Interaction of sigma factor σ^N with *Escherichia coli* RNA polymerase core enzyme

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The equilibrium binding and kinetics of assembly of the DNA-dependent RNA polymerase (RNAP) σ^N -holoenzyme has been investigated using biosynthetically labelled 7-azatryptophyl-(7AW) σ^N . The spectroscopic properties of such 7AW proteins allows their absorbance and fluorescence to be monitored selectively, even in the presence of high concentrations of other tryptophan-containing proteins. The 7AW σ^N retained its biological activity in stimulating transcription from σ^N -specific promoters, and in *in vitro* gel electrophoresis assays of binding to core RNAP from *Escherichia coli*. Furthermore, five Trp → Ala single mutants of σ^N were shown to support growth under conditions of nitrogen limitation, and showed comparable efficiency in activating the σ^N -dependent *nifH* promoter *in vivo*, indicating that none of the tryptophan residues were essential for activity. The equilibrium binding of 7AW σ^N to core RNAP was examined by analytical ultracentrifugation. In sedimentation

equilibrium experiments, absorbance data at 315 nm (which reports selectively on the distribution of free and bound 7AW σ^N) established that a 1:1 complex was formed, with a dissociation constant lower than 2 μ M. The kinetics of the interaction between 7AW σ^N and core RNAP was investigated using stopped-flow spectrofluorimetry. A biphasic decrease in fluorescence intensity was observed when samples were excited at 280 nm, whereas only the slower of the two phases was observed at 315 nm. The kinetic data were analysed in terms of a mechanism in which a fast bimolecular association of σ^N with core RNAP is followed by a relatively slow isomerization step. The consequences of these findings on the competition between σ^N and the major sigma factor, σ^{70} , in *Escherichia coli* are discussed.

Key words: analytical ultracentrifugation, protein–protein interactions, transcription, tryptophan analogues.

INTRODUCTION

An essential step in gene expression is the assembly of specific DNA-dependent RNA polymerase (RNAP) complexes at promoter sites on DNA, where RNA synthesis is initiated. In prokaryotes, gene expression is primarily controlled at the level of transcription initiation, and a crucial role is played by the association of specific sigma (σ) factors with core RNAP ($\alpha_2\beta\beta'$) to form the transcriptionally competent holoenzyme ($\alpha_2\beta\beta'\sigma$). The reversible binding of alternative sigma factors allows RNAP to recognize different groups of promoters and co-ordinate the expression of different sets of genes in response to varying cellular needs [1,2]. The σ factors of bacterial systems fall into two classes. The larger family comprises factors with close structural and functional similarities to the major σ^{70} factor of *Escherichia coli*, which is responsible for transcribing the major ‘housekeeping’ bacterial genes [3]. The σ^N (also known as σ^{54}) protein is the only known sigma factor in bacteria that is not a member of this family; its amino acid sequence bears very little resemblance to that of σ^{70} and there are important mechanistic differences in the action of RNAP when associated with σ^{70} or σ^N [4]. The complex formed by RNAP– σ^{70} and the DNA promoter sequence can undergo transition from the initial ‘closed’ complex to form the transcriptionally competent ‘open’ complex, in which the DNA is unwound, without any other regulatory factor being necessary [5]. However, the RNAP– σ^N complex with the promoter is a closed complex that is usually kinetically and thermodynamically stable; it requires contact with an enhancer-binding activator protein (AP) and NTP hydrolysis to promote opening of the DNA, in a process which is reminiscent of eukaryotic transcription [6,7]. Binding of the AP upstream, or

occasionally downstream, of the polymerase–promoter complex is followed by looping of the DNA, which brings the AP and polymerase into direct contact; in some systems the looping is mediated by integration host factor (‘IHF’) [8]. It is not known how this protein–protein contact with the AP at one end of the polymerase elicits DNA unwinding, but it may be supposed that conformational changes in RNAP– σ^N are involved [9]. The σ^N subunit will bind to strong promoters as an isolated subunit, although association with core RNAP changes the nature of the σ^N –DNA contacts and the stability of the complex [10]. This is unlike σ^{70} , which is unable to bind to promoter DNA as an isolated protein; in this case, the DNA binding determinants, which are masked in the isolated protein [11], are only revealed on binding to core RNAP, presumably due to conformational changes in the σ^{70} subunit on association with the core enzyme [12,13]. It is probable that competition among sigma factors is responsible for differential regulation of gene expression in bacteria, and for controlling overall levels of expression. We have recently demonstrated, using gel electrophoresis to monitor the binding of σ to core RNAP, that σ^N and σ^{70} do compete with each other for core RNAP, implying that the two σ -binding sites are either coincident or overlapping; we have further shown that the affinity of σ^N for core RNAP is not weak, even in the absence of DNA [14]. The σ^N factor interacts with core RNAP, promoter DNA and distally-bound transcription factors by an array of linked interactions and associated conformational changes that determine the commitment of the σ^N –RNAP complex to productive transcription. To provide information about the details of these protein–protein and protein–DNA interactions, we report in the present paper the results of studies in which a specific label, in the form of a tryptophan analogue, 7-azatryptoph-

Abbreviations used: AP, activator protein; 7AW, 7-azatryptophyl-; DTT, dithiothreitol; 5OHW, 5-hydroxytryptophan; RNAP, DNA-dependent RNA polymerase; M_w^0 , whole-cell molecular mass.

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phyl-(7AW), has been incorporated biosynthetically, in place of tryptophan, into σ^N . This confers on the protein a distinct spectroscopic signature that can be selectively monitored in the presence of tryptophan-containing proteins, even those as large as the core RNAP. The UV absorption band of 7AW is red-shifted by approx. 20 nm with respect to tryptophan, allowing selective monitoring of absorbance and also excitation of the protein fluorescence at 315 nm. The advantages of using such protein analogues with either 5-hydroxytryptophan (5OHW) [15] or 7AW as labels, sometimes termed 'spectrally-enhanced proteins' ('SEPs'), have been amply demonstrated [16,17]. 7AW has been used to study several other proteins [18–20]; it does not appear to alter structure or function significantly, and is preferred over 5OHW as a more structurally conservative substitution. The present paper describes the production and functional characterization of 7AW σ^N , studies of its binding to core enzyme using the analytical ultracentrifuge, and equilibrium and kinetic studies using stopped-flow spectrofluorimetry to assess the binding and conformational changes in the protein that occur on assembly of the σ^N -RNAP complex.

MATERIALS AND METHODS

Reagents

All reagents were purchased from Sigma (Poole, Dorset, U.K.), unless otherwise stated.

Plasmids

Plasmids used in this work were as follows: pMKC28 corresponds to pTE103::P_{nifH}*R. meliloti*, a template for *in vitro* nifH transcription assays (M. Chaney and M. Buck, unpublished work); pTE103 [21]; pMM83, for mutagenesis of the *rpoN* gene [22]; pHSG575, encoding wild-type *rpoN* for growth in minimal media [23]; pMB1, containing the *nifH-lacZ* fusion for *in vivo* β -galactosidase assays [24]; pMSP5 (pHSG576::*rpoNW88A*), pMSP6 (pHSG576::*rpoNW126A*), pMSP7 (pHSG576::*rpoNW212A*), pMSP8 (pHSG576::*rpoNW285A*) and pMKC21 (pHSG576::*rpoNW328A*), containing the *rpoN* Trp → Ala mutant genes (the present work); and pWVC93025 for over-expression and preparation of σ^N [9].

Purification of core RNAP

Mixed holo and core RNAP were purified from *E. coli* MRE 600 according to the method of Burgess and Jendrisak [25], and the modifications described in [26]. Core polymerase was prepared by removal of the σ factor from the holoenzyme by chromatography through a Bio-Rex 70 column (Bio-Rad Laboratories, Hemel Hempstead, Herts., U.K.). Purified core enzyme was subsequently stored at -80°C in storage buffer [10 mM Tris/HCl (pH 8.0), 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 200 mM NaCl and 50% (v/v) glycerol]. Protein purity was greater than 95%, as judged by SDS/PAGE, and the level of residual σ^{70} was $< 2\%$. Concentrations of core RNAP were determined spectrophotometrically using an absorption coefficient of $2.1 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ [25].

Expression and purification of 7AW σ^N

The pWVC93025 expression plasmid was transformed into the *E. coli* tryptophan auxotroph strain CY15077 (donated by Professor C. Yanofsky). Cells were grown at 37°C in 500 ml of Terrific Broth (12 g/l tryptone, 24 g/l yeast extract, 10 ml/l glycerol, 2.31 g/l KH_2PO_4 and 12.54 g/l K_2HPO_4), supplemented

with 200 $\mu\text{g/ml}$ ampicillin, until D_{600} was between 0.8 and 1.0. Cells were collected by centrifugation in a Sorvall GS-3 rotor at 5000 rev./min (4225 g) for 5 min. The cell pellets were resuspended in 500 ml of M9 medium supplemented with 0.4% casamino acids, 5 $\mu\text{g/ml}$ vitamin B1, 0.4% glucose, 0.1 mM CaCl_2 , 2 mM MgSO_4 and 200 $\mu\text{g/ml}$ ampicillin. After shaking for 45 min at 37°C , 7AW was added at a concentration of 50 $\mu\text{g/ml}$, and protein expression was induced by adding 1 mM isopropyl β -D-thiogalactoside. The cells were then shaken for a further 3 h. The cells were subsequently collected and stored at -80°C until required. 7AW σ^N was purified according to the method of Cannon et al. [27]. Using SDS/PAGE, purity was demonstrated to be $> 95\%$.

The AP of the σ^N -RNAP holoenzyme used *in vitro* was PspFAHTH, prepared as a histidine-tagged fusion protein [9].

Native-gel holoenzyme assembly assays

These were performed as described previously [28], but in Tris/NaCl buffer [40 mM Tris/HCl (pH 8.0), 10% (v/v) glycerol, 0.1 mM EDTA, 1 mM DTT and 100 mM NaCl]. Assays contained 250 nM *E. coli* core RNAP and various amounts of wild-type σ^N and 7AW σ^N . In order to resolve σ^N , core RNAP and the holoenzyme, gels [4.5% (w/v) polyacrylamide] were run at 50 V for 2 h at room temperature in buffer containing 25 mM Tris and 200 mM glycine, pH 8.6.

In vitro AP-dependent transcription assay in supercoiled DNA

Reactions (30 μl) in STA buffer [25 mM Tris/acetate (pH 8.0), 8 mM Mg-acetate, 3.5% (w/v) poly(ethylene glycol), 10 mM KCl and 1 mM DTT] contained 100 nM holoenzyme (formed by mixing 100 nM core RNAP with 200 nM σ^N), 20 units of RNasin (Promega) and 10 nM supercoiled DNA template (pMKC28). When used, PspFAHTH activator was added at 4 μM and GTP at 4 mM. The reactions were incubated for 20 min at 30°C (PspFAHTH and/or GTP were added after incubation of the holoenzyme and DNA for 10 min) before the addition of the following elongation mixture: 0.1 mM ATP, 0.1 mM CTP, 0.1 mM UTP, 0.1 mM GTP (omitted in reactions where 4 mM GTP was used as a hydrolysable nucleotide), 0.3 μCi [α - ^{32}P]UTP (20 $\mu\text{Ci}/\mu\text{l}$) and 100 $\mu\text{g/ml}$ heparin. Following a further 10 min incubation at 30°C , the reactions were placed on ice, precipitated using 0.25 vols. of 10 M ammonium acetate and 2.5 vols. of ethanol, and the pellets were then washed with 80% ethanol. Dried pellets were resuspended in 8 μl of 10 mM Tris/HCl (pH 8.0)/0.1 mM EDTA and 4 μl of formamide sequencing dye. Samples (5 μl) were electrophoresed in a 6% (w/v) polyacrylamide, denaturing, sequencing gel, run at 50 W for 2 h, and were analysed on a PhosphorImager (Molecular Dynamics).

Site-directed mutagenesis

Site-directed mutagenesis of the *rpoN*-containing plasmid, pMM83, was carried out by PCR using the Quickchange site-directed mutagenesis kit (Stratagene). The DNA was then transformed into *E. coli* JM109 and recovered for sequencing. The residues Trp⁸⁸, Trp¹²⁶, Trp²¹², Trp²⁸⁵, Trp³²⁸ of *Klebsiella pneumoniae* σ^N were changed to alanine.

Screening by growth

A qualitative assessment of *rpoN* activity was made by examining the growth of *K. pneumoniae* UNF2792 (pMSP5, pMSP6, pMSP7, pMSP8, pMKC21, pHSG575 or pMM83) on M9 salt-based minimal media with 0.5 or 1 mg/ml arginine as the limiting nitrogen source and supplemented with trace elements, 125 $\mu\text{g/ml}$

histidine and 20% (w/v) glucose as the carbon source. Growth was scored as the ability to grow at 37 °C overnight.

β -galactosidase assays

Expression of a *K. pneumoniae* *nifH-lacZ* gene fusion (pMB1) in the *K. pneumoniae* strain UNF2792, an *rpoN* strain, carrying pMM83 or mutant derivatives was determined by β -galactosidase assays carried out in NFDM (0.1 mg/ml MgSO_4 , 25 $\mu\text{g/ml}$ NaMoO_4 , 25 $\mu\text{g/ml}$ FeSO_4 , 12.06 mg/ml K_2HPO_4 , and 3.2 mg/ml KH_2PO_4) supplemented with 125 $\mu\text{g/ml}$ histidine, 2% (w/v) glucose as the carbon source and 100 $\mu\text{g/ml}$ aspartic acid as the nitrogen source, in the absence of oxygen.

Western blot analysis

K. pneumoniae UNF2792 (pMSP5, pMSP6, pMSP7, pMSP8, pMKC21, pHSG575 or pMM83) were grown for 10 h in broth, and 2% (v/v) of the crude cell lysate was resolved by SDS/PAGE [7.5% (w/v) polyacrylamide] at 200 V for 40 min. Western blotting, using an antibody raised against *E. coli* σ^N [1], was carried out according to the manufacturer's instructions (Bio-Rad).

Sample preparation

Binding experiments in the analytical ultracentrifuge and fluorescence measurements were conducted at 20 °C in buffer containing 50 mM Tris/HCl (pH 8.0), 0.1 mM EDTA, 0.1 mM DTT, 200 mM NaCl and 10% (v/v) glycerol. Protein samples were dialysed extensively before use.

Analytical ultracentrifugation

Analytical ultracentrifugation experiments were carried out on a Beckman XL-A ultracentrifuge (Beckman, Palo Alto, CA, U.S.A.) using an AN-Ti 60 rotor. Sedimentation velocity experiments on σ^N were carried out at 40 000 rev./min in Epon two-channel centrepieces, with data collected at approximately 5 min intervals. Velocity experiments involving RNAP were carried out at 30 000 rev./min. Sedimentation equilibrium experiments were carried out at 5000, 6000, 7000, 8000, 9000, 12 000, 16 000, 22 000 and 28 000 rev./min in Epon six-channel centrepieces. Radial scans were taken 4 h apart, and were overlaid to check that equilibrium had been attained at each speed. Data were collected at 280 and 400 nm for wild-type proteins, and an additional scan was performed at 315 nm for experiments involving the spectrally-enhanced 7AW σ^N protein. Scans at 400 nm were used to detect for the presence of Wiener skewing arising from the protein gradient evident near the bottom of the centrifuge cell [29,30]. After the final scan, the rotor speed was increased to 36 000 rev./min for 8 h in order to clear the meniscus. Radial scans at the three wavelengths were then taken of each cell so as to determine the true optical baseline.

Determination of molecular mass of individual components

Data from sedimentation equilibrium experiments involving individual components were used to derive molecular-mass information using the program NONLIN [31]. A variety of models were used to fit the data and the best model was judged by examination of the residuals and the statistics of the fit.

Determination of whole-cell molecular mass (M_w^0)

M_w^0 (app) was determined using the program MSTARA [32,33]. The M^* function is an operational point average molecular mass given by:

$$\frac{C(r) - C_a}{M^*(r)} = kC_a(r^2 - a^2) + 2k \int_a^r r(C(r) - C_a) dr$$

where $C(r)$ is the concentration at radius r , C_a is the concentration at the radial position of the meniscus (a), M^* is the operational point average molecular mass and $k = (1 - \bar{v}\rho)\omega^2/2RT$. Here $\bar{v}$ is the partial specific volume, ρ is the density, ω is the angular velocity, R is the universal gas constant, and T is the thermodynamic temperature. The virtue of the M^* function is that when it is extrapolated to the cell base it returns the value for M_w^0 (app) with greater accuracy than conventional methods [33].

Stopped-flow spectrofluorimetry

Rapid mixing fluorimetry experiments were performed using a HiTech SF-61 DX2 stopped-flow spectrofluorimeter (HiTech, Salisbury, Wilts., U.K.), with an instrumental dead time of 2 ms. Equal volumes of 7AW σ^N and core RNAP were mixed, and fluorescence changes were recorded over a time period of up to 20 s. Excitation wavelengths of 280 nm and 315 nm were used in conjunction with a 360 nm cut-off filter positioned before the emission photomultiplier. Rate constants were determined by non-linear least-squares fitting from the average of at least three traces. Due to the small change in fluorescence observed on excitation at 315 nm and the consequent high noise-to-signal ratio, results were obtained by averaging from multiple traces (6–8) before fitting the data.

RESULTS

Incorporation of 7AW into σ^N

Figure 1 shows the UV absorbance spectra for wild-type σ^N and 7AW σ^N . For 7AW σ^N there is significant absorbance above 300 nm, indicating a high level of incorporation of 7AW. The

Figure 1 UV absorbance spectra of wild-type σ^N (continuous line) and 7AW σ^N (broken line)

Significant absorption can be seen at 315 nm for the 7AW-containing protein. The degree of incorporation for this sample was found to be 82% and the resulting absorption coefficient was $29858 \text{ M}^{-1} \cdot \text{cm}^{-1}$.

Figure 2 Native gel shift holoenzyme assay

Lanes are as follows: (1) 250 nM RNAP; (2) 250 nM RNAP + 500 nM σ^N ; (3) 250 nM RNAP + 1.25 μ M σ^N ; (4) 250 nM RNAP + 500 nM 7AW σ^N ; and (5) 250 nM RNAP + 1.25 μ M 7AW σ^N .

Figure 3 Results of *in vitro* activator-dependent transcription assays

Lanes are as follows: (1) RNAP- σ^N ; (2) RNAP- σ^N + PspF Δ HTH; (3) RNAP- σ^N + GTP; (4) RNAP- σ^N + PspF Δ HTH + GTP; (5) RNAP-7AW σ^N ; (6) RNAP-7AW σ^N + PspF Δ HTH; (7) RNAP-7AW σ^N + GTP; and (8) RNAP-7AW σ^N + PspF Δ HTH + GTP. The results show that the 7AW σ^N protein supports wild-type transcription.

level of incorporation was determined using linear combination of spectra ('LINC') analysis:

$$\alpha_t(\lambda) = \sum_{i=1}^n m_i(\lambda) \alpha_i(\lambda)$$

where $\alpha_t(\lambda)$ = total absorbance, $\alpha_i(\lambda)$ = absorbance of basis spectra, $m_i(\lambda)$ = amplitude of basis spectra (each as a function of wavelength). The wavelength range used for fitting each of the spectra was 260–350 nm. The basis spectra used were those of tyrosine, tryptophan and 7AW. Typical levels of incorporation were found to be in the range 70–90%. Absorption coefficients of the protein were then determined based upon the level of incorporation, and protein concentrations were determined from this data as detailed in the legend to Figure 1.

Activity of 7AW σ^N

The results of the native-gel holoenzyme-assembly assays show that incorporation of 7AW in place of the five naturally occurring tryptophans in the protein (at positions 88, 126, 212, 285 and 328) did not affect the ability of σ^N and core RNAP to associate (Figure 2). In each case 7AW σ^N combined with the core protein to produce a distinct holoenzyme species. Figure 3 shows the

Table 1 β -galactosidase assay of alanine-substitution mutants of σ^N

The *K. pneumoniae* gene fused to *lacZ* was assayed in *K. pneumoniae* UNF2792 under nitrogen-limiting anaerobic conditions for activation by the *nifH* protein. 100% activity represents 15000 Miller units. Measurements were made in triplicate. Errors were approx. 5%.

σ^N -bearing plasmid	% of wild-type activity
None	0.6
pMM83 (wild-type)	100
Trp ⁸⁸ → Ala	81
Trp ¹²⁶ → Ala	89
Trp ²¹² → Ala	90
Trp ²⁸⁵ → Ala	97
Trp ³²⁸ → Ala	73

Figure 4 Western blot analysis of cell extracts from cells expressing plasmids containing the wild-type *rpoN* gene and mutant *rpoN* genes for the tryptophan-substitution mutants

Lanes are as follows: (1) prestained marker; (2) σ^N marker; (3) pHS675 (vector); (4) pMM83 (as control); (5) Trp⁸⁸ → Ala; (6) Trp¹²⁶ → Ala; (7) Trp²¹² → Ala; (8) Trp²⁸⁵ → Ala; and (9) Trp³²⁸ → Ala. Proteins were expressed in *K. pneumoniae* UNF2792, an *rpoN*⁻ strain.

results of an activator-dependent transcription assay. Levels of transcript show that the incorporation of 7AW did not appear to affect initiation in the presence of the AP (lanes 4 and 8), or result in any significant transcription independent of its activator (lanes 3 and 7). The conclusion from this is that analogue incorporation does not compromise the activity of the σ^N protein in maintaining the closed complex or in allowing activator-dependent responsiveness.

Role of tryptophan residues in σ^N

To assess whether any of the tryptophan residues in the protein had a crucial role in protein activity, a series of mutations were made in which each of the five tryptophan residues was changed to alanine. The five single, Trp → Ala mutants were able to support σ^N -dependent growth, and were able to activate the σ^N -dependent *nifH* promoter *in vivo* with similar efficiency to the wild-type protein (Table 1). The levels of expression of the mutants and the wild-type were determined by immunoblotting and were shown to be comparable (Figure 4). These findings imply that none of the single Trp → Ala mutations had any marked effects on activity or stability, implying that the five tryptophan residues are not individually crucial for holoenzyme assembly or activity, and that 7AW substitutions were likely to be

Table 2 Derived hydrodynamic properties of σ^N , 7AW σ^N and RNAP

$s_{20,w}^0$ corresponds to the sedimentation coefficient corrected to 20 °C in water at infinite dilution. Loading concentrations of protein were 0.5, 1.0 and 1.5 mg/cm³. M_w^0 (app) is the apparent molecular mass derived from the sedimentation equilibrium experiments. Initial protein concentrations were 0.25, 0.5 and 0.75 mg/cm³. The last column shows the model used to derive values of M_w^0 (app).

Sample	$s_{20,w}^0$ (svedbergs)	M_w^0 (app) (kDa)	Model
σ^N	3.1 ± 0.2	54 000 ± 500	Single species
7AW σ^N	3.0 ± 0.2	54 000 ± 600	Single species
Core RNAP	14.0 ± 0.3	376 000 ± 17 000	Monomer/dimer

tolerated well, consistent with the core binding and transcription assays (Figures 2 and 3).

Sedimentation studies of individual components

The results of sedimentation velocity and equilibrium experiments on wild-type σ^N , the 7AW analogue and core RNAP are shown in Table 2. The sedimentation coefficients of both sigma factors are the same ($s_{20,w}^0 = 3.1$ S) indicating that they have the same overall shape in solution. Equilibrium data show that both proteins are completely monomeric under the conditions used, yielding a molecular mass of 54 000 ± 500 Da (mean ± S.D.), compared with a theoretical value of 53 926 Da. Combined with the activity data described above, these findings indicate that the incorporation of 7AW into σ^N does not cause any gross conformational changes in its structure leading to loss of activity, implying that in this case 7AW incorporation can be regarded as a conservative change.

Sedimentation velocity studies on the core RNAP show that its sedimentation coefficient is 14 S, which is close to the value of 13.1 S, which would be expected for a monomeric protein [34]. However, sedimentation equilibrium data show that the core is present as a mixture of monomers and higher-order aggregates in a rapidly-reversible equilibrium. The simplest model that could be fitted to the data was a monomer/dimer equilibrium, for which non-linear least-squares fitting yielded a dissociation constant, K_d , of 4.4 ± 2.0 μ M. It can be estimated that at the highest loading concentration of 1 mg/ml (equivalent to 2.4 μ M monomer units) the dimeric form would comprise approximately 30 % of the total protein species. The consequences of this in the binding experiments are discussed below.

Selection of the correct rotor speed to visualize both σ^N and core RNAP

As the core RNAP is approx. 7 times larger than σ^N , there is a problem in selecting the correct rotor speed so as to be able to measure the molecular mass of mixtures of both proteins if either is present free in appreciable amounts. If the rotor speed is too low the distribution of σ^N across the centrifuge cell will be too shallow to allow accurate molecular-mass determination. Conversely, if it is too high the core polymerase will sediment into a narrow concentrated band at the bottom of the centrifuge cell. To alleviate this problem a series of experiments was carried out at 5000, 6000, 7000, 8000 and 9000 rev./min on core and σ^N proteins on their own. It was found that the speed that allowed the best determination of both protein molecular masses was between 6000 and 8000 rev./min. This gave an accurate value for the core monomer molecular mass (376 000 ± 15 000 Da), but

under these conditions, the molecular mass of σ^N could only be measured to an accuracy of approx. ± 30 %.

Sedimentation equilibrium experiments of core polymerase and 7AW σ^N

A titration experiment was performed where 7AW σ^N , at a concentration of 7.1 μ M, was incubated with increasing concentrations of core RNAP. At this concentration of 7AW σ^N the absorbance at 315 nm was approx. 0.1. The mixtures were analysed by sedimentation equilibrium in the analytical ultracentrifuge at 6000, 7000 and 8000 rev./min. M_w^0 (app), for each of the mixtures, was determined using the program MSTAR [32,33]. There are contributions to the value of M_w^0 (app) from each of the species observed in the equilibrium distribution. Consequently, the value of M_w^0 (app) determined at 280 nm reflects the molecular-mass distribution of all of the components in the cell, whereas the same procedure carried out at 315 nm reflects only the distribution of 7AW σ^N . The data from both sets of determinations can be seen in Figures 5(A) and 5(B) respectively. An increase in the value of M_w^0 (app) at 280 nm is apparent due to the increased amount of core RNAP present. The two curves overlaid on the data (Figure 5A) are the theoretical distributions that would be expected for the two extreme hypotheses of (i) a tight binding interaction between σ^N and core (continuous line), and (ii) no interaction between the two species (broken line). It can be seen that, within the limits of error, the data can be described by either curve, making it impossible to determine whether an interaction is occurring between the core protein and 7AW σ^N . The corresponding results with the data at 315 nm, however, do allow a ready distinction between the two hypotheses (Figure 5B). Since the core enzyme is transparent at 315 nm, it is trivial to determine the non-interacting case: a value of approx. 54 kDa would be returned irrespective of the polymerase concentration. However, the determined values of M_w^0 (app) rise from a value of 54 kDa to a value of approx. 400 kDa, close to that expected for the holoenzyme (430 kDa), demonstrating that binding is occurring. Additionally, the data establish unequivocally that σ^N and core RNAP bind in a 1 : 1 complex, as was previously suggested from the results of gel filtration experiments [35]. The broken lines shown in Figure 5(B) indicate the concentration dependence of M_w^0 (app) that would be expected with different values of the dissociation constant for the interaction between 7AW σ^N and core RNAP. As can be seen from Figure 5, although this method is accurate enough to determine the stoichiometry of the interaction it does not have the accuracy required to determine a precise value for the K_d . The best estimate that can be determined is that the K_d is tight and less than 2 μ M, a finding that is consistent with results obtained from native-gel electrophoresis [14]. Therefore we turned to kinetic measurements in an attempt to determine the K_d indirectly.

Fluorescence studies of 7AW σ^N binding to core RNAP

Equilibrium fluorescence titrations showed that the changes in fluorescence were not large (1–2 %). The data were found to be not accurate enough to warrant analysis, as there was the need for a blank correction arising from scattering from the far larger polymerase species (results not shown). However, in stopped-flow experiments the polymerase is at a constant concentration and therefore any change in background will occur during the mixing time of the experiment. Consequently time-resolved

Figure 5 Results of equilibrium titration of wild-type σ^N and 7AW σ^N in the analytical ultracentrifuge determined at rotor speeds of 6000 rev./min (filled triangles), 7000 rev./min (filled squares) and 8000 rev./min (filled circles)

(A) Data at 280 nm. Two theoretical curves are shown: the tight-binding limit for a 1:1 binding event (continuous line) and no interaction (broken line). It can be seen that using data at this wavelength it is not possible to distinguish between either type of behaviour. (B) Data at 315 nm. The theoretical distributions for a 1:1 binding event have been overlaid on top of the data. The top continuous line is the tight-binding limit. The bottom continuous line is the distribution for no interaction between 7AW σ^N and RNAP [M_w^0 (app) = 54 kDa]. The intermediate distributions are for the following values of K_d , (in descending order): 1 μ M, 2 μ M and 5 μ M.

Figure 6 Stopped-flow spectrofluorimetry traces of the binding of RNAP to 7AW σ^N . The concentrations of RNAP and 7AW σ^N were 0.13 and 0.02 μ M respectively

Excitation wavelengths were 280 nm (left panel) and 315 nm (right panel). It can be seen that the data obtained with excitation at 280 nm is biphasic, whereas that obtained at 315 nm excitation is monophasic (see the text). The two rate constants obtained from fitting the data at 280 nm were $1.1 \pm 0.24 \text{ s}^{-1}$ and $0.11 \pm 0.02 \text{ s}^{-1}$, and for the data at 315 nm a value of $0.11 \pm 0.025 \text{ s}^{-1}$ was obtained. Values are means $\pm$ S.E.M.

changes in fluorescence can be attributed to changes in the fluorescence characteristics of the species, rather than light scattering or background effects. Figure 6 shows the changes in

fluorescence in stopped-flow experiments upon mixing 7AW σ^N with core RNAP and exciting at either 280 nm (left panel) or 315 nm (right panel). Fits and residuals are shown. The overall total decrease in fluorescence was approx. 1–2 %, a small change that is consistent with the results of the equilibrium fluorescence spectra. The trace at 280 nm shows a biphasic decrease in fluorescence, which was fitted to the following biexponential function:

$$\Delta F = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$$

where A_1 and A_2 are the amplitudes, t is time, and τ_1 and τ_2 are the relaxation times of the two effects. The left panel of Figure 6 shows the residuals of the fits for a double and a single exponential fit to the traces at 280 nm. It can be seen from this that the double exponential function describes the data better than the single exponential function. Because of the small changes in fluorescence observed at 315 nm the results from multiple traces were averaged to increase the signal-to-noise ratio and provide more reliable results for kinetic analysis. The right panel of Figure 6 shows the data obtained by averaging results from eight experiments with a core RNAP concentration of 130 nM. This panel also shows the residuals obtained on fitting the data to a single exponential and a double exponential function. It is evident that a satisfactory fit is obtained with a single exponential function, and that a double exponential does not significantly improve the fit. There is no evidence of an observable fluorescence effect at 315 nm corresponding to the fast effect seen upon excitation at 280 nm. As a control, it was established that no change in fluorescence was observed on excitation at 280 nm when core RNAP was mixed with buffer in the stopped-flow experiment (results not shown) indicating that the origin of the fluorescence change seen at 280 nm excitation did not arise from the dissociation of core RNAP dimers, but rather from a 7AW σ^N -linked binding event. The results indicate that two types of behaviour are being observed at the two exciting wavelengths: the data at 280 nm reflect changes in the fluorescence of the whole system upon binding, whereas the data at 315 nm report only the changes in the fluorescence of the sigma factor. For the

Figure 7 Variation of the observed reciprocal relaxation times with concentration of RNAP

The traces are as follows: (open squares) fast phase at 280 nm excitation (open circles) slow phase at 280 nm excitation (open triangles) single phase 315 nm excitation. The error bars for the fast phase are 67% confidence limits for the linear fit.

data at 280 nm it was observed that the rate of the first fast phase of the fluorescence change increased with increasing concentrations of polymerase, whereas there was little change in the slower phase of the effect. From this it can be concluded that the initial concentration-dependent fast phase represents the bimolecular association of the sigma factor with the core enzyme, whereas the slow phase corresponds to a subsequent unimolecular change. The following kinetic scheme can be used to represent this two step bimolecular association:

where E represents the core enzyme and $E\sigma^N$ and $E\sigma^{N*}$ represent different conformational forms of the σ^N holoenzyme.

The variation in the reciprocal relaxation time as a function of core RNAP concentration is illustrated in Figure 7. It can be seen that $1/\tau_1$ for the first fast phase at 280 nm increases linearly with increasing concentrations of polymerase. The bimolecular rate constant (k_1) given by the slope of the graph was found to be $(5.1 \pm 0.2) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$, close to that expected for a diffusion-controlled reaction [12,36]. The intercept of the line, corresponding to the back rate constant (k_{-1}) for this process, had a value of $0.3 \pm 0.1 \text{ s}^{-1}$. The ratios of the forward and reverse rate constants gave a K_d value of $60 \pm 30 \text{ nM}$ for the initial association. The relaxation time for the unimolecular isomerization step is related to the rate constants for the individual processes by the following expression, where [E] is the concentration of RNAP:

$$1/\tau_2 = k_{-2} + k_{+2}/\{1 + k_{-1}/(k_{+1}[E])\}$$

According to this equation, values of $1/\tau_2$ would be expected to increase from k_{-2} at low values of [E] to limiting values of $k_{+2} + k_{-2}$ at high values of [E]. Within the limits of error, the relaxation times (τ_2) for the slow phase observed at 280 nm were the same as those observed at 315 nm, suggesting that the same

process was being monitored at the two wavelengths. The reciprocal relaxation times appeared to be independent of polymerase concentration above a concentration of 53 nM (the lowest concentration that gave reliable relaxation effects) and had a value of $0.2 \pm 0.1 \text{ s}^{-1}$. Since in the present work with σ^N , the slow effect could not be measured at low concentrations of polymerase, it was not possible to derive reliable values of the individual kinetic parameters k_{+2} and k_{-2} for the isomerization step. A similar situation was observed in a previous study with σ^{70} [12]. However, on the reasonable assumption that the isomerization step favours the final form of the holoenzyme ($E\sigma^{N*}$), i.e. $k_{+2} > k_{-2}$, we consider the most likely explanation is that the observed isomerization ($0.2 \pm 0.1 \text{ s}^{-1}$) essentially represents the forward rate constant k_{+2} , and that the back rate constant k_{-2} is small. Further support for this hypothesis is that complexes of σ^N with core have a relatively long lifetime in native-gel binding experiments (see the Discussion section).

DISCUSSION

The results presented in this paper show that it is possible to obtain kinetic and equilibrium binding information on the formation of the σ^N -RNAP holoenzyme using 7AW σ^N . This protein has 7AW incorporated biosynthetically to a high level, in place of tryptophan, into σ^N . The resulting protein maintains the ability of the wild-type protein to assemble the holoenzyme, as revealed by native-gel assays (Figure 2), and supports σ^N -dependent promoter activation (Figure 3). These findings suggest that the five modified tryptophans in σ^N are not essential for activity, a conclusion that is reinforced by the results of our studies in which each of the tryptophan residues was mutated singly to alanine; none of these mutations affected σ^N -holoenzyme transcription activity *in vivo* (Table 1). The substitution of 7AW for tryptophan therefore represents a conservative change and provides a selective spectroscopic probe to examine the details of holoenzyme assembly. The ultracentrifugation results demonstrate the usefulness of tryptophan analogues in analysing protein-protein interactions. From the data at 280 nm it is impossible to obtain any useful information about the interaction between σ^N and core, as the expected change in M_w^0 (app) with core concentration for a interacting and a non-interacting system would be expected to be very similar (Figure 5A). However, the additional information provided by selectively monitoring the radial distribution of the 7AW σ^N at 315 nm in solution enabled the interaction to be characterized (Figure 5B); the stoichiometry of the complex is unequivocally 1:1 and the binding is relatively strong ($K_d < 2 \mu\text{M}$). Additionally, it should be noted that previous attempts to analyse the σ^N -RNAP interaction have used gel electrophoresis and size-exclusion chromatography, which unlike ultracentrifugation are not true solution methods, and can be affected by the matrix producing so-called 'cage' effects. Our results demonstrate clearly that σ^N does bind RNAP tightly in free solution, a fact that was controversial [2]. Under the conditions used to investigate its interaction with σ^N , core polymerase did associate to form higher aggregates. The association could be satisfactorily fitted to a monomer-dimer association-dissociation equilibrium with a K_d of $4.4 \pm 2.0 \mu\text{M}$. This finding accords with sedimentation velocity results obtained previously [34,37], where a small amount of self-association is seen at these salt concentrations. However, under the limited range of conditions studied in the present paper, it was not possible to see any of the higher-order aggregates ($n > 2$) from previously predicted binding models [34]. At a concentration of 1 mg/ml it follows that approximately 30% of the core polymerase is present as a dimer (or higher-order aggregate). From

Table 3 Values of derived rate constants for the 7AW σ^N -RNAP interaction

The values for σ^{70} are taken from reference [12].

Rate constant	σ^N	σ^{70}
k_1	$(5.1 \pm 0.2) \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$	$3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$
k_2	$0.2 \pm 0.1 \text{ s}^{-1}$	0.24 s^{-1}
k_{-1}	$0.3 \pm 0.1 \text{ s}^{-1}$	0.23 s^{-1}

the limiting value of M_w^0 (app) in Figure 5(B) (approximately 430 kDa, which is the value expected for a monomeric holoenzyme) it appears that σ^N binds preferentially to the monomeric form of RNAP, as is the case with σ^{70} [38]. If σ^N bound equally well to the monomeric and dimeric forms of the core polymerase, it would not change the position of monomer-dimer equilibrium, and the limiting value of M_w^0 (app) would be expected to be 560 kDa. Binding of 7AW σ^N to core RNAP is accompanied by only a small change (1–2 %) in the 7AW fluorescence of the sigma factor. This suggests that there is little change in the solvent accessibilities of the five tryptophan residues in sigma when core RNAP binds, since the quantum yield of the 7AW residue decreases very dramatically, by 27-fold, in going from a hydrophobic to a hydrophilic environment [18]. Previous work has shown that four out of the five tryptophan residues are in the fragment of sigma found to be a 'core binding' domain [27]: it is not known the extent to which any of these residues are solvent exposed, although proteolysis data suggests that Trp³²⁸ is on the surface while the others are not as solvent accessible [9]. It appears, therefore, that none of the tryptophan residues directly contact the core in the holoenzyme. As is discussed below, analysis of the kinetics of the binding makes it more likely that the change in fluorescence is due to a conformational change in σ^N upon core binding rather than exclusion of the fluorophore from the solvent by being at a binding interface. This could explain the small changes in fluorescence observed. Although the change in fluorescence is small, it is sufficient to enable stopped-flow spectrofluorimetry to be used to study binding. Additionally, by exciting at 280 and 315 nm it is possible to examine the response of the whole system and 7AW σ^N respectively. The kinetic data obtained at 280 nm for the interaction of 7AW σ^N with core polymerase are consistent with a two-step mechanism in which a rapid bimolecular association is followed by a relatively slow unimolecular isomerization. The best-fit parameters for the kinetic constants are shown in Table 3. The value of the bimolecular rate constant ($5 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) is close to the corresponding value for the interaction of σ^{70} with core polymerase ($3 \times 10^6 \text{ M}^{-1} \cdot \text{s}^{-1}$) also monitored by stopped-flow spectrofluorimetry, but using N-pyrene maleimide attached to σ^{70} as a label [12]. This value is close to that observed for elementary rates of association in protein-protein interactions [12,36], and probably represents the diffusion-limited rate when allowance is made for the orientation factor due to the limited extent of the two interacting regions on the protein surfaces. It is noteworthy that the fast phase of the relaxation following mixing is only observed following excitation at 280 nm and not at 315 nm where the signal reports on the 7AW σ^N fluorescence. This suggests that the initial rapid association process does not involve changes in the solvent accessibility of the 7AW residues on sigma, which would be expected to produce significant changes in the sigma-specific fluorescence; the subsequent conformational change induced by sigma binding does, however, encompass both sigma and the core. The initial association of σ^N and core

RNAP is relatively tight ($60 \pm 30 \text{ nM}$), and the complex presumably becomes more stable due to the subsequent conformational transition. Although the value of k_2 , the forward rate for this conformational change, represented by the plateau in $1/\tau_2$ at high concentrations of core is well established, it is difficult to gauge values of k_{-2} from the present data. The results of native-gel binding experiments suggest that this is likely to be a slow process, since complexes of radiolabelled σ^N with core have a long lifetime when challenged with excess unlabelled σ^N [14]. Since the kinetic parameters characterizing the binding of σ^N and core RNAP are strikingly similar to those obtained with σ^{70} [12], we conclude that σ^N , despite being a minor sigma factor, is able to compete successfully for core RNAP under physiological conditions and so form the σ^N holoenzyme which recognises rare promoters [14]. Despite these similarities in forming holoenzyme, there are important differences between σ^{70} and σ^N . The transcriptionally inactive σ^N holoenzyme needs specific APs for activity. This is presumably reflected, in part, in differences in the precise nature of the conformational changes in core elicited by σ^N and σ^{70} and their interaction with promoter DNA. Our previous data on the sequences of σ^N that define core-binding regions suggest that these are contained in two functionally distinct regions: a 95-amino-acid-sequence (amino acids 120–215) within region III of σ^N , which is responsible for providing the contacts that anchor sigma and core RNAP; and a second region within the N-terminal region (amino acids 1–56), which regulates the activity of the holoenzyme [14]. We are presently studying the interaction of domains of σ^N with core to dissect the contribution of the two regions to the overall stability of the complex, and to assess the contribution of the N-terminal region to productive holoenzyme assembly.

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