Location of Aromatic Amino Acids and Helix Content in *Escherichia coli* Ribonucleic Acid Polymerase

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1. The perturbing effect of glycerol on the direct spectrum of *Escherichia coli* DNA-dependent RNA polymerase has been studied. 2. By comparison with model compounds and with the unfolded polymerase in 3.8 M-urea it was possible to determine the ratio of tyrosine and tryptophan residues present. On reduction of the urea-treated enzyme with 2-mercaptoethanol, no further change in the difference spectrum occurred. 3. The amino acid composition of the enzyme is given. 4. In the intact protein approx. 30% of the tryptophan and 54% of the tyrosine residues were exposed. In conjunction with the extinction value and molecular weight this corresponded to 7 tryptophan residues and 57 tyrosine residues on the surface and 16 tryptophan residues and 48 tyrosine residues 'buried'. 5. The optical rotatory dispersion of the enzyme was unaffected by 20% glycerol. 6. The helix content calculated from Moffit plots over 560–300 nm was 13%, and from the 233 nm trough 13%.

Studies so far on the RNA polymerase (EC 2.7.7.6) of *Escherichia coli* B have indicated the presence of a subunit structure (Fuchs, Zillig, Hofschneider & Preuss, 1964). The existence of the 2α , β , β' subunits was shown by Burgess (1969) by polyacrylamide-gel electrophoresis of the sodium dodecyl sulphate-treated enzyme. This subunit structure has also been observed on gel electrophoresis and density-gradient centrifugation of the enzyme denatured by a variety of agents (King, 1970). Little information is available, however, on the location and function of amino acid residues within the enzyme.

In this paper the ORD* results are presented and the state of the aromatic amino acids has been examined by solvent-perturbation spectroscopy. This latter technique, which has proved so useful for studying the location of chromophoric residues in proteins (Williams, Herskovits & Laskowski, 1965; Donovan, 1964; Cross & Fisher, 1966), is based on the small red-shift of the absorption maximum that occurs when proteins are exposed to solvents of higher refractive index than water. The magnitude of the shift depends on the location of the aromatic chromophore within the tertiary structure; thus those residues on the surface of the molecule are easily perturbed by solvent, whereas those buried in the interior of the molecule are not.

The amino acid analysis of this preparation is

* Abbreviation: ORD, optical rotatory dispersion.

given. This has been reported before by Preiss & Zillig (1967) and by Maitra & Hurwitz (1967) for RNA polymerase from *E. coli* W.

MATERIALS AND METHODS

The preparation, storage and assay of RNA polymerase from *E. coli* B has been described (Nicholson & Peacocke, 1965). The enzyme was further purified on hydroxyapatite and equilibrated with 0.4 M-KCl–0.01 M-tris-HCl buffer, pH 8.0, on Sephadex G-25 and used immediately.

The enzyme moved as a single band on electrophoresis in 5% (w/v) polyacrylamide gels in 0.05 M-tris-HCl–0.1 M-2-mercaptoethanol. Although no phosphocellulose step was included, the amount of sigma was less than 2% (w/w) (King, 1970) indicating that the product was almost entirely core polymerase ($\alpha_2\beta\beta'$).

Spectrophotometric measurements were made with a Unicam SP.800 with a Bryans chart recorder for scale expansion. Base lines were set to within 0.002 *E* and matched 2 mm or 5 mm path-length silica cells were used for all measurements. The temperature of the cell holder was maintained at 10°C by the forced circulation of water from a thermostat bath, which was cooled by circulating antifreeze from a –20°C bath through a copper coil submerged in the thermostat bath. Temperature was monitored in the cell by the thermistor (F23-STC) arm of a bridge circuit whose output was fed to a recorder. Control was better than $\pm 0.01^\circ\text{C}$. The cell compartment was continuously flushed with air dried by passage through a silica-gel column and subsequently cooled through the coil of a glass condenser submerged in the –20°C bath.

The main bore of the condenser served as an intake for the antifreeze circulating pump. This prevented condensation in the cell compartment and made for temperature stability. On runs with the enzyme N_2 was used instead of air.

The buffer used throughout for spectrophotometry was 0.4M-KCl-0.01M-tris-HCl, pH 8.0. The model-compound solutions were prepared in the same buffer. Both model-compound and enzyme concentrations were arranged to give a direct E_{280} value of about 0.8; this gave good accuracy for the difference spectra while being below the concentration at which deviation from Beer's Law occurred. These stock solutions and the enzyme solutions were divided into two. To equal volumes of each stock solution was added glycerol to give a final concentration of 20% (v/v), and to the other sufficient buffer to give the same final volume as the glycerol solution. Urea additions were made either by adding preweighed solid urea, with addition of buffer to the reference (non-glycerol) sample for volume equality, or by the addition of 10M-urea solutions with and without 20% (v/v) glycerol. All dilutions were made with Agla syringes with due allowance for the partial molar volumes of the components. For serially increasing urea concentrations, appropriate volumes of 10M-urea solutions were placed in 2ml stoppered plastic containers in ice. The enzyme solution was removed from the cell, mixed, returned to the cell and the process repeated to ensure thorough mixing.

Protein reduction was carried out essentially by the method of Sela, White & Anfinsen (1957). The reducing agent was 2-mercaptoethanol. Typically 0.01 ml of 1.2M-2-mercaptoethanol was added to each cuvette containing 0.6 ml of enzyme solution. The cuvette was flushed with N_2 and the thermostat adjusted to 25°C. After 4h the temperature was reset to the original thermistor reading before the spectra were recorded.

ORD curves were recorded on a Bendix-Ericsson Polar-matic 62 with slave recorder. A 10mm-path-length quartz cell was used between 560 and 300nm, and a 1mm cell below 300nm. Both entrance and exit slits were set at 1.8mm, and the rotation was calibrated on the basis of $[m]_{540}^{40m^\circ}$ for 5.2% (w/v) sucrose. Enzyme samples were prepared to give concentrations in the range 0.10–0.15g/100ml. The refractive index, n , for the buffer was taken as 1.34 throughout the wavelength range studied. For glycerol solutions, n was taken to be 1.36. Noise/signal ratio was less than 5% throughout.

For the amino acid analysis RNA polymerase was dialysed against degassed 0.05M-KCl, pH 8.0, and freeze-dried. Then 1ml of 6M-HCl containing 10 μ l of 0.1M-phenol/ml was added to each sample before it was sealed under vacuum. Hydrolysis was at 100°C for 24h and 72h. The amino acid analysis was performed by Dr H. Van Emden. Tryptophan was determined by difference spectroscopy and by Edelhoch's (1967) method in 6.0M-guanidine hydrochloride–0.02M-phosphate buffer, pH 6.5.

Urea (Aristar grade; BDH Chemicals Ltd., Poole, Dorset, U.K.) was recrystallized from ethanol-water, and solutions were prepared shortly before use.

N-Acetyl-L-tryptophan amide and the N-acetyl ethyl esters of tyrosine and phenylalanine were from Sigma Chemical Co., St Louis, Mo., U.S.A. Glycerol (BDH Chemicals Ltd.) and all other chemicals were A.R. grade.

RNA polymerase concentration was determined from $E_{280}^{0.1\%} = 0.541$ (King 1970).

RESULTS

The direct spectrum of RNA polymerase in 0.4M-KCl-0.01M-tris-HCl buffer, pH 8.0, is shown in Fig. 1. The use of a high-ionic-strength buffer is necessary to prevent association of the enzyme into oligomers. The absorption maximum in this buffer occurs at 278nm, and the E_{280}/E_{260} ratio is 1.69. The shoulder at 285nm also indicates the presence of tryptophan. The E_{280} and the molecular weight have been determined to be as follows: $E_{280}^{0.1\%}$ 0.541; mol.wt. 4.40×10^5 (A. M. Q. King & B. H. Nicholson, unpublished work). On this basis ϵ is 2.40×10^5 . A typical solvent-perturbation difference spectrum of native RNA polymerase is shown in Fig. 2 (curves A). The characteristic tryptophan peak is at 290–292nm. The 282–284nm peak, to which both tyrosine and tryptophan contribute, and the tyrosine peak at 277–279nm are clearly discernible. Lower-wavelength inflexions (below 270nm) are due to phenylalanine. The production of a difference spectrum with native enzyme indicates that some tyrosine, tryptophan and phenylalanine residues are on the surface of the enzyme molecule. Glycerol (20%, v/v) gives the expected positions for the difference maxima with the model compounds, although these are 0.5–1.0nm below their normal wavelength in each case owing to the low temperature (10°C) at which the spectra were determined. The use of low temperatures decreased the possibility of any thermally induced denaturation, enabled a constant temperature to be maintained accurately and had the slight advantage of increasing the magnitude of the difference spectra by 10–20%. On raising the

Fig. 1. Direct spectrum of RNA polymerase in 0.4M-KCl-0.01M-tris-HCl buffer, pH 8.0. The RNA polymerase concentration was 15.3 μ M. Silica cells, path length 2mm, were used for all measurements.

Fig. 2. Difference spectra of RNA polymerase and model compounds induced by 20% (v/v) glycerol in 0.4 M-KCl-0.01 M-tris-HCl buffer, pH 8.0. Curves A: —, native enzyme (15.3 μ M); ----, spectrum calculated for 0.87 mM-*N*-acetyl-L-tyrosine ethyl ester and 0.10 mM-*N*-acetyl-L-tryptophan amide. Curves B (in 3.8 M-urea): —, denatured enzyme; ----, spectrum calculated for 2.15 mM-*N*-acetyl-L-tyrosine ethyl ester and 0.467 mM-*N*-acetyl-L-tryptophan amide (these curves have been corrected for dilution on addition of urea, and are therefore comparable with curves A and C). Curve C: theoretical difference spectrum with the tyrosyl content based on the amino acid analysis and the tryptophyl content based on the tyrosine/tryptophan ratio 4.6:1 in curves B; the concentrations were 1.61 mM-*N*-acetyl-L-tyrosine ethyl ester and 0.35 mM-*N*-acetyl-L-tryptophan amide.

temperature, both $\lambda_{\max}$ and $\Delta\epsilon$ for $\lambda_{\max}$ for the model compound approached the literature values, although slight differences still remained due to differential thermal expansions. Similar increases in ΔE at decreased temperatures have been observed by other workers (Brandts, 1964). Since phenylalanine contributes little or nothing in the absorption region of tyrosine and tryptophan, i.e. above 270 nm, and has a small molar difference spectrum below 270 nm (the relative ΔE values at their respective $\lambda_{\max}$ values for phenylalanine, tyrosine and tryptophan derivatives are 1:17.5:57), it has not been included in the model-compound mixtures.

The determination of the number of exposed tyrosyl and tryptophyl residues was made by using the individual difference spectra of *N*-acetyl-L-tyrosine ethyl ester and *N*-acetyl-L-tryptophan amide separately determined at known concentrations. These were then multiplied by appropriate factors before being added together to form a

Fig. 3. Urea-dependence of the degree of exposure of tyrosyl and tryptophyl residues in RNA polymerase. The concentration of RNA polymerase was 6.35 μ M; the perturbant was 20% (v/v) glycerol in 0.4 M-KCl-0.01 M-tris-HCl buffer, pH 8.0.

theoretical difference spectrum for RNA polymerase. The closeness of the fit of this theoretical difference spectrum to the experimentally determined difference spectrum depends on the selection of the required factor, and this was done by trial and error. This method of matching spectra is definitely more satisfactory than the use of simultaneous equations with E values substituted from the difference spectral peaks, since these, as shown in Fig. 2, are invariably shifted to the lower wavelengths in the model compounds as compared with RNA polymerase where the chromophores are inserted in the polypeptide chain. The difference spectrum for native RNA polymerase is shown in Fig. 2 (curves A) together with the matching model-compound spectrum. The concentrations in the model-compound mixture were 0.87 mM-*N*-acetyl-L-tyrosine ethyl ester and 0.10 mM-*N*-acetyl-L-tryptophan amide, giving a tyrosine/tryptophan ratio 8.7:1. These then represent the concentrations of residues that may be considered as surface residues since they are readily accessible to the glycerol, which has an effective molecular diameter of 5.4 Å (Herskovits & Laskowski, 1962).

Since the difference-spectral maxima are proportional to the number of accessible residues, their magnitude can be used to indicate the state of the protein under different conditions. Fig. 3 shows the effect produced by the stepwise addition of solid urea to both normal and perturbed solutions of enzyme. As the urea concentration rises the $\Delta E_{286-287}/E_{278}$ ratio increases rapidly between 1.5 M- and 3.5 M-urea. The addition of urea results in a large increase in volume and this has been corrected for in the graph.

It is clear that at 3.8 M-urea a denatured form of the enzyme has been produced in which all

aromatic residues are accessible to glycerol. On this basis 3.8M-urea was chosen for an accurate difference spectrum of denatured enzyme, rather than 8M-urea, since this was sufficient and higher concentrations could lead to inaccuracy since urea itself perturbs.

The difference spectrum of RNA polymerase in 3.8M-urea is shown in Fig. 2 (curves *B*). No further change was brought about by reduction with 2-mercaptoethanol over 4h, nor was any turbidity apparent, but the high $\Delta E_{286-287}/E_{278}$ ratio, 0.115, indicated the occurrence of some aggregation and/or other side effects that have been observed with urea denaturation at neutral pH (Frensdorff, Watson & Kauzmann, 1953).

On raising the temperature of the cuvettes to 30°C the $\Delta E_{286-287}/E_{278}$ ratio fell to 0.064, a much greater fall than anticipated on the basis of the individual model compounds (e.g. ΔE for tyrosine falls by about 20%). Both the 30°C and 10°C difference spectra were, however, closely matched by model mixtures containing tyrosine and tryptophan in the ratio 4.6:1, although, of course, in different total amounts. Fig. 2 (curve *C*) shows the theoretical difference spectrum of a model mixture with the tyrosine content based on the amino acid analysis and the tryptophan content fixed by the 4.6:1 ratio. The mixture was 1.61mM-*N*-acetyl-L-tyrosine ethyl ester and 0.350mM-*N*-acetyl-L-tryptophan amide. If the individual direct spectra for these concentrations are added to that for *N*-acetyl-L-phenylalanine, the resulting theoretical direct spectrum is in close agreement with that for RNA polymerase, further justifying the use of the ratio from the urea data.

The $\Delta E_{284-286}/E_{275}$ ratio for the mixture shown in Fig. 2 (curve *C*) was 0.066; the $\Delta E_{286-287}/E_{278}$ ratio for native RNA polymerase was 0.032, giving the $\Delta E_{284-286}/E_{275-278}$ ratio $0.032/0.066 = 0.48$.

The ratios of the concentrations of the model mixtures in Fig. 2 (curves *A* and *C*) give the proportion of exposed tyrosyl and tryptophyl residues in the native enzyme, which is 54% for tyrosine and 29% for tryptophan. By dividing the concentration of model compound by that of RNA polymerase (15.3 μ M) found from the molar extinction coefficient the number of residues involved can be approximated. For tyrosine, there are 105 residues/RNA polymerase molecule, of which 57 are on the surface and 48 are buried. For tryptophan, there are 23 residues/molecule, of which 7 are on the surface and 16 are buried.

An independent determination of the total amount of tryptophan and tyrosine by Edelhoch's (1967) method is in reasonable agreement with this. The values obtained were 22 tryptophan residues and tyrosine, for which the method is less accurate, 91 residues. No correction for cystine absorption at

Table 1. *Amino acid composition of RNA polymerase*

Conditions for hydrolysis are as described in the Materials and Methods section. Threonine, serine and methionine values were extrapolated to zero time. Tryptophan was found by difference spectroscopy and included in the final column.

Amino acid	Amino acid composition (mol/100 mol)		
	24h	72h	Mean
Asp	10.6	10.0	10.2
Thr	5.3	5.3	5.3
Ser	6.7	6.7	6.7
Glu	13.6	13.7	13.6
Pro	3.4	3.4	3.4
Gly	7.4	7.5	7.4
Ala	8.8	8.8	8.8
Val	6.7	6.7	6.7
Met	2.9	2.9	2.9
Ile	5.2	6.0	5.6
Leu	11.1	10.7	10.9
Tyr	2.6	2.7	2.6
Phe	2.7	2.8	2.8
His	1.6	2.6	1.6
Lys	5.5	5.4	5.4
Arg	5.8	5.8	5.8
Trp	—	—	0.57

the two required wavelengths (280 and 288nm) was made, but the presence of 50 cystine residues/RNA polymerase molecule would alter the absorption by 2%.

The results of the amino acid analysis are shown in Table 1. The analysis is the average of the result of 24h and 72h hydrolyses, except for those for serine, threonine, methionine and tryptophan. The values for serine, threonine and methionine were corrected for destruction by a linear extrapolation from the 24h and 72h values. The value for tryptophan has been calculated from that of tyrosine on the basis of the molar ratio determined by difference spectroscopy. Cysteine was not determined. The results are generally similar to those reported by Preiss & Zillig (1967) with their preparation from *E. coli* B, except that their values for serine and glutamic acid were about 20% lower, and proline, methionine and arginine over 20% higher. The mean residue weight on the basis of the analysis reported here is found to be 111.

The ORD spectrum of RNA polymerase is shown in Fig. 4. The rotation values were low throughout the wavelength range 222–589nm. A single Cotton-effect minimum is present at 233nm, and no aromatic bands are discernible.

Analysis of the curve in the range 560–300nm by the procedure of Moffit & Yang (1956) gave a straight-line plot for native RNA polymerase

Fig. 4. ORD curve of RNA polymerase in 0.4M-KCl-0.01M-tris-HCl buffer, pH 8.0, showing the Cotton effect at 233nm. The concentration of the enzyme was 1.38 mg/ml; n^{20}_D was taken as 1.34 at all wavelengths. $[m']_\lambda = [3/(\lambda^2 + 2)] \cdot 10^{-2} \text{MRW} \cdot [\alpha]_\lambda$, where $[\alpha]_\lambda$ is the rotation at wavelength λ and MRW is the mean residue weight (111).

Fig. 5. Data of Fig. 4 plotted according to the Moffit-Yang equation $[m']_\lambda = a\lambda_0^2/(\lambda^2 - \lambda_0^2) + b\lambda_0^4/(\lambda^2 - \lambda_0^2)^2$. With λ_0 set at 212nm, the intercept gives $a_0 = -218$ and the slope gives $b_0 = -81$.

(Fig. 5). The constants a_0 and b_0 gave $a_0 = -218$ and $b_0 = -81$. The b_0 value for the region 250–300nm was -56 , with λ_0 arbitrarily set at 216nm. The value of $[m']_{233}$ at the Cotton-effect minimum (Fig. 4) is -3.30° . The refractive index, n , for the polymerase in 0.4M-KCl-0.01M-tris-HCl buffer, pH 8.0, was assumed to be 1.34 over the entire range. Assuming the absence of β structures in the polymerase molecule, the b_0 values of -81 (300–550nm) and -56 (250–300nm) correspond to 12.8% and 9.8% helicity respectively, taking $b_0 = -630$ as 100% helix. The trough value of $[m']_{233}$ gives 13% helix by the method of Simmons, Cohn, Szent-Gyorgi, Wetlaufer & Blout (1961), taking 100% helical polypeptide as $[m'] = -12.70^\circ$ and random coil as $[m'] = 1.80^\circ$.

The plot for RNA polymerase in 20% glycerol gave identical slope, indicating that no structural change is induced in the RNA polymerase molecule by the presence of glycerol in the concentration used for solvent-perturbation experiments.

DISCUSSION

The results presented show that at pH 8.0 and 1.0M approx. 30% of the tryptophan and 54% of the tyrosine residues are exposed. It must, however, be pointed out that no distinction can be inferred between 30% of fully exposed residues and 60% half-exposed. Some of the residues are almost certainly only partially exposed, as has been concluded for other proteins investigated with perturbants of different molecular radius. Thus Herskovits & Laskowski (1962) found that for bovine serum albumin 50% of tyrosyl residues are accessible to small perturbants and only 30% to bulky perturbants. Similarly, ribonuclease has 50% exposed, four residues being partly exposed and two residues buried.

Partial exposure appears to come about in two ways: the group may be situated in a crevice and only capable of interacting with small-radius perturbants, as happens with bovine serum albumin, or it may be due to the 'range' effect of the perturbants on chromophores situated near, but not on, the surface. Glycerol was chosen as the perturbant in the present study since it does not interact strongly with the tyrosyl residues of protein and thus cannot seriously weaken the protein structure by possible hydrogen-bond formation or breakage or exchange of existing hydrogen-bonds (Williams *et al.* 1965). It has a molecular radius of 5.4 Å, intermediate between that of sucrose and dimethyl sulphoxide (two other commonly used perturbants), has medium-range effects (Williams *et al.* 1965) and has been found to preserve enzyme activity.

In α -glutamate dehydrogenase, previously the largest molecule to which this technique had been applied (Cross & Fisher, 1966), 78% of the tryptophan, 42% of the tyrosine and 25% of the phenylalanine residues are at least partially exposed, and about half of these are completely exposed. These authors also state that 39% of tryptophyl residues are located in crevices. Whether such tryptophan-containing crevices exist in RNA polymerase is not certain, but RNA polymerase does bind the fluorescent probe aniline naphthalene sulphonate at several hydrophobic sites (F. Zaheer & B. H. Nicholson, unpublished work), indicating that crevices do exist. In view of the hydrophobic nature of tryptophan it might be expected that at least some residues will be at these sites.

The number of exposed tryptophyl residues is surprising. In the general case the ratio of the surface area to internal hydrophobic regions would be expected to decrease as the size of the molecule increases, and hence it might be expected to find proportionately fewer hydrophobic amino acid residues on the surface of a large molecule than on a smaller one. In addition, the tendency of the hydrophobic residues to be shielded from water would leave fewer residues on the surface. In relation to other proteins, RNA polymerase with molecular weight 440000 and 23 tryptophyl residues, 30% exposed, is in between aldolase (molecular weight 142000; 10–11 tryptophyl residues), which has 10% of its tryptophan totally exposed (Donovan, 1964), and glutamate dehydrogenase (molecular weight 310000; 54 tryptophyl residues), which has 40–78% of these residues exposed (Cross & Fisher, 1966), indicating that there is little tendency to bury these groups completely. Again, the existence of crevices would explain this anomaly.

The tyrosyl groups of these proteins are also considerably exposed: aldolase, 45%; glutamate dehydrogenase, 23% fully exposed and a further 20% partly exposed in crevices; RNA polymerase, 54% exposed. Tyrosyl residues have been shown to be involved in subunit linkages in glutamate dehydrogenase (Cross & Fisher, 1966). It is of note that the urea concentration of the $\Delta E/E$ transition just precedes that at which dissociation on polyacrylamide gels is observable (King, 1970). Since further urea produces no further increase in ΔE , unless all the aromatic buried groups are situated at or near the subunit interfaces, this transition must indicate unwinding of the chains and not just a separation of these interfaces with consequent exposure to solvent.

The amino acid analysis has already been commented on. Of the residues present 34% were mainly hydrophobic in character, corresponding to the amino acids alanine, leucine, isoleucine, methionine, phenylalanine, and tyrosine and tryptophan. Estimates based on this analysis of the number of tyrosyl and tryptophyl residues present give 105 and 23 respectively.

Because of its low difference extinction coefficient, the extent of exposure of phenylalanine could not be determined accurately in the present experiment. By taking the amino acid analysis values of approximately equal concentrations of tyrosine and phenylalanine, calculations show that the amount of surface phenylalanyl residues on the

basis of the difference spectra must be less than 20% of the total.

The results of disulphide-bond reduction are not surprising. Glycerol appears to have a medium-range effect, and is relatively unaffected by steric constraints, such as disulphide bonds, that interfere with short-range strongly interacting perturbants like dimethyl sulphoxide (Williams *et al.* 1965).

The ORD is reasonably straightforward; no aromatic inflexions are visible, but some are apparent on circular-dichroism measurements (D. Dalgleish, B. H. Nicholson & A. R. Peacocke, unpublished work). The helix content as measured by the depth of the 233 nm trough and the b_0 values at 250–300 nm and 300–560 nm agree quite well at 13, 10 and 13% respectively.

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