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Intrasubunit Nucleotide Binding in Ribonucleic Acid Polymerase

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1. Periodate oxidation of the ribose ring was used to synthesize derivatives of nucleoside triphosphates. 2. These oxidized nucleoside triphosphates are competitive inhibitors of RNA polymerase. 3. On incubation, together with $NaBH_4$, these oxidized labelled nucleotides are covalently bound to *Escherichia coli* RNA polymerase. 4. Nucleoside triphosphate substrates decrease the extent of labelling. 5. A lysine residue in an α-subunit is labelled. 6. The significance of these results in relation to the location of the nucleotide-binding site is discussed.

In uninfected *Escherichia coli*, one enzyme is responsible for the synthesis of all the cell's RNA. This enzyme, RNA polymerase (EC 2.7.7.6), must therefore be subject to a variety of control mechanisms and effectors, since the nature of the RNA species being synthesized is not constant. It is generally agreed that RNA polymerase is an allosteric enzyme (Travers, 1976), whose activity is modulated by molecules as varied as guanosine tetraphosphate, fMet-tRNA and the σ-subunit. Since little is known of the nature of the active site, virtually nothing is known about the molecular nature of the conformational changes presumed to be involved in the control of gene expression.

The use of various chemical reagents has suggested that the side chains of lysine (Venegas *et al.*, 1973; Bull *et al.*, 1975), arginine (Graham *et al.*, 1976; Armstrong *et al.*, 1976*a*), cysteine (Nicholson & King, 1973; Yarbrough & Wu, 1974) and tryptophan (Wasylyk & Malcolm, 1975) may be involved in the enzyme's mechanism. Nucleotide analogues have been shown to label either the β- or β'-subunits or sometimes both (Nixon *et al.*, 1972; Frischauf & Scheit, 1973; Wu & Wu, 1974; Armstrong *et al.*, 1976*b*). The present paper reports the synthesis of four affinity labels for RNA polymerase and describes their interaction with the enzyme.

Materials

E. coli M.R.E. 600 was purchased as the frozen cell paste from M.R.E., Porton Down, Wilts., U.K.; ATP and GTP were from Boehringer (U.K.), Lewes, Sussex, U.K., and CTP and UTP were the products of P–L Chemicals, Milwaukee, WI, U.S.A. [8-^{3}H]-ATP (sp. radioactivity approx. 25 Ci/mmol) and [5,6-^{3}H]UTP (sp. radioactivity approx. 50 Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K. Calf thymus DNA was from Koch–Light, Colnbrook, Bucks., U.K. (catalogue 1492-01) and poly[d(A-T)] came from Miles, Stoke Poges, Bucks., U.K. Sephadex G-10 was from Pharmacia, London W.5, U.K., and poly(ethyleneimine) thin-layer plates were made by Schleicher and Schuell, Dassel, Germany. Soluene 350 was from Packard, Caversham, Berks., U.K. All other chemicals were from normal commercial sources and were used without further purification.

Methods

RNA polymerase holoenzyme was extracted from *E. coli* essentially by the method of Burgess & Jendrisak (1975). Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Laemmli, 1970) failed to show the presence of polypeptides other than with molecular weights corresponding to α (40000), σ (155000) and β^1 (165000) subunits. Enzyme assays were carried out as previously described (Malcolm *et al.*, 1975), except that poly[d(A-T)] was used as a template in place of calf thymus DNA.

Periodate-oxidized nucleotides were all prepared by published methods (Powell & Brew, 1976; Easterbrook-Smith *et al.*, 1976). The radioactive derivatives were usually synthesized by causing 1 nmol of nucleotide to react with 100 μl of 87 mM-KIO_4 for 60 min at 25°C. The oxidized nucleotide was separated from the other reactants on Sephadex G-10, with the use of radioactive labelling to detect it. The leading 60% of the peak was pooled and freeze-dried. Chromatography on poly(ethyleneimine) thin layer plates in 0.8 M-NH_4HCO_3 showed that the product (R_F 0.0) was free from non-oxidized nucleotide (R_F 0.70). The non-radioactive derivatives were usually prepared by causing 0.34 mmol of nucleotide to react with 10 ml of 0.37 M-KIO_4 and measuring the A_{260} to detect the product eluted from the Sephadex column.

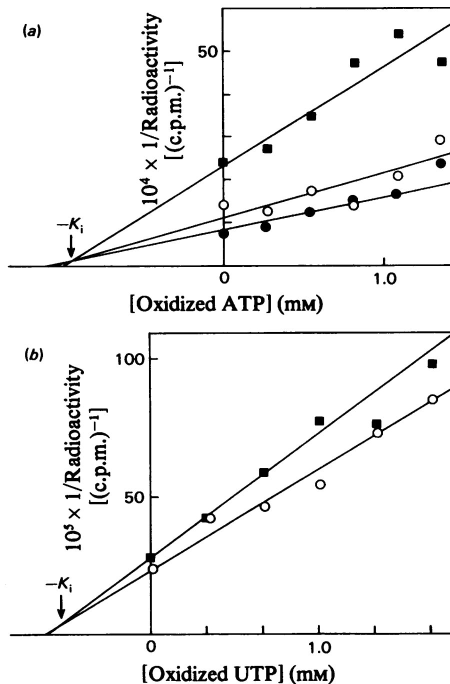

Fig. 1. *Dixon plots for the inhibition of RNA polymerase by periodate-oxidized nucleotides*

(*a*) Inhibition by oxidized ATP. ATP concentrations are: 20μM (■), 80μM (○) and 320μM (●). All other concentrations are as described in the text. (*b*) Inhibition by oxidized UTP. UTP concentrations are 11μM (■) and 100μM (○). All other conditions are as described in the text.

The inhibition experiments were carried out in the normal assay volume of 250μl and contained 40mM-Tris/HCl, pH7.9, 12mM-$MgCl_2$, 1mM-$MnCl_2$, 200mM-KCl, 0.1mM-EDTA, 0.4mM-potassium phosphate, dithiothreitol (1mM), 75μg of poly[d(A-T)], fixed nucleoside triphosphates at 0.8mM each, and variable nucleoside triphosphate as indicated in Fig. 1; the specific radioactivity of the variable nucleoside triphosphate was 28d.p.m./pmol for UTP and 11d.p.m./pmol for ATP. In addition each assay contained 0.13mM-ApU, which stimulated initiation and thus simplifies the kinetics (Downey & So, 1970).

In a typical labelling experiment 0.5mg of RNA polymerase was dialysed overnight against 0.1M-borate buffer, pH9.0, and then allowed to react for 120min at 25°C with 0.5μM radioactive oxidized nucleotide in borate buffer, pH9.0, containing 0.3mg of calf thymus DNA/ml. The volume of the incubation mix was 0.5ml. $NaBH_4$ was then added to a final concentration of 530mM, together with 1μl of n-octan-1-ol to decrease protein denaturation from foaming.

After 120min the reaction was quenched by the addition of 50μl of 10mM-L-lysine. The mixture was then dialysed overnight against 1% sodium dodecyl sulphate. Polyacrylamide gels (5%) were run as described by Laemmli (1970). Some early samples (not shown) were run on single concentration sodium dodecyl sulphate/polyacrylamide gels (Weber & Osborn, 1969), but in our hands these produced diffuse protein bands together with a comparatively high and irregular background. Slices of the gels 2mm thick were obtained with a Gilson gel slicer. The slices were dissolved by heating with 300μl of Soluene 350 at 60°C for 120min, and then counted in 4.5ml of 0.4% 2,5-diphenyloxazole in toluene in a Hewlett–Packard Tri-Carb scintillation counter. A gel was always run in parallel containing about 25μg of changed RNA polymerase, which was subsequently stained with Coomassie Blue to indicate the positions of the RNA polymerase subunits. These were scanned on a Gilford 250 spectrophotometer with gel-scanning attachment, reading at 600nm.

Results and Discussion

Periodate-oxidized derivatives of all four ribonucleotides were synthesized and shown to inhibit RNA polymerase. The kinetics of inhibition on calf thymus DNA as template are complicated since nucleotides are involved in both initiation and elongation steps (Downey & So, 1970). However, with poly[d(A-T)] as a template and in the presence of ApU, both oxidized UTP and oxidized ATP behave as competitive inhibitors with K_i values of 0.5mM and 1.1mM respectively (Fig. 1).

A comparison of the profiles of Coomassie Blue-stained gels with those of the sliced, radioactively labelled gels (Fig. 2) shows that it is the α-subunit that reacts with the affinity labels. (The small difference between the two gels arises because insertion of the gel into the tube of the slicer results in an elongation of the gel by about 5%. Thus the apparent migration distance of bands will be artificially increased when the gels are sliced.)

The extent of labelling is low. We estimate a stoicheiometry of between 1 and 5% per α-subunit. This cannot be due to insufficient time being allowed for the reaction to occur. It is well known that the reaction between a carbonyl group and an amine group is rapid and this is confirmed by the inhibition experiments described in the present paper. Presumably shorter incubation times than 120min would also have been sufficient, but this was not tested. The concentrations of both enzymes and oxidized nucleotide are low in these labelling experiments. If the kinetically determined K_i values can be used as an estimate of the equilibrium constant for Schiff-base formation, it can readily be calculated that only about 1% of the enzyme will be labelled. When the

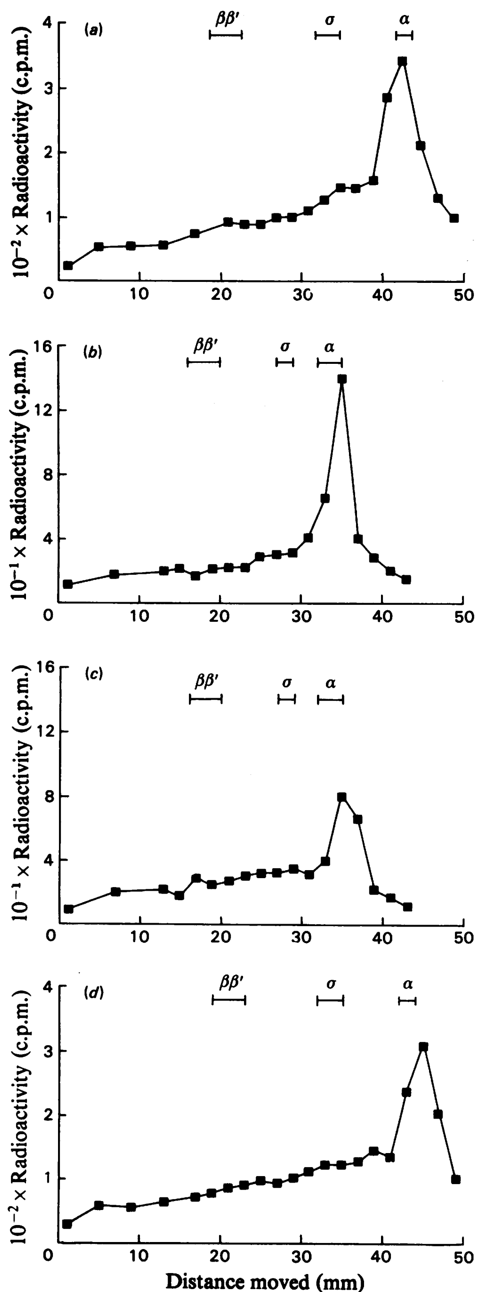

Fig. 2. *Profile of RNA polymerase ^{3}H-labelled with oxidized nucleotides and run on sodium dodecyl sulphate/ 5% polyacrylamide gels*

Radioactivity was determined after solubilizing slices and counting in toluene/2,5-diphenyloxazole. The positions of the subunits were determined from a gel run in parallel and stained with Coomassie Blue. (*a*) Labelled with oxidized ATP; (*b*) labelled with oxidized CTP; (*c*) labelled with oxidized GTP; (*d*) labelled with oxidized UTP.

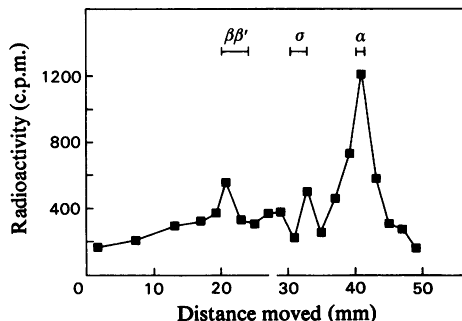

Fig. 3. *Profile of RNA polymerase labelled with oxidized [^{3}H]ATP when lysine was not used to quench the reaction*

Table 1. *Protection by substrate against affinity labelling*

RNA polymerase and oxidized nucleotides were incubated in the presence of substrates as indicated together with $NaBH_4$ as described in the text. Incorporated radioactivity was measured after 72h dialysis to remove unchanged affinity label.

Affinity label (oxidized nucleotide)	Substrate added	Radioactivity incorporated (%)
ATP	None	100
ATP	18.3mM-UTP	12.7
ATP	18.3mM-ATP	12.8
UTP	None	100
UTP	18.3mM-UTP	13.8
UTP	18.3mM-ATP	14.5

borohydride is added both the –CH=N– group of the bound affinity label and the –CHO group of the free label will be reduced. Once the –CHO group has been reduced it will not of course be possible to bind it covalently to the enzyme.

To confirm that this labelling is specific, several controls were carried out. A sample of oxidized ATP, run by itself on a gel, diffused out of the gel during fixing and therefore could not be confused with any of the protein bands. When oxidized ATP alone was treated with borohydride and then dialysed, none remained in the sac, showing that it is only protein-bound ligand that is retained during dialysis. If the reaction was not quenched by lysine, all the subunits were labelled (Fig. 3). The extent of this non-specific labelling was small, presumably because most of the carbonyl groups of the affinity label had been reduced by the borohydride before the dodecyl sulphate denatured the enzyme.

The specificity of the labelling reaction is further confirmed by protection experiments. If the labelling is carried out in the presence of nucleoside triphosphate substrates, the amount of radioactivity

incorporated is greatly decreased (Table 1). Previous workers (Armstrong *et al.*, 1976*b*) have shown that 5-formyl-UTP (in which the reactive carbonyl group is on the pyrimidine ring) labels the β-subunit (and also the β'-subunit at longer times of incubation). This suggests that this nucleotide-binding site is located in the contact region between two subunits. Such substrate binding has previously been observed in glyceraldehyde 3-phosphate dehydrogenase (Malcolm & Coggins, 1974), where the nucleotide coenzyme binds between two subunits and is responsible for the observed allosteric properties of the enzyme. RNA polymerase is generally considered to undergo conformational changes (Travers, 1976), but data relating to the nature of such changes have not yet been published.

The enzyme has two nucleotide-binding sites (Anthony *et al.*, 1969; Wu & Goldthwait, 1969), one generally ascribed to initiation of RNA chains and the other to chain elongation. Since the former binds only purine nucleotides and we are able to label the protein equally well with all four oxidized ribonucleotides, we conclude that it is the elongation site that is being studied in these experiments. This suggestion is confirmed by the fact that UTP protects against labelling by oxidized ATP and ATP protects against oxidized UTP (Table 1).

The fact that the closely related nucleotide analogues 6-methylthioinosine dicarbaldehyde (Nixon *et al.*, 1972) and its *N*-(acetylaminoethyl)-5-aminonaphthalene-1-sulphonate derivative (Wu & Wu, 1974) have been reported to label the β-subunit may have either of two explanations. The first is that they label the other nucleotide site, the initiation site, and this is proposed by both groups of authors. The other possibility is that, since the binding site lies close to both subunits, a small alteration to the binding, such as the naphthylamine group might cause, could affect which subunit was labelled. Many molecules (e.g. rifampicin, the σ-subunit etc.) are thought to alter the conformation of RNA polymerase. It will be interesting to see whether any of these molecules can affect the labelling by these oxidized nucleotides.

We are not yet able to conclude whether one or both of the α-subunits are labelled. This is particularly important in view of a model (Coggins *et al.*, 1977) based on cross-linking experiments, which suggests that the two α-subunits are not close together in the quaternary structure of the enzyme.

After infection by bacteriophage T4, the α-subunit is modified by the addition of ADP-ribose (Seifert *et al.*, 1971; Goff, 1974). This modification prevents the enzyme from transcribing *E. coli* DNA (Mailhammer *et al.*, 1975). Arginine-265 is the residue modified (Ovchinnikov *et al.*, 1977). We cannot yet say what the relationship is between the site modified by oxidized nucleotides and this regulatory site.

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