EDTA. The entire mixture from each reaction was spotted on Whatman 3MM paper in 5–6-μL aliquots with interim drying, and the papers were developed with ascending chromatography in WASP solvent [H_2O/saturated $(NH_4)_2SO_4$ (pH 8.0)/2-propanol, 18:80:2 v/v/v, containing 5 mM EDTA]. The chromatograms were dried at room temperature, cut into strips, and analyzed for radioactivity by Cerenkov counting. The R_f value of the product (pApU) was approximately 0.30.

The procedure of McClure (1980) was used to obtain estimates of the isomerization constant, k_2, and the association constant, K_{assoc}. This approach, which is based on a two-step model for initiation, is briefly summarized below. As the rate of abortive product formation approaches a final steady-state rate, the observed lag time (τ_{obs}) is given by eq 1, where [R] is the enzyme concentration. Measurements of the lag time, τ, as a function of enzyme concentration are made. The values of the two constants k_2 and K_{assoc} can be determined directly from a plot of τ_{obs} versus 1/[R], i.e., a τ plot. Reaction mixtures contained, in a volume of 150 μL, 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 M KCl, 1 mM DTT, 2 mM AMP, 0.05 mM [α-^{32}P]UTP at a specific activity of approximately 360 cpm/pmol, 1 nM plasmid pAR1707 or pBR322 DNA, and various amounts of enzyme. Two sets of reactions were performed at each enzyme concentration. In one case, the reaction was initiated by the addition of nucleotides. In the other case, it was initiated by the addition of enzyme. For the initiation of the reactions by the addition of nucleotides, 75 μL of cocktail C [66.7 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 M KCl, 2 nM plasmid pAR1707 or pBR322, and 1.7 mM DTT] was added to 25 μL of enzyme solution at the appropriate concentration. After a 5-min incubation at 37 °C, the reactions were initiated by the addition of 50 μL of cocktail D [6 mM AMP, 0.15 mM [α-^{32}P]UTP at a specific activity of approximately 360 cpm/pmol, 10 mM $MgCl_2$, and 0.1 M KCl]. The mixtures were incubated at 37 °C. Aliquots (10 μL) were removed at timed intervals over a period of 12 min, and the reaction was stopped by the addition fo 10 μL of 0.2 M EDTA. The samples were analyzed by ascending chromatography as described above. Data were analyzed by linear regression. For the initiation of the reaction by the addition of enzyme, 75 μL of cocktail C was mixed with 50 μL of cocktail D. After a 5-min incubation at 37 °C, the reactions were initiated by the addition of 25 μL of an enzyme solution at the appropriate concentration. Aliquots (10 μL) were removed at timed intervals over a period of 12 min, and the reaction was stopped by the addition of 10 μL of 0.2 M EDTA. The mixtures were spotted on Whatman 3 MM paper in 5–6-μL aliquots with interim drying and resolved with ascending chromatography in WASP solvent. The chromatograms were analyzed as described above. Linear least-squares analyses of the experimental data in the steady-state portion of the curves after the lag yielded values of τ_{obs} with their associated error.

$$\tau = 1/k_{obs} = 1/k_2 + 1/K_{assoc}k_2[R] \quad (1)$$

Chain Elongation Assay. The procedure of Kingston et al. (1981) was used to measure the rate of chain elongation. Reaction mixtures (200 μL) contained 44 mM Tris-HCl (pH 8.0), 14 mM $MgCl_2$, 20 mM NaCl, 14 mM 2-mercaptoethanol, 40 μM EDTA, 40 μg/mL acetylated BSA, 0.4 mM each of the required nucleoside triphosphates (ATP, GTP, UTP, and CTP) with [^{32}P]UTP at a specific activity of approximately 360 cpm/pmol, 0.3 mM (in nucleotides) T7 DNA, and 0.4 μg of enzyme. A 10-min preincubation at 30 °C was carried out on a mixture (160 μL) containing enzyme, DNA,

salts, and 10 μM each of the four unlabeled ribonucleoside triphosphates. After the 10-min preincubation period, 40 μL of a solution containing the four ribonucleoside triphosphates with [^{32}P]UTP at a specific activity of 360 cpm/pmol was added to 160 μL of the reaction mixture to give the indicated final concentrations of reagents in a volume of 200 μL. The reaction was incubated at 30 °C. At 2 and 4 min later, 50-μL aliquots were removed. The reaction was stopped by the addition of the 50-μL aliquot to 100 μL of carrier solution containing 50 mM sodium pyrophosphate, 50 mM EDTA, and 0.5 mg/mL yeast RNA. This was followed immediately by the addition of 2.5 mL of ice-cold 10% trichloroacetic acid. Samples were kept on ice for at least 30 min and then were filtered using Whatman GF/C filters which were previously soaked with 0.1 M pyrophosphate. Each filter was washed with about 35 mL of an ice-cold solution containing 1 M HCl and 0.1 M sodium pyrophosphate, and then with 10 mL of ethanol. After being dried in an oven, the filters were counted using scintillation fluid. The rate of incorporation of UTP over these two time points was then determined. This value was corrected to reflect the overall rate of incorporation of nucleotides into RNA by assuming that, to a first approximation, the rate of incorporation of UTP is one-fourth the total rate of nucleotide incorporation. This final corrected value is taken as the velocity of chain elongation.

Transcript Analysis. The procedure of Briat and Chamberlin (1984) was used to determine if the pyrenyl derivative is capable of producing full-length transcripts and also to determine the efficiency of chain termination. RNA synthesis was performed in a reaction mixture (50 μL) containing 40 mM Tris-HCl (pH 8.0), 10 mM 2-mercaptoethanol, 10 mM $MgCl_2$, acetylated BSA at 0.1 mg/mL, 0.4 mM each of GTP, ATP, CTP, and [α-^{32}P]UTP (at 500–1000 cpm/pmol), *Sal*I-digested pAR1707 DNA at 20 μg/mL, and holoenzyme at 40 μg/mL. There was a 10-min preincubation at 37 °C carried out in the absence of nucleotides. Heparin was added (final concentration, 100 μg/mL) 10 min after the initiation of transcription to block reinitiation of RNA chains, and incubation at 37 °C was continued for 10 min to allow runoff of elongating transcripts. The reactions were stopped by the addition of 100 μL of a solution containing 1.5 M ammonium acetate, 37.5 mM EDTA, and 45 μg/mL yeast RNA. The samples were phenol-extracted and then precipitated with 0.38 mL of ethanol. The pellets were suspended in 0.3 M sodium acetate (pH 5.2) and 0.2% SDS. Prior to analysis by gel electrophoresis, the samples were once again ethanol-precipitated. Precipitated RNA products were suspended in loading buffer (80% formamide, 9 mM Tris, 9 mM borate, 0.25 mM EDTA, 0.1% bromophenol blue, and 0.1% xylene cyanol), heated for 5 min at 65 °C, quenched on ice, and separated by electrophoresis. An 8% polyacrylamide/7 M urea gel as described by Maniatis et al. (1975) was used to separate the transcripts. Gels were dried and autoradiographed with Kodak XAR5 film. The relative amounts of the two transcripts present were determined by scanning the autoradiographs with an LKB laser densitometer in the 160- and 502-nucleotide regions. It was established that the intensities of the bands were in the linear range of the instrument by loading various amounts of the sample onto the gel and determining the relationship between intensity and amount added. After correction for differences in length, the amount of read-through was calculated from the amount of the 502-nucleotide transcript relative to the amount of the 160-nucleotide transcript.

Spectroscopic Measurements. Absorption spectra were recorded with an IBM Model 9430 spectrophotometer interfaced to an IBM 9000 computer. Circular dichroism spectra were recorded with a Jasco J-40C spectropolarimeter. The spectropolarimeter was calibrated with a solution of *d*-10-camphorsulfonic acid. The cell path length was 0.1 cm. All reported values of [θ] are expressed as mean residue ellipticities assuming a residue weight of 114 for the holoenzyme, 113 for the core enzyme, and 115 for the σ subunit. Corrected fluorescence emission spectra were obtained with a Spex Model 1681 spectrofluorometer in the ratio mode. The spectrofluorometer was interfaced to a Spex DM1B minicomputer. Spectra were generated by 90° transverse excitation of the sample. Data were recorded every 0.5 nm with an integration time of 2 s, and with excitation and emission bandwidths of 1.8 and 4.5 nm, respectively. All samples had an absorbance of less than 0.05 to prevent inner filter effects. The excitation wavelength was 330 nm. All spectra shown were obtained at 25 °C with samples in buffer A + 0.1 mM DTT.

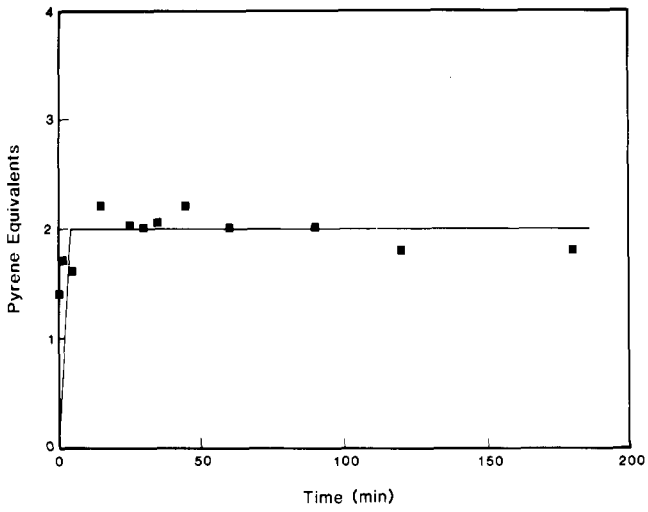

FIGURE 1: Modification of holoenzyme by *N*-(1-pyrenyl)iodoacetamide as a function of time. Aliquots (0.5 mL) were removed from the reaction mixture at various times, and the reaction was stopped by the addition of 2 μL of 2-mercaptoethanol to each aliquot. Excess unreacted *N*-(1-pyrenyl)iodoacetamide was removed by centrifugation. The samples were extensively dialyzed against buffer A + 0.1 mM DTT. Protein concentration was determined by the method of Lowry (1951). Pyrene content was determined from the absorption at 344 nm using an extinction coefficient of 2.2×10^4 M^{-1} cm^{-1}.

Dynamic quenching of various pyrenyl derivatives of RNA polymerase by acrylamide was determined by titrating the protein solution (2 mL) with 20-μL aliquots of a 5 M solution of acrylamide. *N*-(1-Pyrenyl)iodoacetamide was titrated in a similar fashion. All quenching data were analyzed by Stern–Volmer plots (Stern & Volmer, 1919).

Results

Conjugation of N-(1-Pyrenyl)iodoacetamide to RNA Polymerase. The reaction of RNA polymerase holoenzyme (4.6 μM) with a 50-fold molar excess of *N*-(1-pyrenyl)iodoacetamide was monitored as a function of time (Figure 1). After 15 min, 2.2 equiv of the fluorescent label was found attached per enzyme molecule. There was no further increase in the extent of modification over a period of 2.75 h. The effect of the molar ratio of *N*-(1-pyrenyl)iodoacetamide to enzyme on the extent of modification was also investigated. The reaction time in this case was 1 h. At a molar ratio of 10:1, 1.5 pyrene molecules were introduced per enzyme molecule, whereas at a molar ratio of either 25:1 or 50:1, 2.4 pyrene molecules were introduced. On the basis of these results, all further chemical modifications were carried out at a molar ratio of 50:1 for 1 h. As can be seen from Table I, this led to the reproducible

Table I: Pyrene Distribution in Chemically Modified Holoenzyme[a]

sample	no. of preparations	pyrene equiv
holoenzyme	7	2.1 ± 0.3
core enzyme	4	1.0 ± 0.2
σ	3	0.9 ± 0.2
β	2	0.6 ± 0.1
β'	2	0.6 ± 0.1
α	2	<0.1

[a] The core polymerase and individual subunits were isolated from modified holoenzyme derivatives that had been prepared with a 60-min incubation and a 50-fold molar excess of *N*-(1-pyrenyl)iodoacetamide.

modification of the holoenzyme and resulted in the introduction of approximately 2 pyrene equiv per enzyme molecule.

The sulfhydryl content of pyrenyl RNA polymerase was analyzed by using Ellman's reagent. In control studies using the native enzyme, the sulfhydryl content was found to be 33.7 ± 0.4. This value agrees well with the reported value of 33 which is based on a combination of amino acid and nucleic acid sequence analyses (Ovchinnikov et al., 1977, 1982; Burton et al., 1981). The average sulfhydryl content per enzyme molecule for the modified enzyme was found to be 30.4 ± 0.4. Thus, there is a reduction in the number of sulfhydryl groups present in the pyrenyl derivatives of between 2 and 3. This indicates, as expected, that sulfhydryl groups in the enzyme are the sites of modification by *N*-(1-pyrenyl)iodoacetamide. In the presence of either calf thymus DNA (0.1 mg/mL) or nucleotides (ATP and CTP at 0.4 mM each), there was no reduction in the extent of modification of the holoenzyme. Thus, the sites of modification do not appear to occur in regions of binding for either polynucleotides or substrate molecules.

In determination of the subunits in the holoenzyme which were modified by *N*-(1-pyrenyl)iodoacetamide, the σ subunit was first removed from the pyrenyl holoenzyme derivative. Analysis of this subunit indicated that it had undergone close to stoichiometric modification (Table I). The core polymerase was also shown to have undergone close to stoichiometric modification. The subunits in the core polymerase were next isolated and analyzed. The β subunit contained approximately 0.6 equiv of pyrene, whereas the α subunit contained less than 0.1. Because of the large amount of light scattering that was present in the absorption spectrum of β', it was not possible to directly determine the extent of modification of this subunit. On the basis of the pyrene content originally determined for the core polymerase, however, it was possible to obtain an estimate of 0.6 for the pyrenyl content of β'. Confirmation that this subunit does indeed contain pyrene was obtained from fluorescence spectroscopy.

Spectral Analysis. The absorption spectrum of pyrenyl holoenzyme is given in Figure 2. The introduction of pyrene into the enzyme gives rise to a peak at 344 nm and a shoulder at 330 nm. The peak at 280 nm is due almost exclusively to the protein. Pyrenyl core polymerase, σ, and β all display comparable absorption spectra with the shoulder and peaks in identical positions (data not shown). As stated previously, the light scattering observed for the pyrenyl β' subunit obscured the absorbance due to the fluorescent probe. Thus, it was not possible to analyze the absorption spectrum of this pyrenyl derivative. It should be noted that this intense light scattering is a phenomenon commonly observed for isolated β'. Relative to the spectrum of *N*-(1-pyrenyl)iodoacetamide free in solution (Figure 2) which has a peak at 340 nm, there is a bathochromic (red) shift of the peak for the pyrene molecule which is covalently attached to the enzyme. This shift is consistent with apolar microenvironments for the covalently attached pyrene molecules.

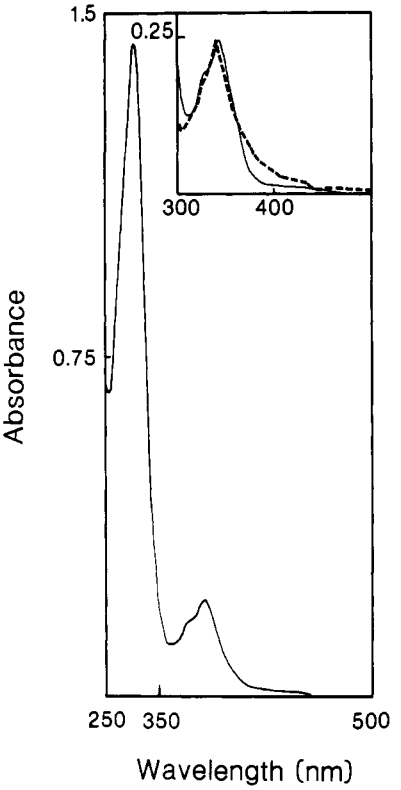

FIGURE 2: Absorption spectrum of pyrenyl RNA polymerase holoenzyme. The spectrum was recorded at a pyrene adduct concentration of 11.2 μM. The pyrene content per enzyme molecule was 2. The solid line in the inset represents an enlargement of the spectrum of pyrenyl holoenzyme between 300 and 500 nm. The dashed line is the absorption spectrum of *N*-(1-pyrenyl)iodoacetamide free in solution (buffer A + 0.1 mM DTT) at a concentration of 11.2 μM.

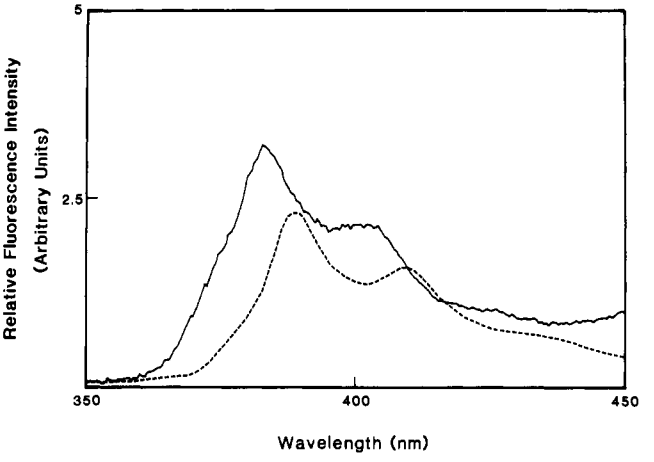

FIGURE 3: Fluorescence spectrum of pyrenyl holoenzyme. The fluorescence spectrum was recorded at a pyrene adduct concentration of 100 nM (solid line). The fluorescence spectrum of *N*-(1-pyrenyl)iodoacetamide was recorded at a concentration of 100 nM (dashed line).

The fluorescence spectrum of pyrenyl RNA polymerase is given in Figure 3. This spectrum, which was obtained with an excitation wavelength of 330 nm, has been normalized to the concentration of pyrene equivalents present in the protein. It is characterized by a peak at 383 nm and a shoulder at 402 nm. Except for differences in relative intensities, pyrenyl core polymerase, σ, β, and β' all display similar fluorescence spectra (data not shown). Relative to the fluorescence spectrum of *N*-(1-pyrenyl)iodoacetamide, which is characterized by a peak at 389 nm and a shoulder at 410 nm (Figure 3), there is a hypsochromic (blue) shift in the pyrene spectrum for pyrenyl

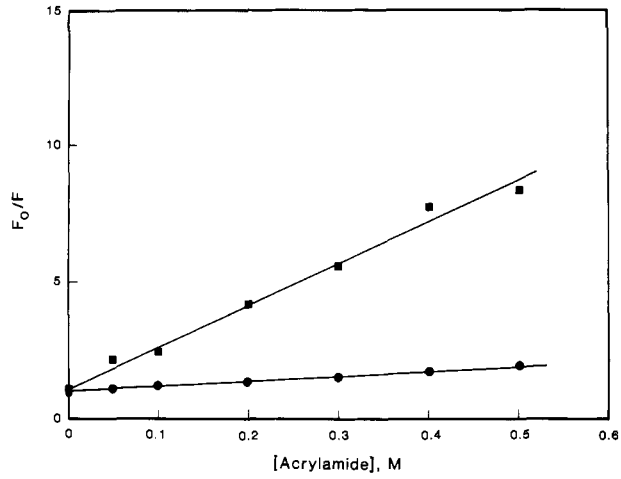

FIGURE 4: Quenching of pyrene fluorescence in pyrenyl holoenzyme. The ratio of the relative fluorescence intensity as a function of acrylamide concentration for pyrenyl holoenzyme [100 nM (●)] and *N*-(1-pyrenyl)iodoacetamide [100 nM (■)]. To 2 mL of each respective solution were added aliquots of a 5 M acrylamide solution in buffer A + 0.1 mM DTT. The excitation wavelength was 330 nm, and the fluorescence emission was measured at 383 nm for the pyrenyl adduct and at 389 nm for pyrene free in solution.

Table II: Mean Residue Ellipticities at 220 nm

protein	$[\theta]_{220}$ ($\times 10^{-3}$ deg cm^2 dmol^{-1})
holoenzyme	−13.9
pyrenyl holoenzyme	−10.8
core	−11.8
pyrenyl core	−7.9
σ	−26.6
pyrenyl σ	−20.3

RNA polymerase holoenzyme. These results are also consistent with apolar microenvironments for the pyrene molecules.

Stern–Volmer Analysis. The fluorescence exhibited by pyrenyl holoenzyme is quenched by acrylamide. Stern–Volmer plots for *N*-(1-pyrenyl)iodoacetamide free in solution and attached to the holoenzyme are given in Figure 4. The Stern–Volmer constant (k_{ST}) for *N*-(1-pyrenyl)iodoacetamide free in solution is 15. The Stern–Volmer plot for pyrenyl holoenzyme is linear, and the calculated k_{ST} constant is 1.8. The lack of curvature in this plot indicates that the pyrene molecules attached to the enzyme are equally accessible to acrylamide. Relative to the value of the Stern–Volmer constant observed for the pyrene molecule free in solution, the Stern–Volmer constant in the case of pyrenyl holoenzyme indicates that the covalently attached pyrene molecules are shielded from the aqueous environment. The corresponding Stern–Volmer constants for pyenyl σ, β, and β' are 2.3, 2.4, and 2.5, respectively. Thus, the pyrene molecules in each of these subunits are equally accessible to acrylamide. This accessibility is only slightly altered upon the formation of the core polymerase (k_{ST} of 2.1) or the holoenzyme.

Physicochemical Properties. CD studies were undertaken to investigate possible alterations in the structure of RNA polymerase upon the introduction of pyrene molecules. The CD spectra for native holoenzyme, core polymerase, and σ subunit (Figure 5) are all comparable in magnitude and shape as to those previously reported by Levine et al. (1980). The CD spectrum for the pyrenyl derivative of each species displays a decrease in magnitude as well as a change in shape (Figure 5). Values for the mean residue ellipticity at 220 nm are given in Table II. In the case of the pyrenyl derivatives of the holoenzyme, core polymerase, and σ subunit, the observed

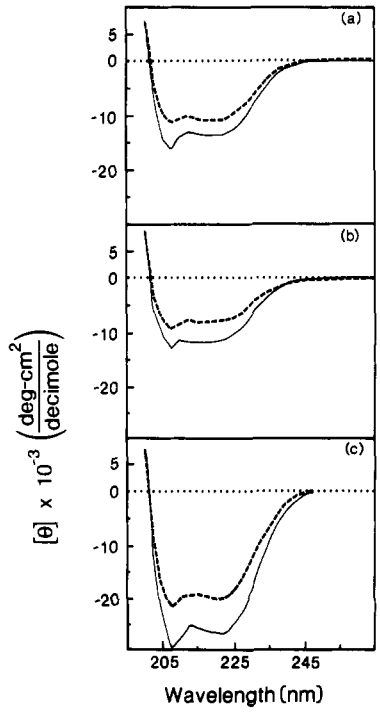

FIGURE 5: Far-UV CD spectra. The solid lines represent the CD spectra of the (a) holoenzyme, (b) core polymerase, and (c) σ subunit. The CD spectra for the corresponding pyrenyl derivatives are given by the dashed lines.

reductions in the mean residue ellipticity at 220 nm are 22, 33, and 24%, respectively.

DNA Binding Ability. A typical elution profile for pyrenyl RNA polymerase applied to a DNA–cellulose column is given in Figure 6. The peaks of enzyme activity and OD_{280} occur at a salt concentration of approximately 0.41 M NaCl. This is in the range in which the native enzyme elutes from a DNA–cellulose column (Burgess & Jendrisak, 1975). As is apparent from this figure, all of the modified enzyme is capable of binding to DNA.

Enzymatic Activity. The activity of pyrenyl holoenzyme and core polymerase with a variety of templates is given in Table III. These assays were conducted under conditions similar to those used to analyze the nonspecific interaction of the enzyme with DNA in the following paper (Johnson, 1991). In all cases, the modified derivative displayed reduced activity. (It should be noted that both the derivative and native enzyme contained approximately the same number of active enzyme molecules, i.e., approximately 50%). There was some fluctuation in the enzymatic activity depending upon the conditions and template used. The values ranged from 58 to 93% residual enzymatic activity in the case of pyrenyl holoenzyme, and from 25 to 81% for the pyrenyl core polymerase.

Abortive Initiation. The abortive initiation assay was carried out for two purposes: first, to obtain estimates of the number of active enzyme molecules present; second, to obtain estimates of the isomerization constant and the association constant for the interaction of pyrenyl holoenzyme with the A1 promoter of bacteriophage T7.

For the first purpose, the ability of pyrenyl holoenzyme to catalyze the abortive initiation reaction was investigated with plasmid pAR1435 as the template. This plasmid contains a 107 bp fragment of DNA from bacteriophage T7 cloned into the *Bam*HI site of pBR322. Relative to the A1 promoter which is located on this fragment, promoters P1, P2, and P3 of the pBR322 vector are much weaker (Studier, personal communications). Furthermore, they contain different starting

Table III: Transcriptional Activity[a]

buffer	enzyme	CT-DNA 37 °C	CT-DNA 25 °C	poly[d(A-T)] 37 °C	poly[d(A-T)] 25 °C	poly[d(G-C)] 37 °C	poly[d(G-C)] 25 °C
B[b]	holoenzyme	426	349	1662	494		
	pyrenyl holoenzyme	255 (60)	204 (58)	1053 (63)	428 (87)		
C[c]	holoenzyme	709	458	1319	501	334	235
	pyrenyl holoenzyme	458 (65)	345 (75)	1008 (76)	365 (73)	316 (95)	178 (76)
D[d]	holoenzyme	626	439	1554	257		
	pyrenyl holoenzyme	306 (49)	290 (66)	1145 (74)	238 (93)		
B	core	222	166	453	438		
	pyrenyl core	91 (41)	69 (42)	304 (67)	187 (43)		
C	core	309	229	439	360	293	210
	pyrenyl core	145 (47)	95 (41)	355 (81)	101 (28)	131 (45)	84 (40)
D	core	210	169	399	458		
	pyrenyl core	120 (55)	99 (59)	289 (72)	190 (41)		

[a] Values listed are in terms of units of enzyme activity as defined under Materials and Methods; values in parentheses represent the percent residual activity. [b] 50 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.1 M KCl, 10 mM $MgCl_2$, and 0.1 mM DTT. [c] 10 mM HEPES (pH 8.0), 0.1 mM EDTA, 0.1 M KCl, 10 mM $MgCl_2$, and 0.1 mM DTT. [d] 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 0.1 M KCl, 10 mM $MgCl_2$, and 0.1 mM DTT.

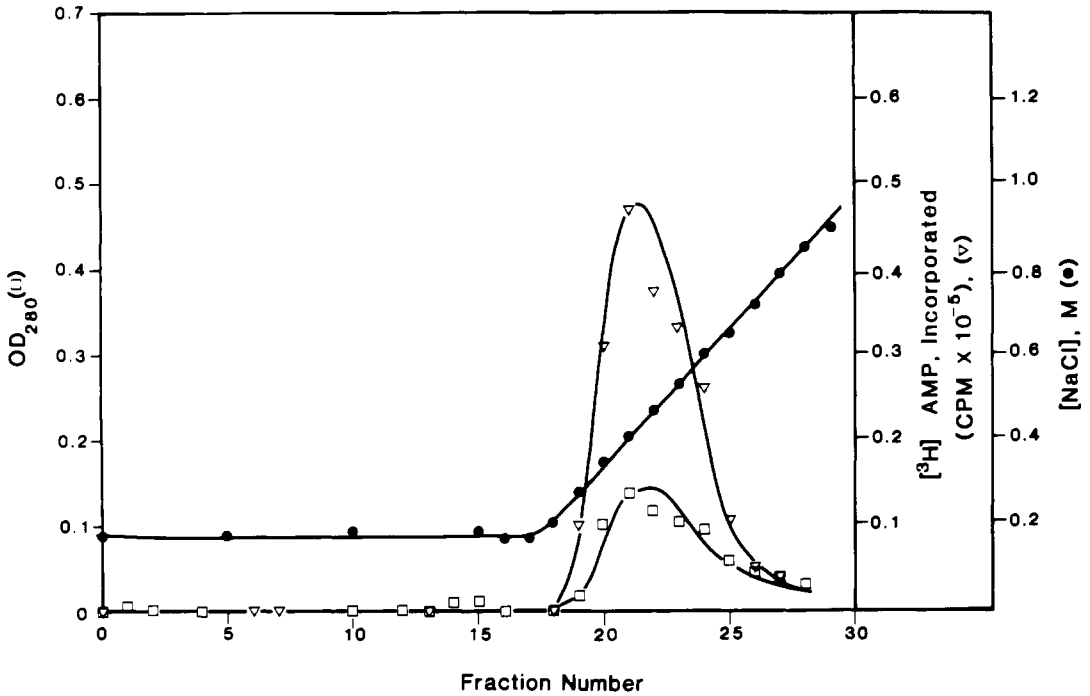

FIGURE 6: Elution profile of pyrenyl holoenzyme from a DNA–cellulose column. The flow rate was 10 mL/h, and the fraction size was 4.5 mL. The salt concentration was determined by using a Bio-Rad conductivity monitor and standard flow cell. (□) OD_{280}; (▽) enzyme activity; (●) NaCl concentration.

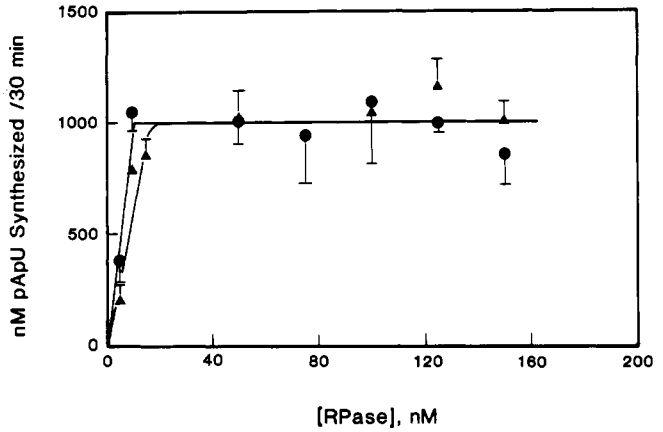

FIGURE 7: Abortive initiation assay for the native enzyme and the pyrenyl derivative. This assay was performed according to the protocol given under Materials and Methods using a plasmid concentration of 5 nM and various enzyme concentrations. (●) Native enzyme; (▲) pyrenyl RNA polymerase.

sequences. With plasmid pAR1435 as the template, the rate of dinucleotide (pApU) formation was monitored as a function of enzyme concentration for both the native enzyme and the pyrenyl derivative (Figure 7). As a control, the rate of dinucleotide formation using plasmid pBR322 as the template was also monitored. In the case of the pyrenyl derivative, at a concentration of enzyme of 10 nM, the overall production of dinucleotide observed in the presence of pBR322 was 11% of that observed with pAR1435. At an enzyme concentration of 150 nM, this value increased to 67%. For the native enzyme, the observed values were 18% at 10 nM and 72% at 150 nM. These results suggest that there are promoter-like sites present on pBR322 that are capable of synthesizing the dinucleotide pApU. The relative amounts of the dinucleotide produced in the case of both plasmids indicate that these promoter-like sites are significantly weaker than the A1 promoter site. The data presented in Figure 7 have been corrected for the contribution of the background DNA in the plasmid pBR322 to the generation of dinucleotide product. In the case of the native enzyme, the sharp break in the curve at a protein concentration of approximately 10 nM under conditions where the concentration of plasmid was 5 nM indicates that approximately 50% of the enzyme molecules are active in pro-

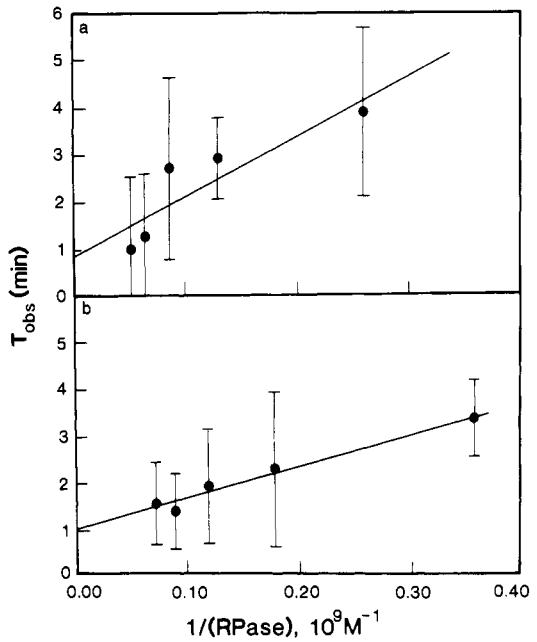

FIGURE 8: τ plots for the interaction of the native enzyme and the pyrenyl derivative with the A1 promoter. Each data point was determined with abortive initiation assays as described under Materials and Methods. The activity due to the background DNA in plasmid pAR1707 has been subtracted out. τ is plotted as a function of estimated active enzyme concentration. The errors associated with the data points are those propagated from linear regression of the results of lag assays. (a) τ plot for the native enzyme. (b) τ plot for the pyrenyl derivative.

moter binding and initiation. At saturating levels of enzyme, the total amounts of pApU synthesized by both the native enzyme and pyrenyl derivative are comparable. Because the maximal rate (V_{max}) of synthesis of the abortive transcript is proportional to the concentration of active enzyme molecules bound at the promoter site and the maximal rate is limited only by the isomerization constant (k_2), the results indicate that the pyrenyl derivative contains the same percentage of active enzyme molecules as the native enzyme from which it was prepared, that is, 50%. It should be noted that analyses of τ plots indicate that the values of the isomerization constant for the native enzyme and the derivative are comparable. On the basis of the data in Figure 7 at subsaturating concentrations of enzyme, it appears as if the pyrenyl derivative has a comparable or slightly weaker affinity than the native enzyme for the A1 promoter.

Estimates of the isomerization constant, k_2, and the association constant, K_{assoc}, for the interaction of the native enzyme and pyrenyl derivative with the A1 promoter of bacteriophage T7 were obtained from τ plots according to the procedure of McClure (1980). Plasmid pAR1707, which contains the A1 promoter as well as the T_e termination site from bacteriophage T7 cloned into the *Bam*HI site of pBR322, was used for this purpose. The reaction was monitored as indicated under Materials and Methods. For each reaction performed with plasmid pAR1707, a control reaction using pBR322 was also carried out. In each case, the control was used to correct for the contribution to dinucleotide formation due to the background DNA in plasmid pAR1707. τ plots for the native enzyme and derivative are given in Figure 8. Analysis of the data yields a value for k_2 of 1.2 ± 0.8 min^{-1} for the native enzyme and 1.0 ± 0.1 min^{-1} for the pyrenyl derivative. (The indicated errors are those generated by a linear least-squares analysis of the data.) Dayton et al. (1984) reported a value of 5.4 ± 4.8 min^{-1} for the interaction of RNA polymerase with a linear fragment of DNA containing the A1 promoter, and Rosenberg et al. (1982) reported a value of 2.8 ± 0.7 min^{-1} for the interaction of RNA polymerase with the A1 promoter in T7 D111 DNA. There is reasonable agreement between the values obtained in the three studies, considering the associated errors. A value of (1.0 ± 0.5) × 10^8 M^{-1} was obtained in the case of the association constant for the native enzyme and (1.6 ± 0.3) × 10^8 M^{-1} for the pyrenyl derivative. These values correspond well with the value of 3 × 10^8 M^{-1} reported by Dayton et al. (1984) for the interaction of the native enzyme with the A1 promoter. The method of Rosenberg et al. (1982) does not provide a means of determining the association constant for the formation of the closed complex. These results demonstrate that the introduction of pyrene molecules into RNA polymerase does not decrease the efficiency of initiation of transcription at the A1 promoter site as monitored by the abortive initiation assay.

Elongation Rate. The ability of pyrenyl RNA polymerase to catalyze the chain elongation step during transcription was analyzed with T7 DNA as the template as outlined under Materials and Methods. The rate constant for the reaction catalyzed by the native enzyme (38% active enzyme molecules) was found to be 2.86 ± 0.35 nmol/h. Kingston et al. (1981) obtained a value of 3.45 nmol/h for the native enzyme (38% active enzyme molecules). This value was extracted from Figure 7C in the paper by Kingston et al. (1981). It should be noted that a total protein concentration of 2.35 μg/mL was used in the studies by Kingston et al. (1981), whereas in this study a concentration of 2.00 μg/mL was used. If the difference in the concentrations is taken into account, there is reasonable agreement between these two values. In the case of the pyrenyl derivative (33% active enzyme molecules), a value of 1.55 ± 0.28 nmol/h was observed. This represents a 46% decrease in the rate of chain elongation observed for the native enzyme. This decrease far exceeds the difference in the number of active enzyme molecules present for the native enzyme and the derivative, and indicates that the introduction of pyrene molecules into RNA polymerase leads to a reduction in the rate of chain elongation.

Transcript Analysis. The ability of pyrenyl RNA polymerase to produce full-length transcripts was analyzed with *Sal*I-linearized pAR1707. With this plasmid, the principal transcript produced should contain 160 nucleotides corresponding to initiation from the A1 promoter site and termination at the T_e site present on this plasmid (Briat, & Chamberlin, 1984). If read-through occurs at the T_e site, then a 502-nucleotide runoff transcript should be present. As is apparent from the autoradiogram in Figure 9, the principal transcript generated using pyrenyl RNA polymerase contains 160 nucleotides. As with the native enzyme, a certain percentage of pyrenyl RNA polymerase molecules read-through the termination site and generate a 502-nucleotide transcript. Analysis of data from several gels indicates that 21 ± 2% of the enzyme molecules read-through the termination site in the case of the pyrenyl derivative and 26 ± 2% in the case of the native enzyme. Briat and Chamberlin (1984) observed a 35% read-through in the case of the native enzyme. Thus, the introduction of pyrene molecules into RNA polymerase does not alter the efficiency of termination. Moreover, the absence of discrete bands other than the ones corresponding to 160 and 502 nucleotides indicates that pyrenyl RNA polymerase is not undergoing premature termination.

Discussion

In the introduction of fluorescent probes into *E. coli* RNA polymerase, the reaction of *N*-(1-pyrenyl)iodoacetamide with

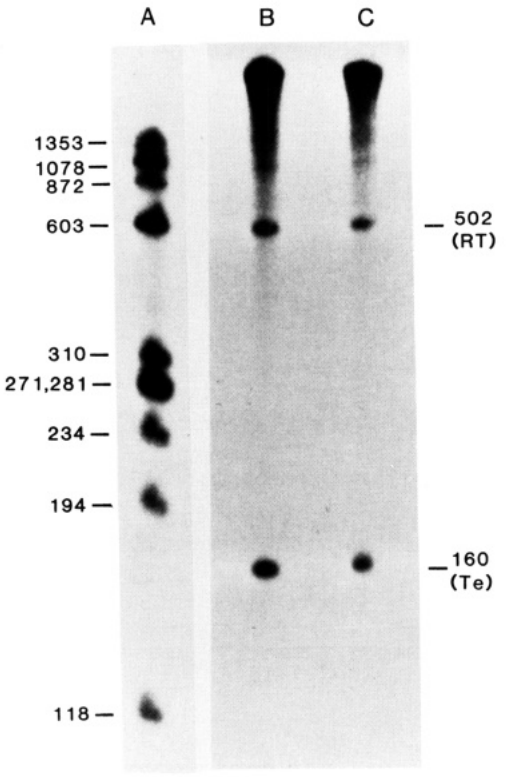

FIGURE 9: In vitro transcription of *Sal*I-digested pAR1707. RNA transcripts were prepared and analyzed on an 8% polycrylamide/7 M urea gel as described under Materials and Methods. Approximately 3000 counts were loaded per well in the case of the native enzyme (B) and pyrenyl derivative (C). The DNA ladder in lane A was generated from the *Hae*III digestion of ϕX174 RF DNA. The DNA sample was subjected to heating at 65 °C for 5 min in 80% formamide, and thus was in the single-stranded form when applied to the gel. The ^{32}P-labeled fragments were generously provided by Dr. J. A. McCubrey and L. S. Steelman (Department of Immunology and Microbiology, East Carolina University School of Medicine). Sizes are shown in nucleotides. RT, read-through; T_e, terminated.

RNA polymerase displays a relatively high degree of specificity in that only a net of approximately 2 of the 33 sulfhydryl groups present in the holoenzyme are modified. This contrasts with the results obtained by Yarbrough and Wu (1974) upon the modification of the holoenzyme with iodoacetamide. They observed that approximately seven sulfhydryl groups were modified after a 60-min incubation. This difference may be due to the bulky pyrene moiety which restricts the accessibility of the modifying reagent for certain sulfhydryl groups. Neither calf thymus DNA nor nucleotides (ATP and CTP) protected the holoenzyme from modification, thereby indicating that the modified sulfhydryl groups are not directly involved in the binding of either the polynucleotide or the nucleoside triphosphates.

Although there appears to be a high degree of specificity in the modification of the holoenzyme, when the individual subunits were isolated and analyzed for pyrene content, it was found that only the σ subunit had undergone stoichiometric modification. The second pyrene equivalent appears to be distributed equally between the β and β' subunits. It is apparent from the data presented above that the reaction of *N*-(1-pyrenyl)iodoacetamide with the holoenzyme is near completion after approximately 15 min. Thus, the pattern of modification observed for the holoenzyme after an incubation of 60 min suggests that one pyrene molecule binds to a region in the enzyme such that either the β or β' subunit, but not both in the same enzyme molecule, is modified.

CD studies in the far-UV indicate that the introduction of pyrene molecules into RNA polymerase alters its structure. Although CD spectra of proteins below 240 nm have contributions from both the peptide backbone and the side chains of aromatic amino acid residues, they are dominated by the peptide backbone chromophore (Bell, 1981). Furthermore, alterations of the local environments of a few aromatic side chains generally lead to changes in the mean residue ellipticity of less than 5% (Bell, 1981; Woody et al., 1987). Alterations in the CD spectra of the pyrenyl derivatives of the holoenzyme, core polymerase, and σ subunit range between 22 and 33%. Thus, the magnitude of the perturbations observed is consistent with alterations in the secondary structure of RNA polymerase upon chemical modification with *N*-(1-pyrenyl)iodoacetamide. Because the exact origin of the alterations of the CD spectra is not known, no attempt was made to determine the helical content of the pyrenyl derivatives.

The introduction of groups as bulky as pyrene molecules into RNA polymerase may have a dramatic effect on its biochemical properties. It has been demonstrated that the sulfhydryl groups that are modified by *N*-(1-pyrenyl)iodoacetamide are not directly involved in the interaction of the enzyme with either the ribonucleoside triphosphate substrate molecules or DNA. However, on the basis of CD studies, it is clear that there are alterations in the secondary structure of the enzyme upon chemical modification. These alterations in the structure of the enzyme could affect the active site in such a manner that the enzymatic properties are altered. In studies on the transcriptional activity of pyrenyl RNA polymerase using synthetic polydeoxyribonucleotides, it was observed that the overall enzymatic activity of the pyrenyl derivative was less than that observed for the native enzyme (Table III). Abortive initiation assays, however, indicate that the isomerization constant and association constant for the interaction of the native enzyme and the pyrenyl derivative with the A1 promoter are comparable. The pyrenyl derivative and native enzyme display similar termination efficiencies. Moreover, the pyrenyl derivative does not appear to undergo premature termination. The origin for the decrease in enzymatic activity appears to be the elongation step; i.e., the rate of elongation for the pyrenyl derivative is approximately 46% less than that observed for the native enzyme.

Conclusions

This study demonstrates that it is possible to introduce fluorescent probes into RNA polymerase and obtain an enzymatically active derivative. Although this derivative carries out the elongation step at a slower rate than the native enzyme, it is capable of recognizing the A1 promoter site and initiating transcription at this site with the same overall efficiency as the native enzyme. Furthermore, it does not appear to undergo premature termination, and it undergoes termination at the T_e site with the same efficiency as the native enzyme.

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Nonspecific Interaction of *Escherichia coli* Pyrenyl RNA Polymerase Holoenzyme with Synthetic Polynucleotides As Monitored by Fluorescence Spectroscopy

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ABSTRACT: A derivative of RNA polymerase containing approximately 2 pyrene equiv per enzyme molecule has been used to study the interaction of RNA polymerase with poly[d(A-T)]·poly[d(A-T)] and poly[d-(G-C)]·poly[d(G-C)]. As monitored by fluorescence spectroscopy, pyrenyl RNA polymerase displays a unique set of conformational changes with each synthetic polynucleotide as a function of temperature. An increase in the fluorescence intensity was observed for both polynucleotides at 5 °C. A decrease was observed in the case of poly[d(A-T)]·poly[d(A-T)] at 25 and 37 °C, whereas no discernible perturbation was observed in the case of poly[d(G-C)]·poly[d(G-C)]. Different salt dependencies were observed for the interaction of pyrenyl RNA polymerase with these polynucleotides at 5 and 25 °C. Further characterization of these interactions as well as correlation of the observed fluorescence changes to the corresponding open and closed complexes was carried out with heparin. The interaction between pyrenyl RNA polymerase and poly[d-(A-T)]·poly[d(A-T)] at 25 °C was quantified by using two different methods. The graphical method of Schwarz and Watanabe [Schwarz, G., & Watanabe, F. (1983) *J. Mol. Biol. 163*, 467-484] yielded values of 115-228 for the apparent cooperativity parameter, 31-43 for the apparent lattice number, (0.9-1.4) × 10^7 M^{-1} for the observed association constant, and (4.8-7.8) × 10^4 M^{-1} for the apparent intrinsic association constant, whereas the curve-fitting method of McGhee and von Hippel [McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469-489] yielded values of 116-228, 35-37, (0.9-1.1) × 10^7 M^{-1}, and (5.0-7.6) × 10^4 M^{-1} for the corresponding constants. All values are in terms of base pairs for DNA concentration.

The process of transcription involves the intricate and specific interplay between RNA polymerase and the DNA template. The importance of DNA structure in this process has been well documented in terms of conserved sequences centered at −10 and −35 base pairs which define promoter sites (Hawley & McClure, 1983; von Hippel et al., 1984; McClure, 1985), the distance between the −10 and −35 base pair sequences required for optimal transcriptional activity (Brosius et al., 1985;

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