# A Study of the Quaternary Structure of *Escherichia coli* RNA Polymerase Using Bis(imido esters)†

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ABSTRACT: The quaternary structure of *Escherichia coli* RNA polymerase has been studied by cross-linking with a periodate-cleavable bis(imido ester), *N,N'*-bis(2-carboximidoethyl)tartaramide dimethyl ester dihydrochloride (CETD). The cross-linked holoenzyme gives a characteristic five-band pattern after electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. The components of each band have been unambiguously identified by (a) molecular-weight measurements, (b) comparisons of cross-linking patterns of holoenzyme and core enzyme, and (c) periodate cleavage of cross-links followed by a second dimension sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The bands are (1) $\alpha\beta$ and $\alpha\beta'$, (2) $\sigma\beta$ and $\sigma\beta'$, (3) $\alpha\sigma\beta$ and $\alpha\sigma\beta'$, (4) $\beta\beta'$, and (5) $\sigma\beta\beta'$. Bands 2 and 4 are the most prominent at low reagent concentrations (up to 2.5 mM) but band 5 becomes the most prominent at higher concentrations. There are no bands corresponding to $\alpha\alpha$ and $\alpha\sigma$; a faint band has been tentatively identified as $\alpha\beta\beta'$. Shorter bis(imido esters) are much less effective cross-linking reagents than CETD and they do not give rise to any other cross-linked species. On the basis of these observations, a model for the subunit arrangement of RNA polymerase is proposed.

DNA-dependent RNA polymerase of *Escherichia coli* (EC 2.7.7.6) core enzyme consists of four subunits, $\alpha_2\beta\beta'$ (Burgess, 1969; King and Nicholson, 1971). The holoenzyme contains a fifth subunit, $\sigma$, which binds to the core enzyme and causes specific start points on the DNA template to be recognized (Burgess and Travers, 1971). The reported subunit molecular weights are $\alpha$ 39 000–41 000, $\beta$ 145 000–155 000, $\beta'$ 150 000–165 000, and $\sigma$ 86 000–95 000 (Chamberlin, 1974). Little is so far known about the spatial arrangement of these subunits.

The cross-linking method using bis(imido esters), introduced by Davies and Stark (1970) for determining the number of subunits in a multimeric protein, can also provide information about the arrangement of the subunits (Cohlberg et al., 1972; Hucho and Janda, 1974; Hucho et al., 1975; Bickle et al., 1972; Kornberg and Thomas, 1974; Hitchcock, 1975). Cross-linking studies on RNA polymerase using bis(imido esters) have been reported (King et al., 1974; Schramm and Dulfer, 1976). Experiments with dimethyl suberimidate on the core enzyme and the holoenzyme (King et al., 1974) showed that the major products of the cross-linking reaction were $\beta\beta'$ and $\sigma\beta\beta'$, respectively. No cross-linked species involving the $\alpha$ subunit were reported nor was it established whether the $\sigma$ subunit was more readily cross-linked to $\beta$ or to $\beta'$. We have extended these studies using a wider range of cross-linking reagents to obtain more detailed information about the subunit arrangement.

With multimeric proteins containing several different kinds of subunit, it is usually impossible to decide what the subunit composition of a particular cross-linked species is on the basis of molecular weight alone. For this reason, reversible cross-linking reagents have been introduced to allow the components of an individual cross-linked band to be identified by reversing the cross-linking and analyzing the components (Sun et al., 1974; Lutter et al., 1974; Wang and Richards, 1974; Ruoho et al., 1975). We have recently introduced a periodate-cleavable bis(imido ester), *N,N'*-bis(2-carboximidoethyl)tartaramide dimethyl ester dihydrochloride, for studying the subunit arrangement of multienzyme complexes (Coggins et al., 1976). In this paper, we describe the application of CETD[1] to RNA polymerase.

## Experimental Procedure

*Materials.* RNA polymerase was isolated from *E. coli* strain MRE 600 (obtained from the Microbiology Research Establishment, Porton, Wiltshire, U.K.) essentially according to the method of Burgess and Travers (1971). Cells were broken using several short high-speed bursts on a Waring blender, instead of by treatment with glass beads in a homogenizer. The holoenzyme used in these experiments contained 0.6 mol of $\sigma$/mol of core enzyme, as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Core enzyme was prepared from holoenzyme by chromatography on phosphocellulose (Burgess, 1969). Ovalbumin was obtained from Sigma. Dimethyl suberimidate dihydrochloride and the shorter-chain analogues were prepared from the corresponding dinitriles (purchased from Aldrich) by the method of Davies and Stark (1970). The reagents, which are hygroscopic and subsequently hydrolyzed at room temperature, were stored in tightly stoppered bottles at −20 °C. CETD was synthesized on the day of use as described previously (Coggins et al., 1976).

*Cross-linking.* RNA polymerase was exhaustively dialyzed against 0.1 M triethanolamine hydrochloride, pH 8.0, 0.1 mM DTT. Stock solution of cross-linking reagent (0.1 M) were prepared immediately before use in a solvent made by mixing 1 volume of 1.0 M triethanolamine hydrochloride, pH 8.0, and 1 volume of 0.4 M NaOH; if necessary, the final pH was adjusted to 8.0 with 1.0 NaOH or 1.0 M HCl. Cross-linking reactions were carried out at 20 °C with a final concentration of 1.0 mg/mL enzyme ($E_{1cm}^{0.1\%}$ = 0.65 for RNA polymerase at 280 nm) and a buffer concentration of 0.1 M. The NaCl

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[1] Abbreviations used are: CETD, *N,N'*-bis(2-carboximidoethyl)tartaramide dimethyl ester dihydrochloride; DTT, dithiothreitol.

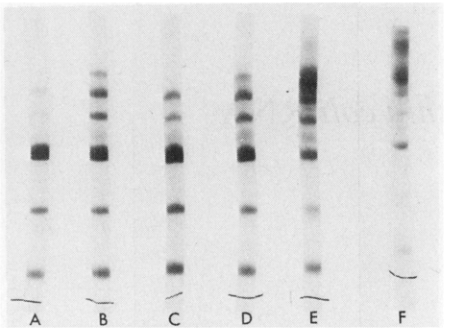


FIGURE 1: Cross-linking of RNA polymerase holoenzyme with dimethyl suberimidate and with CETD. Sodium dodecyl sulfate-polyacrylamide gels (3%) stained with Coomassie brilliant blue. RNA polymerase (1.0 mg/mL) was cross-linked for 1 h at 20 °C in 0.1 M triethanolamine hydrochloride, pH 8.0, 0.44 M NaCl with various concentrations of reagent as described under Experimental Procedure. (A) Control; (B) 0.5 mM dimethyl suberimidate; (C) 0.5 mM CETD; (D) 2.5 mM CETD; (E) 10.0 mM CETD; (F) as for E, except the NaCl concentration was 0.04 M.

concentrations were 0.04 M for the low-salt experiments and 0.44 M for the high-salt ones. After 1 h, the cross-linking reaction was terminated by the addition of 1 M ammonium bicarbonate to a final concentration of 0.05 M. After a further 30 min at 20 °C, 0.1 volume of 10% sodium dodecyl sulfate and 0.01 volume of 2-mercaptoethanol were added, and the samples were placed in a boiling water bath for 5 min. The denatured protein solutions were dialyzed against 0.1 M sodium phosphate (pH 6.5), 0.1% sodium dodecyl sulfate, 0.01% 2-mercaptoethanol for 3 h and then subjected to polyacrylamide gel electrophoresis.

*Polyacrylamide Gel Electrophoresis.* Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out according to Shapiro and Maizel (1969). Gels (0.6-cm diameter) contained 3% acrylamide, 6 M urea, 0.1 M sodium phosphate (pH 6.5), and 0.1% sodium dodecyl sulfate. Fifty microliters of sample, 10 μL of 0.1% bromophenol blue, and 10 μL of glycerol were applied to each gel, and electrophoresis was carried out at 4 mA/gel for 20 min and then 6 mA/gel for 5–5.5 h. For two-dimensional experiments, the first-dimension gels were 7.0 × 0.4 cm and electrophoresis was at 3.5 mA/gel. Following electrophoresis, these gels were soaked for 2 h at 20 °C in three changes of 20 mM sodium phosphate (pH 7.5), 0.1% sodium dodecyl sulfate to remove the urea. The cross-linked species were then cleaved by transferring the gels to fresh buffer containing 30 mM sodium periodate and incubating for a further 4 h at 20 °C (based on the method of Lutter et al., 1974). Each tube gel was then laid along the top of a slab gel (7.2 × 7.2 cm, containing 5% acrylamide, 0.1% sodium dodecyl sulfate, 0.1 M sodium phosphate, pH 6.5) and sealed in place with a layer of 1.4% agarose. Electrophoresis in the second dimension was carried out for 16 h in a Pharmacia GE-4 apparatus at 18 mA/slab.

Tube gels were stained for 1 h and slab gels for 2 h at 40 °C in 0.1% (w/v) Coomassie brilliant blue G250 (Serva, Heidelberg, West Germany) in methanol–acetic acid–water, 5:1:4 (v/v), and destained, also at 40 °C in methanol–acetic acid–water, 1:1:8 (v/v). Gels were scanned at 600 nm in a Gilford spectrophotometer fitted with a Model 252 gel scanner. Cross-linked ovalbumin, for use as a molecular-weight standard, was prepared as described by Carpenter and Harrington (1972).

## Results

*Cross-linking of the Holoenzyme.* Sodium dodecyl sulfate-polyacrylamide gels of RNA polymerase holoenzyme, cross-linked in high-salt conditions (0.44 M NaCl) at pH 8.0 with dimethyl suberimidate and three different concentrations of CETD, are shown in Figure 1. Cross-linking with the intermediate concentration of CETD (2.5 mM) and with 0.5 mM dimethyl suberimidate gave very similar results (Figure 1B,D). There is a characteristic five-band pattern which is most clearly illustrated in the densitometer trace of gel D (Figure 2). The bands are numbered 1 to 5 in order of increasing molecular weight; an additional faint band, which is sometimes detected on the low-molecular-weight side of band 5, is designated 5′. The effect of increasing the CETD concentration is shown in Figure 1C,D,E. At 0.5 mM CETD (C) only bands 2 and 4 are present. These bands remain the most prominent at 2.5 mM CETD (D), while at 10 mM CETD (E) band 5 has become the most prominent. Similar results were obtained when these experiments were repeated at low salt (0.04 M NaCl), except in the case of 10 mM CETD which gave several additional high-molecular-weight bands (Figure 1F). These bands had molecular weights larger than one complete holoenzyme molecule and must therefore be derived from cross-linking of aggregates of RNA polymerase. This is consistent with the reported aggregation of RNA polymerase at low salt concentrations (Chamberlin, 1974).

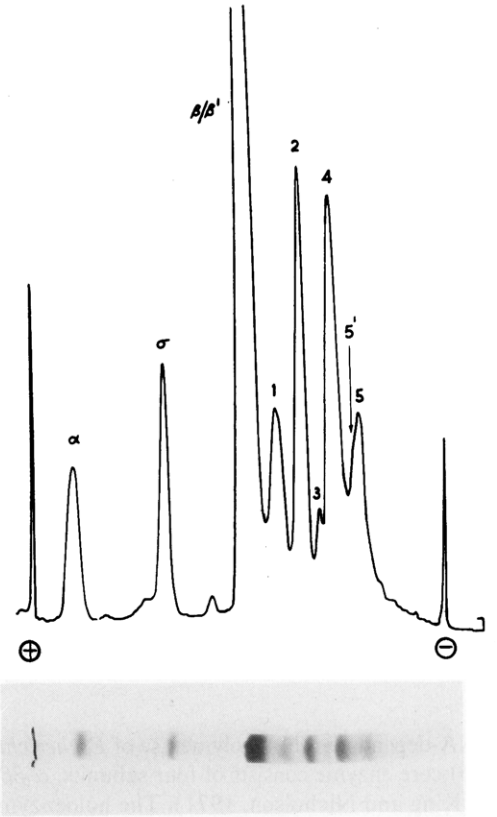


FIGURE 2: Densitometer trace of gel D in Figure 1.

To deduce the arrangement of the subunits, we decided to study RNA polymerase cross-linked with 2.5 mM CETD in high salt. This material contained sufficient quantities of each of the five cross-linked bands for further analysis, but was free of products formed by intermolecular cross-linking. To establish the molecular weight of the cross-linked species, a plot was made of mobility ($R_f$) vs. logarithm of molecular weight for a series of cross-linked ovalbumin markers and for the uncross-linked RNA polymerase subunits. This plot was linear over the molecular weight range 40 000–400 000. The mo-

TABLE I: Molecular Weights of the Species Obtained by Cross-linking RNA Polymerase Holoenzyme with CETD and Their Possible and Established Subunit Compositions.[a]

| Band No. | Obsd Mol Wt ($\times 10^{-3}$) | Possible Cross-linked Species (predicted mol wt $\times 10^{-3}$) | | Composition of Band Established by Electrophoresis after Cleavage of Cross-links |
|---|---|---|---|---|
| 1 | 200–215 | $\alpha\beta$ (184–196) | $\alpha\beta'$ (189–206) | $\alpha\beta$ and $\alpha\beta'$ |
| 2 | 240–270 | $\alpha_2\beta$ (223–237) | $\alpha_2\beta'$ (228–247) | $\sigma\beta$ and $\sigma\beta'$ |
| | | $\sigma\beta$ (231–250) | $\sigma\beta'$ (236–260) | |
| 3 | 280–305 | $\alpha\sigma\beta$ (270–291) | $\alpha\sigma\beta'$ (275–301) | $\alpha\sigma\beta$ and $\alpha\sigma\beta'$ |
| 4 | 305–345 | $\beta\beta'$ (295–320) | | $\beta\beta'$ |
| 5 | 375–415 | $\alpha\beta\beta'$ (334–361) | $\alpha_2\beta\beta'$ (373–402) | $\sigma\beta\beta'$ |
| | | $\sigma\beta\beta'$ (381–415) | | |
| | 90 | $\alpha_2$ (78–82) | $\sigma$ (86–95) | $\sigma$ |

[a] The conditions for cross-linking were as described for Figure 1, gel C, the bands are numbered as in Figure 2, and the observed molecular weights are those derived from Figure 3.

lecular weights of bands 1 to 5 as estimated from this plot, together with the predicted molecular weights of some of the possible products of cross-linking RNA polymerase holoenzyme, are shown in Table I. From the third column of this table, it is apparent that, on the basis of molecular weight alone, the composition of the cross-linked species cannot be deduced. For example, in the case of band 2, the observed molecular weight is consistent with its being composed of either $\alpha_2\beta$, $\alpha_2\beta'$, $\sigma\beta$, $\sigma\beta'$, or possibly a mixture of some or all of these species. To resolve this problem, it was necessary to cleave the cross-links formed with CETD by oxidation with periodate and then to identify the resulting subunits by electrophoresis on a 5% sodium dodecyl sulfate–polyacrylamide gel. The results of this experiment are shown in Figure 3. The stained first-dimension gel on the left was run under identical conditions to that in Figure 1D. A duplicate unstained gel was soaked in phosphate–sodium dodecyl sulfate buffer to remove the urea and then in sodium periodate to cleave the cross-links. This gel was laid along the top edge of a 5% slab gel and the cleaved protein bands electrophoresed into it. The resulting pattern is shown on the left side of Figure 3. $\beta$ and $\beta'$ are present in apparently equal amounts in all of the cross-linked species, but $\sigma$ is present only in bands 2 and 5. At the loading used here (25 $\mu$g of protein/gel), $\alpha$ was not detected in any of the cross-linked bands, although the spot for uncross-linked $\alpha$ showed up clearly. It seemed possible that $\alpha$ was not detected because insufficient sample had been applied and so a second set of gels was run with four times the loading of protein (100 $\mu$g/gel). To ensure optimal separation of the cross-linked bands, these heavily loaded gels were electrophoresed longer than usual. As a result, by the end of the first dimension, the uncross-linked $\alpha$ and $\sigma$ bands had run off the gel and the $\beta/\beta'$ band was approaching the bottom. In the resulting slab gel (right side of Figure 3) $\sigma$ was clearly seen to be a component of band 3, as well as of bands 2 and 5, and a distinct $\alpha$ spot was seen to be derived from band 1. As before, $\beta$ and $\beta'$ were seen in equal amounts from all the bands. Faint $\alpha$ spots, which do not show up in the photograph, were also seen to be derived from bands 3 and 5′.

A close examination of Figure 3 reveals that the bands in the first-dimension gels and in the slabs are not perfectly aligned. This is because the first-dimension tube gels had to be slightly compressed in order to fit tightly between the glass plates containing the slab gels so that the tube gels could not move during the second-dimension electrophoresis. This lack of perfect alignment did not cause any ambiguities in the interpretation of the slab-gel patterns, which are shown diagrammatically in the lower part of Figure 3 and are listed in Table I.

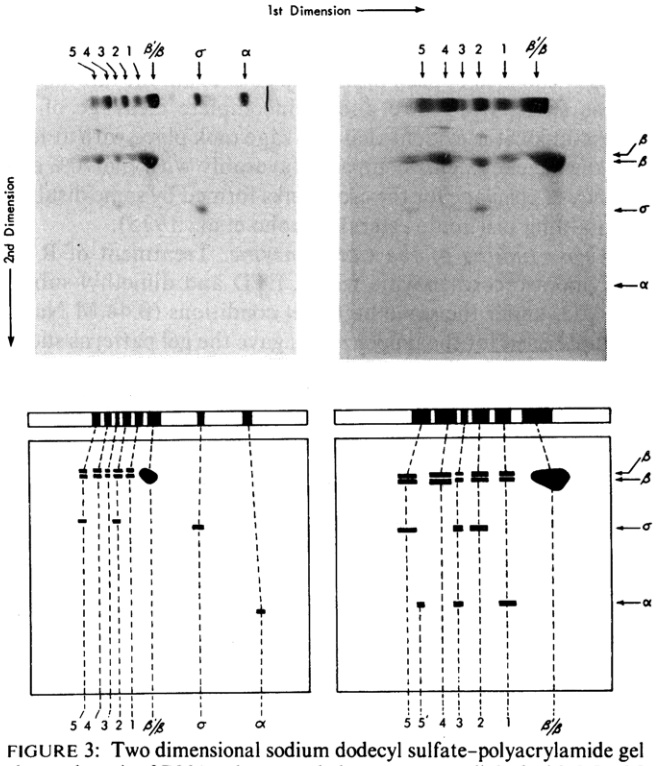


FIGURE 3: Two dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis of RNA polymerase holoenzyme cross-linked with 2.5 mM CETD. The first dimension of electrophoresis was carried out in 3% tube gels. Duplicate first-dimension gels were run; one was stained for protein and the other was soaked in 30 mM sodium periodate for 4 h at 20 °C to cleave the cross-links. The periodate-treated gel was then laid across the top of a 5% sodium dodecyl sulfate–polyacrylamide slab gel and the components of the cross-linked bands were electrophoresed into the slab gel so that they could be separated for identification. The upper part of the figure shows, at the top, the first-dimension tube gels stained for protein and, below, the corresponding second-dimension slab gels. The lower part of the figure shows a diagrammatic interpretation of these gels; some faint bands which do not show up in the photographs of the slab gels are also included. The gels on the left were loaded with 25 $\mu$g of protein and the first-dimension electrophoresis was for 250 min, while the gels on the right were loaded with 100 $\mu$g of protein and the first dimension-electrophoresis was for 875 min. Further details are given under Experimental Procedure.

In the slab gels, the spots for uncross-linked $\alpha$, $\sigma$, and $\beta/\beta'$ serve as markers for identifying the components derived from the cross-linked bands by periodate cleavage. These spots lie

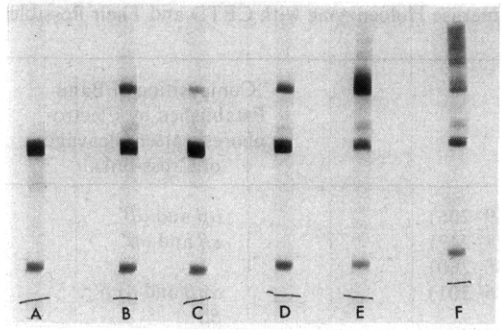


FIGURE 4: Cross-linking of RNA polymerase core enzyme with dimethyl suberimidate and with CETD. Sodium dodecyl sulfate–polyacrylamide gels (3%) stained with Coomassie brilliant blue. Cross-linking conditions as in Figure 1. (A) Control; (B) 0.5 mM dimethyl suberimidate; (C) 0.5 mM CETD; (D) 2.5 mM CETD; (E) 10 mM CETD; (F) as for E, except the NaCl concentration was 0.04 M.

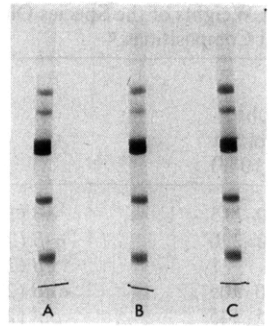


FIGURE 5: Cross-linking of RNA polymerase holoenzyme with a series of short bis(imido esters). Sodium dodecyl sulfate–polyacrylamide gels (3%) stained with Coomassie brilliant blue. RNA polymerase (1.0 mg/mL) was cross-linked for 1 h at 20 °C in 0.1 M triethanolamine hydrochloride, pH 8.0, 0.44 M NaCl with 10 mM reagent. (A) Dimethyl malonimidate; (B) dimethyl succinimidate; (C) dimethyl glutarimidate.

on a shallow curve, rather than on a diagonal, because the acrylamide concentrations for the two dimensions of electrophoresis were different. Some faint spots of high-molecular-weight can also be seen in the slab gels. From the faintness of these spots, which are due to incomplete cleavage of the cross-links, it is evident that cleavage took place with at least 90% efficiency. This compares favorably with the 70% efficiency of cleavage for the cross-links formed by some disulfide containing bis(imido esters) (Ruoho et al., 1975).

*Cross-linking of the Core Enzyme.* Treatment of RNA polymerase core enzyme with CETD and dimethyl suberimidate, under the same high-salt conditions (0.44 M NaCl) and pH used for the holoenzyme, gave the gel patterns shown in Figure 4. At 0.5 mM CETD (C), there is a single band in the same position as band 4 in the holoenzyme. At 10 mM CETD (E), there is a faint double band in the position of band 1, a faint single band in the position of 5′, while the band in position 4 has become very intense. In all cases, bands 2, 3, and 5, which contain $\sigma$ in the holoenzyme, are absent.

RNA polymerase core enzyme cross-linked with 10 mM CETD at high salt (Figure 4E) gives rise to two very faint high-molecular-weight bands, which suggest that even 0.44 M NaCl is not enough to prevent aggregation. More extensive formation of high-molecular-weight species occurs when the cross-linking is carried out at low salt concentrations (Figure 4F).

*Studies with Different Length Cross-linking Reagents.* It has been shown that cross-linking studies on multimeric proteins with a single bis(imido ester) reagent can sometimes give very misleading results about the number of subunits in the structure and about its overall symmetry (Hucho et al., 1975). It is preferable to carry out experiments at several reagent concentrations and, more important, it is essential to find out if the length of reagent used affects the nature and the relative yields of the products formed. Our own experience with aldolase amply confirms this earlier work (Coggins, manuscript in preparation). We have therefore carried out experiments on RNA polymerase with a series of simple bis(imido esters) of formula $(MeO)HN{=}C(CH_2)_nC{=}NH(OMe)$ where $n$ varied from 1 to 6.

Treatment of RNA polymerase holoenzyme with dimethyl adipimidate ($n = 4$) and dimethyl pimelimidate ($n = 5$) gave cross-linking patterns similar to those obtained with dimethyl suberimidate. By comparison, the shorter cross-linking reagents dimethyl malonimidate ($n = 1$), dimethyl succinimidate ($n = 2$), and dimethyl glutarimidate ($n = 3$) were much less effective cross-linkers. The gel patterns obtained when 10 mM solutions of these last three reagents were used under high-salt conditions for 1 h at pH 8.0 are shown in Figure 5.

These patterns are similar to those obtained with 0.5 mM CETD (Figure 1C); only bands 2 and 4 are prominent, while band 3 is absent and bands 1 and 5 are very faint. No new cross-linked species were detected and the relative amounts of the cross-linked species observed were similar to those found with other reagents.

## Discussion

The goal of the experiments described in this paper was to use the cross-linking method to identify the various intersubunit contacts present in RNA polymerase. By using the reversible reagent CETD, we have, for the first time, been able to identify unambiguously the subunit composition of each cross-linked band. To simplify the analysis, conditions which gave protein that was approximately 50% cross-linked were chosen for detailed study. Under these conditions, $\beta\beta'$ was the main cross-linked product. The other major products were $\sigma\beta$ and $\sigma\beta'$, which were formed in equal amounts, and $\sigma\beta\beta'$. $\beta\beta'$ was also the main product at low reagent concentrations, while $\sigma\beta\beta'$ became the main product at high concentrations. Cross-linked species containing $\alpha$ were never obtained in high yields. Nevertheless, at intermediate concentrations of cross-linker direct evidence was obtained for the occurrence of equal amounts of $\alpha\beta$ and $\alpha\beta'$ and, in lower yield, equal amounts of $\alpha\beta\sigma$ and $\alpha\beta'\sigma$. Some weaker evidence (a shoulder on a gel scan at the correct molecular weight position and a very faint $\alpha$ spot on a slab gel) was obtained for the occurrence of the species $\alpha\beta\beta'$. Although the $\alpha$ bands observed after cleavage of the cross-links were in all cases rather weak, it was evident from the molecular weight of the cross-linked band from which they were derived and the sum of the molecular weights of the more readily detected components of the band that one $\alpha$ subunit was present in each case (see Table I).

These data suggest that the $\beta$ and $\beta'$ subunits interact with a large contact area, since they were the most easily cross-linked pair. The $\sigma$ subunit, which can be cross-linked with equal efficiency to either $\beta$ or $\beta'$, must be in direct contact with both of these subunits. Because the species $\alpha\sigma$ and $\alpha\alpha$ were not detected, it is unlikely that the two $\alpha$ subunits have any contact with $\sigma$, or with each other, in the holoenzyme. Cross-links are formed with equal efficiency between $\alpha$ and $\beta$ and between $\alpha$ and $\beta'$. This suggests that one $\alpha$ subunit is in contact with $\beta$ and the other with $\beta'$. A simple two-dimensional model which illustrates these conclusions is shown in Figure 6.

Cross-linking experiments on the core enzyme also resulted mainly in the formation of $\beta\beta'$. As with the holoenzyme, small and approximately equal amounts of $\alpha\beta$ and $\alpha\beta'$ were formed as well. At high reagent concentrations, evidence was obtained for the formation of traces of $\alpha\beta\beta'$ and $\alpha_2\beta\beta'$. These data suggest that the core enzyme has essentially the same arrangement of the $\alpha$ subunits as the holoenzyme. Cross-linking experiments thus provide no evidence for any major rearrangement of the subunits when the holoenzyme–core enzyme transition occurs.

Because the length of the cross-linking reagents used in studies on multimeric proteins may influence significantly the nature and the relative amounts of the products formed (Hucho et al., 1975; Coggins, manuscript in preparation), we also used a series of simple bis(imido esters) having from 1 to 6 methylene groups between the two imido ester functions. All the reagents gave similar patterns to those obtained with CETD. The shorter reagents, with 1, 2, and 3 methylene groups, gave $\beta\beta'$ in good yield and in lower yield $\sigma\beta$ and/or $\sigma\beta'$. They were much less effective cross-linkers than CETD, since at 10 mM concentrations they gave results similar to those obtained with 0.5 mM CETD. The longer reagents were, by comparison, rather more efficient than CETD. The absence of any major differences in the gel patterns given by the various reagents gives us confidence that the experiments with CETD give a true indication of the subunit contacts in RNA polymerase. The low yields of cross-linked products containing $\alpha$ subunits probably indicate a lack of lysines near the contact region between $\alpha$ and its neighbor.

Our failure to observe the species $\alpha_2$ almost certainly means that the $\alpha$ subunits are not on contact with each other in the native enzyme and this has been taken into account in the model shown in Figure 6. It should, however, be realized that this evidence does not constitute absolute proof, since there is at least one example in the literature of a protein (the pyruvate decarboxylase component of *E. coli* pyruvate dehydrogenase) which undoubtedly exists as a dimer in solution but, even though it contains many lysine residues, has not yet been successfully cross-linked with a bis(imido ester) (Coggins et al., 1976).

The presence of the $\alpha\beta$ and $\alpha\beta'$ packing units in RNA polymerase has also been demonstrated by Ishihama (1972) who obtained these subcomplexes by treatment of the enzyme with 2 M urea. $\beta\beta'$ and $\alpha\beta\beta'$ subcomplexes were also obtained by treatment of the enzyme with *p*-chloromercuribenzoate. These results led to a model which is entirely consistent with our proposed subunit arrangement (Ishihama, 1972).

Attempts to reconstitute *E. coli* RNA polymerase core enzyme in vitro (Ishihama and Ito, 1972; Ito and Ishihama, 1973; Ishihama et al., 1973; Fukuda and Ishihama, 1974; Taketo and Ishihama, 1976) have suggested that the order of assembly is: $2\alpha \rightarrow \alpha_2 \rightarrow \alpha_2\beta \rightarrow \alpha_2\beta\beta'$ (inactive) $\rightarrow \alpha_2\beta\beta'$ (active).

Studies on the biosynthesis of RNA polymerase in vivo have yielded evidence for the occurrence of two of these intermediates, $\alpha_2$ and $\alpha_2\beta$, although the latter complex has only been observed in large amounts in a mutant strain (Ts4) which has abnormal $\beta'$ subunits (Taketo and Ishihama, 1976). Another strain (Ts16) with abnormal $\beta'$ has been found to accumulate "premature core", that is, the species $\alpha_2\beta\beta'$ (inactive) in the above scheme (Taketo and Ishihama, 1976).

The proposed scheme for in vitro assembly implies that the $\beta$ subunit binds both the $\alpha$ subunits and, if there were no subsequent rearrangement during assembly and activation, one would have expected to find $\alpha\beta$ and $\alpha_2\beta$ in the cross-linking experiments and not, as we found, $\alpha\beta$ and $\alpha\beta'$. Indeed, we found no convincing evidence for species containing $2\alpha$ subunits.

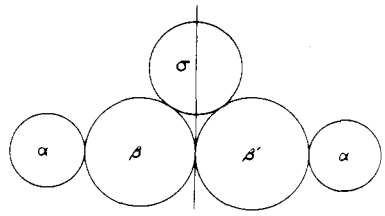


FIGURE 6: A simple model for the arrangement of the subunits in RNA polymerase holoenzyme.

To reconcile our results with the in vitro assembly data and with the in vivo studies, we suggest that, either during the formation of $\alpha_2\beta\beta'$ (inactive) or more likely during the subsequent activation step, an $\alpha$ subunit must move to become more closely associated with $\beta'$ than with $\beta$.

It is known that a high activation enthalpy is involved in the final step of the above scheme (Harding and Beychok, 1974). A major subunit rearrangement, such as we are suggesting, would certainly be expected to have a high activation enthalpy. The lack of activation observed in strain Ts16 may be due to a failure of this rearrangement. Since it is known that the conformation of the $\beta$ subunit in the $\alpha_2\beta$ subassembly resembles that in the native enzyme (Lowe and Malcolm, 1976a,b), we must conclude that the conformational change which we have suggested does not greatly affect the $\beta$ subunit.

Most multimeric proteins are known to have symmetrical structures. Our data suggest that, in spite of the nonidenticality of the $\beta$ and $\beta'$ subunits, both core and holoenzymes show some elements of twofold symmetry, implying that $\beta$ and $\beta'$ are identical packing units. It is interesting in this connection that the amino acid compositions of $\beta$ and $\beta'$ are very similar (Fujiki and Zurek, 1975).

Acknowledgments

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# NADPH–Cytochrome P-450 Reductase from Rat Liver: Purification by Affinity Chromatography and Characterization†

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ABSTRACT: (NADPH)-cytochrome P-450 reductase was purified to apparent homogeneity by a procedure utilizing nicotinamide adenine dinucleotide phosphate (NADP)-Sepharose affinity column chromatography. The purified flavoprotein has a molecular weight of 79 700 and catalyzes cytochrome P-450 dependent drug metabolism, as well as reduction of exogenous electron acceptors. Aerobic titration of cytochrome P-450 reductase with NADPH indicates that an air-stable reduced form of the enzyme is generated by the addition of 0.5 mol of NADPH per mole of flavin, as judged by spectral characteristics. Further addition of NADPH causes no other changes in the absorbance spectrum. A $K_m$ value for NADPH of 5 $\mu$M was observed when either cytochrome P-450 or cytochrome *c* was employed as electron acceptor. A $K_m$ value of 8 ± 2 $\mu$M was determined for cytochrome *c* and a $K_m$ value of 0.09 ± 0.01 $\mu$M was estimated for cytochrome P-450.

The mixed-function oxidase system of liver endoplasmic reticulum, which catalyzes the hydroxylation of a variety of drugs, polycyclic hydrocarbons, alkanes, fatty acids, steroids, and other xenobiotics, is the subject of wide interest and has been reviewed by Brodie et al. (1958), Conney (1967), and Gillette et al. (1972). Omura et al. (1965) demonstrated the role of cytochrome P-450, the carbon monoxide binding pigment of hepatic microsomes (Omura and Sato, 1962), in hydroxylation of various substrates. Lu and Coon (1968) reconstituted the mixed function oxidase system from its resolved components (cytochrome P-450, cytochrome P-450 reductase and a heat-stable lipid factor) using laurate as a substrate, and later Lu et al. (1970) showed a number of other compounds, including drugs, alkanes, and fatty acids, were also hydroxylated by the reconstituted system. Subsequent to the reconstitution of activity from components, efforts were made to purify and characterize the individual components of the mixed-function oxidase system. The lipid component was identified as phosphatidylcholine (Strobel et al., 1970), and cytochrome P-450 has been purified to homogeneity (van der Hoeven et al., 1974; Ryan et al., 1975a). Characterization of the oxidation-reduction properties (Ballou et al., 1974; Guengerich et al., 1975) and the existence of multiple forms of cytochrome P-450 (Ryan et al., 1975b; Haugen et al., 1975) have also been reported. Early efforts to solubilize the reductase with proteolytic (Phillips and Langdon, 1962; Pederson et al., 1973) or lipolytic (Williams and Kamin, 1962) enzymes yielded a flavoprotein capable of reducing electron acceptors such as cytochrome *c* but unable to support cytochrome P-450 dependent substrate hydroxylation (Masters et al., 1975). This flavoprotein, NADPH–cytochrome *c* reductase (EC 1.6.2.4.), has been extensively studied and characterized (Masters et al., 1965a,b; Baggot and Langdon, 1970; Iyanagi et al., 1974; Masters et al., 1975).

Partially purified detergent-solubilized preparations of cytochrome P-450 reductase from rabbit and rat liver have been reported by van der Hoeven and Coon (1974), Satake et al. (1972), and Vermilion and Coon (1974). Recently, our laboratory reported the purification of cytochrome P-450 reductase to apparent homogeneity by solubilization of rat liver microsomes with the detergent Renex 690 and column chro-

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